From Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

Characterization and Applications in Muscle of a Minicircle Vector for Nonviral Gene Therapy

Sofia Stenler

Stockholm 2015

Published by Karolinska Institutet.
Printed by AJ E-PRINT AB

ISBN 978-91-7549-949-9

Characterization and Applications in Muscle
of a Minicircle Vector for Nonviral Gene Therapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Sofia Stenler

Principal Supervisor:
Dr Pontus Blomberg
Karolinska Institutet and
Karolinska University Hospital
Department of Laboratory Medicine
Division of Clinical Research Center

Co-supervisor(s):
Dr Karin Lundin
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Research Center

Professor Edvard Smith
Karolinska Instituet
Department of Laboratory Medicine
Division of Clinical Research Center

Opponent:
Professor Deborah Gill
University of Oxford
Department of John Radcliffe Hospital
Division of Clinical Laboratory Sciences

Examination Board:
Professor Mats Eriksson
Karolinska Institutet
Department of Medicine
Unit of Metabolism

Professor Matti Sällberg
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Professor Gen Larsson
Royal Institute of Technology
School of Biotechnology
Division of Bioprocess Technology

To great things to come.

ABSTRACT

In gene therapy, the aim is to change the behaviour of a cell by introduction of genetic material, often DNA encoding a protein or a therapeutic RNA. The purpose can be to replace a malfunctioning copy of a gene, as in clinical trials for treatment of X-linked severe combined immunodeficiency, or introduce a new gene into the body to help fight a disease, as has been done in clinical trials for e.g. leukaemia and lymphoma where immune cells has been modified to recognize and destroy cancer. In order to alter the behaviour, the genetic material must be transported into the cell and reach the nucleus. The two main ways to achieve this is either using viral vectors, where engineered viruses carry the therapeutic DNA, or nonviral vectors, which are commonly based on plasmids produced in bacteria. This thesis focuses on nonviral vectors.

Nonviral vectors are generally considered safer and more easily produced than viral vectors, but are less efficient in delivery and long term expression. This is thought to be partly due to the plasmid backbone, i.e. sequences needed only for propagation in the bacteria such as origin of replication and selection markers, commonly antibiotics resistance genes. Bacterially produced DNA sequences have a different methylation pattern than eukaryotic DNA. It has been shown that this can induce an immune response, especially in combination with the use of lipids for transfection. Also for naked delivery of plasmids, the expression is transient, which could be due to epigenetic phenomenon. A way to optimize the plasmid vector is to remove the bacterial backbone by recombination in the production bacteria. The resulting vector is called the minicircle (MC).

In one of studies included in this thesis, we investigate how the size of the MC vector affects coiling and relate these findings to analysis of other aspects such as robustness, expression efficiency and transfection. We find that reducing the size of the MC affects the configuration of the vector, causing an increased frequency of dimer and trimer formation during production. We also find that there seems to be a lower size limit for efficient expression. However, the smaller sizes also result in a vector which is more robust than conventional plasmids when exposed to shearing forces, and shows extended expression *in vivo*. In the two other studies, we evaluate the vector for use in muscle. A comparison of the MC to a conventional plasmid for expression of a growth factor in heart and skeletal muscle in the mouse shows that the smaller size allows for a higher effective dose, and thus, higher gene expression. The third study demonstrates that it is possible to use the MC to express small

regulatory RNAs for splice-switching, targeting Duchenne muscular dystrophy, and that treatment with these MCs in mouse muscle results in increased dystrophin levels. However, development of suitable delivery methods is required to realize the full potential of MCs *in vivo*.

Thus, the smaller size enabling a higher dose, prolonged expression and increased robustness, and the fact that the MC construct is devoid of bacterial sequences and antibiotics resistance gene make the MC vector an attractive alternative for nonviral gene. However, for use where systemic treatment is needed, delivery must be enhanced. Consequently, the vector might be more suitable for treatments where only local expression is required, such as single organ treatment, DNA vaccination or *ex vivo* treatments.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Genterapi syftar till att förändra en cells beteende genom att föra in genetiskt material av något slag. Detta kan vara för att ersätta en skadad gen, som i den ärftliga sjukdomen cystisk fibros, eller introducera en ny funktion, till exempel för att lära immunceller att känna igen cancerceller. För att förändra cellens beteende måste det genetiska materialet transporteras in i cellen till kärnan. Ett effektivt sätt att åstadkomma detta är att använda modifierade virus, eftersom virus är evolutionärt anpassade till att föra in genetiskt material i andra celler. Det finns emellertid problem med dessa så kallade virala vektorer, de kan orsaka immunsvar och skador på arvsmassan. Ett alternativ är icke-virala vektorer som ofta baserar sig på plasmid-DNA producerat och framrenat från bakterier. De är billigare att framställa och säkrare än virala vektorer, men har en lägre och mer kortvarig effekt.

I denna avhandling undersöks ett system för att optimera plasmidbaserade vektorer; göra dem effektivare och ännu säkrare genom att ta bort de sekvenser som bara behövs för produktionen i bakterier ifrån den färdiga vektorn. Dessa sekvenser verkar bidra till immunreaktioner och till att hämma effekten av vektorn över tid. Denna minimerade plasmid-vektor för ickeviral genterapi kallas för minicirkel. Minicirkeln undersöks med avseende på vad som händer när storleken på plasmiden minskas och utvärderas för behandling i muskel.

Studierna i denna avhandling visar att minicirkeln är en lovande vektor för terapi i muskel. Den minskade storleken möjliggör en högre dos och kan också ha en positiv inverkan på transporten av vektorn in i cellerna och till kärnan. Försöken visar emellertid också att en alltför kort längd på vektorn hämmar genuttrycket. Studierna visar även att transporten av vektorn måste optimeras för att uppnå en god effekt i muskelcellerna. Slutligen diskuteras olika möjliga framtida användningar av minicirkelsystemet, till exempel som vektor för DNA-vaccinering.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications, which will be referred to in the text by using their Roman numerals:

I. Sofia Stenler, Agneta Andersson, Oscar E. Simonson, Karin E. Lundin, Zhi-Ying Chen, Mark A. Kay, C. I. Edvard Smith, Christer Sylvén, and Pontus Blomberg, *Gene Transfer to Mouse Heart and Skeletal Muscles Using a Minicircle Expressing Human Vascular Endothelial Growth Factor* Journal of Cardiovascular Pharmacology, 2009;53:18–23

II. Sofia Stenler, Oscar P.B. Wiklander, Maria Badal-Tejedor, Janne Turunen, Joel Z. Nordin, David Hallengärd, Britta Wahren, Samir EL Andaloussi, Mark W. Rutland, C.I. Edvard Smith, Karin E. Lundin and Pontus Blomberg, *Micro-minicircle Gene Therapy: Implications of Size on Fermentation, Complexation, Shearing Resistance, and Expression* Molecular Therapy - Nucleic Acids, 2014, 3, e140

III. Sofia Stenler, Samir. EL Andaloussi, Suzan Hammond, Aurélie Goyenvalle, Mark J.A. Wood, Dominic J. Wells, Karin E. Lundin, C.I.Edvard Smith and Pontus Blomberg *Micro-minicircle vectors for splice-switching in Duchenne Muscular Dystrophy* Manuscript

Text from the following paper has been used for writing of the Introduction section of this thesis:

Sofia Stenler, Pontus Blomberg and C.I. Edvard Smith, *Safety and efficacy of DNA vaccines: plasmids vs. minicircles.* Hum Vaccin Immunother. 2014 May;10(5):1306-8.

CONTENTS

LIST OF ABBREVIATIONS

aa	Amino acid
AFM	Atomic force microscopy
AON	Antisense oligonucleotides
AAV	Adeno-associated virus
bp	Base pairs
CMV	Cytomegalovirus
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
GFP	Green fluorescent protein
hVEGF	Human vascular endothelial growth factor
iPS cells	Induced pluripotent stem cells
kbp	Kilobasepairs
MC	Minicircle
miMC	Micro-minicircle
miRNA	Micro-RNA
mRNA	Messenger RNA
pDNA	Plasmid DNA
pre-mRNA	Premessenger RNA
qPCR	Quantitative polymerase chain reaction
q-RT-PCR	Quantitative reverse trancriptase polymerase chain reacton
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
shRNA	Short hairpin RNA
snRNA	Small nuclear RNA

TA	*Tibialis anterior*
TLR9	Toll-like receptor 9

1 INTRODUCTION

1.1 GENE THERAPY - A BRIEF OVERVIEW

Gene therapy aims to change the behaviour of a cell by introduction of genetic material. This can be to replace a malfunctioning copy of a gene, as in clinical trials for treatment of X-linked severe combined immunodeficiency, or introducing a new gene into the body to help fight a disease, as has been done in clinical trials for e.g. leukaemia and lymphoma where immune cells has been modified to destroy cancer. Gene therapy has been used in 2142 clinical trials, with cancer diseases being the most common target.[1] There are to date two commercially available approved gene therapy drugs: Gendicine containing a tumor suppressor gene for treatment of head and neck squamous cell carcinoma was approved in China in 2003, and Glybera targeting a lipoprotein lipase deficiency was approved in Europe in 2012.

In order to alter the behaviour, the genetic material must be transported into the cell and reach the nucleus. There are two main ways to achieve this.

1.1.1 Viral vectors

Viruses have evolved specifically to deliver their genome to a target cell; this is how a virus propagates. By changing the sequence of the genetic material transported by the virus, and altering the virus to prevent it from being replication competent, this trait can be harnessed for gene therapy. Viral vectors are very efficient at delivering their cargo and are therefore used in nearly 70% of gene therapy clinical trials; see Table 1 for the more frequently used vectors.

Commonly used viral vectors[1]	# clinical trials	% of clinical trials
Adeno-associated virus (AAV)	127	5.9
Adenovirus	471	22
Herpes simplex virus	68	3.2
Lentivirus	101	4.7
Poxvirus	68	3.2
Retrovirus	412	19.2
Vaccinia virus	119	5.6

Table 1. Viral vectors commonly used in clinical trials.

However, there are safety issues using viral vectors. As they originate from human pathogens, they can elicit an immune response. Either because the patient has already encountered a native strain of the virus and thus carries antibodies to the vector or because

repeated treatments are needed to sustain the expression of the therapeutic gene. This is the case for adenovirus and AAV derived vectors, where the transgene exists as an episome in the cell and will be diluted with cell division, although the wild type virus can integrate in chromosome 19.

Other viruses, such as retroviruses, integrate their genome into the genome of the target cell, and these can be engineered to do the same with a therapeutic sequence. This enables a constant expression in the cell and all daughter cells without the need for re-administration of the vector. However, the integration event can cause mutations which can have adverse effects. In the treatment of X-linked severe combined immune deficiency using a retroviral vector, the integration caused oncogenes to be activated and five of the in total 20 patients in the clinical trials suffered from leukaemia.[2-4]

Another limitation for viral vectors is the size; there is a physical limit to the amount of genetic material that can be transported in the viral capside.

1.1.2 Nonviral vectors

Nonviral vectors commonly consist of naked double-stranded DNA, often as a plasmid which is a circular, supercoiled DNA construct produced from bacteria. The therapeutic part of a plasmid vector consists of an expression cassette containing a coding sequence, often a protein expressing gene, as well as all regulatory sequences needed for the expression and translation. Furthermore, the plasmid contains sequences needed for propagation in the bacteria, such as origin of replication and selection markers, which commonly is an antibiotics resistance gene. These sequences make up the bacterial backbone of a plasmid vector.

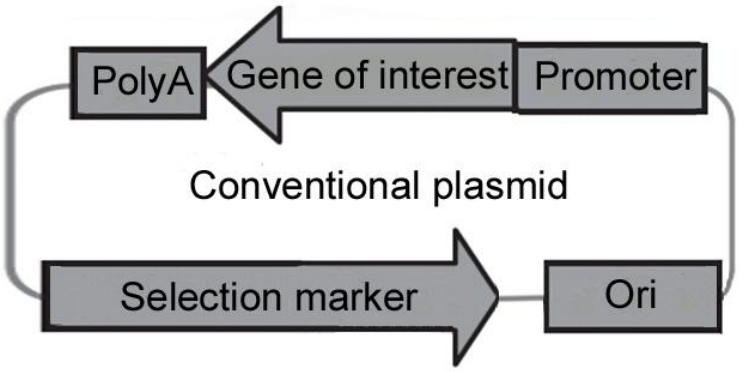

Figure 1. A conventional plasmid carries the expression cassette containing the gene of interest and regulatory sequences such as promoter and polyadenylation signal (PolyA). Furthermore, it needs sequences for propagation in the bacteria, as the origin of replication (Ori) and a selection marker, commonly an antibiotics resistance gene. These sequences are often referred to as the bacterial backbone of the plasmid.

Plasmid construction is relatively straightforward and permits the manipulation of a variety of regulatory elements that has impact on gene expression. It is easy and comparatively inexpensive to produce large quantities, and the possible size range of a plasmid is wide. Plasmid vectors are considered safer than viral vectors as they do not contain proteins from pathogens and thus are less immunogenic. Moreover, plasmids are episomal and non-integrating, which reduces the risk of insertional mutagenesis as compared to e.g. retroviral vectors.

However, as naked DNA lacks active transport systems for delivery into the cell and transfer into the nucleus, a nonviral vector is far less efficient than a viral vector and the expression is low and transient. There are many different approaches to improve the efficiency of these vectors. The plasmid can be packed together with lipids and polymers, which condense the DNA and protect it from degradation as well as facilitates uptake through fusion with the lipid bi-layer of the cell membrane. Other chemical methods to increase transport over the cell- and nuclear membranes is to link the plasmid construct with cell penetrating peptides and nuclear ligand sequences respectively. Physical delivery methods uses different forces to enhance transport into the target cell, such as electroporation, pneumatics and high volume infusions.

Another approach to create more efficient nonviral vectors is to improve the design of the expression cassette itself. The regulatory sequences such as promoters and enhancers can be optimized to achieve high expression, cell type specific expression and long-lived expression.

Gill *et al.* discuss the selection of promoters for long term expression, and the use of tissue specific promoters for improved specificity and safety of non-viral vectors.[5] Other components that can enhance long term expression are the inclusion of splicing elements, or introns, that contribute to higher gene expression.[6] Micro-RNA sequences, on the other hand, can be introduced to limit expression to selected target cells while silencing it in undesired tissues.[7] A method to prevent loss of gene expression due to heterochromatin spread in the nonviral vector is the inclusion of insulators flanking the expression cassette. A special case is the Scaffold/Matrix Attachment Regions (S/MAR).[8] This AT-rich sequence acts as insulator against heterochromatin spread and promotes binding to the transcriptional and translational machinery found in the nuclear matrix. This enhances episomal establishment, enabling a prolonged expression also in dividing cells.[9]

Also the properties of the plasmid vector itself affect the efficiency of a nonviral vector. As stated above, a plasmid needs to contain certain sequences for the production in bacteria. These sequences are however not needed, not even beneficial, in the target cell. Studies have shown that the bacterial sequences present in the vector hamper the gene expression form the plasmid vector.[10,11] For example, the methylation pattern of bacterial DNA and mammalian DNA differs, and unmetylated CG dinucleotides, CpGs, in plasmids can trigger the immune response through the Toll-like receptor nine (TLR9) resulting in the loss of the cells harbouring the plasmid. Elevated levels of inflammatory cytokines have been shown after intravenous injection and after delivery to the lungs of lipocomplexed unmethylated CpG containing DNA *in vivo*.[12] These cytokines have also been shown to inhibit reporter gene expression from several viral promoters.[13] Reducing the number of CpG in a construct leads to a prolonged gene expression in lung whereas having even one CpG dinucleotide results inflammation at levels that are significantly higher than for CpG-free plasmid DNA.[5,14] There is however some debate as to the importance of CpGs in gene silencing.[15,16] Possibly, CpG induced gene silencing is more significant when using lipoplexion as transfection method. One explanation could be that the delivery pathway for lipoplexes to a higher degree puts the construct in contact with the TLR9 receptors located in the endosomal compartments.[17] However, a study investigating the effect of plasmid DNA transfer on immune and tissue response when using electroporation indicates that even here the CpG content is of importance.[18] Although electroporation in itself activated gene pathways associated with muscle function and an immune/inflammatory response, electroporation together with a non-coding plasmid DNA resulted in activation of a larger number genes involved in immune response. When using a CpG-free construct, the duration of the inflammatory response was shorter.

If the plasmid vector is delivered naked, other aspects could be more important. It has been shown that silencing of the vector can be caused by heterochromatin formation in the inactive bacterial sequences of a plasmid, and the heterochromatin then spreads into the promoter and transgene and thus hinders the expression.[19] The vector is still present in the cell, but the gene is no longer active. Thus, even if the vector has been delivered to the nucleus and the expression cassette has been optimized, the expression often diminishes with time. These reasons for plasmid silencing leads us to the minicircle as an alternative to traditional plasmid based vectors.

1.2 MINICIRCLE VECTORS

As discussed in the previous chapter, the sequences needed for propagation of a plasmid vector in the producer bacteria can be hampering or even harmful in the target cell. Removal of the bacterial backbone of the plasmid reduces the CpG-content as well as the risk of heterochromatin spread from inactive sequences. It also results in a smaller construct, which should improve the shuttling into and within the cell, and also enables a higher dose of active DNA.

The minicircle (MC) is the most common vector of this type. It was first developed over 20 years ago and although it has been used in many different areas, which will be discussed below, it has yet to reach the clinic. Possibly, the cumbersome production methods for the earlier systems have contributed to this, but also the newer and more efficient systems has so far only been used *in vitro* and in animal trials. Investigations of the possibilities and limitations of the systems to further develop the use of the vector are of great relevance.

The MC is produced using a parental plasmid where the expression cassette is flanked by specific recombination sites. Production occurs in the bacteria; after amplification, recombination is induced and the parental plasmid forms two constructs: A miniplasmid containing the bacterial sequences and an MC containing the expression cassette with gene of interest and regulatory sequences.

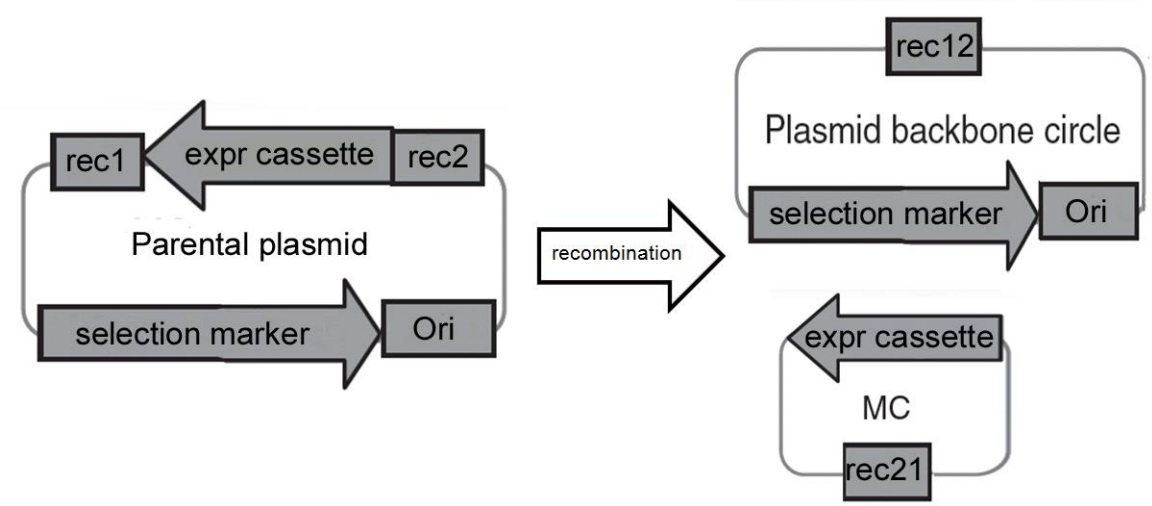

Figure 2. A schematic minicircle production system. Induction of recombination of the parental plasmid at the sites flanking the expression cassette results in the MC and a separate construct containing the bacterial backbone. rec1, rec2: Recombination sites; expr cassette: Expression cassette; Ori: origin of replication; rec12, rec21: post-recombination site-hybrides.

1.2.1 Systems

Several minicircle producing systems have been published. The first publication, from 1997, is based on the lambda phage integrase for recombination of the parental plasmid.[20] A later system is developed by Bigger *et al.* and utilizes a *Cre* recombinase expression system with *LoxP* sites flanking the expression cassette.[21] However, the *loxP*-sites are still active after recombination and the minicircle can be lost by the cassette recombining back into the parental plasmid again and the sites had to be mutated to ensure unidirectionality. Mayrhofer *et al.* have published a system based on the ParA resolvase, and adding the bacterial lactose operator sites in the MC construct they can use affinity chromatography to purify the vector.[22] In all these systems, the parental plasmid and the miniplasmid remain in the bacteria after recombination, and since they contain the origin of replication it is possible that they will continue to amplify in the bacteria, diluting the minicircle.

The system used in this thesis was developed by Chen *et al.* where the *Steptomyces* template integrase ΦC3 is used for recombination and the expression cassette is flanked by the attB and attP recombination sites.[10,23] The resulting attR and attL sites in the minicircle and miniplasmid respectively cannot recombine again to reform the parental plasmid. The problem with separating the MC from the undesired products – the miniplasmid and any unrecombined parental plasmid – is resolved by introducing a rare restriction enzyme

recognition site in the plasmid backbone outside the recombination sites, as well as providing the gene for the restriction enzyme. In the initial studies in this thesis, we used the earlier version of this system where the genes for the integrase and the restriction enzyme are encoded by the parental plasmid. Both genes were placed under the control of an arabinose inducible promoter. This approach resulted in a huge parental plasmid, but the upside is that any recombination competent *Escherichia coli* (*E. coli)* production strain can be used. However, most bacteria have an all-or-none arabinose import, where the level of arabinose inside the bacteria needs to reach a threshold level before active import is induced.[24] This results in a population of bacteria which never reaches this threshold, where neither recombination nor digestion is induced, creating a contamination of undesired plasmids in the final product. The parental plasmid and the miniplasmid contain the origin of replication, so any copies that escape degradation can continue to replicate.

The ΦC31-system was later refined by moving the genes from the parental plasmid to the bacterial genome of a bacteria strain with a constitutively active arabinose transport.[25] This results in a smaller and more stable parental plasmid and purer minicircle fraction. We have utilized this system in our later studies.

1.2.2 Characteristics

The MC is, physically, much alike a normal plasmid vector in that it consists of a circular double-stranded DNA molecule, which is supercoiled. Both the circularity and the supercoiling are of interest, as is of course the fact that the MC is a smaller vector than a conventional plasmid.

1.2.2.1 Size

An MC vector containing a protein expressing gene cassette will most often encompass a few thousand base pairs (bp), whereas a conventional plasmid carrying the same cassette will often have an additional three to four thousand bp of bacterial backbone sequences. These size ranges are interesting in the light of studies of cytoplasmic transport, where it has been shown that mobility decreases with increasing size, and the pDNA fragments became completely immobile at the size of 2 000 bp or more.[26]

The size of the MC vector is entirely dependent on the size of the expression cassette. A cassette for a short peptide would result in a rather small vector. However, the cell rarely expresses active short peptides such as hormones as a single peptide, but produces them as cleavage products from larger polypeptides. This could be to overcome structural constraints on the ribosome caused by the short distance between the initiation and termination codons.[27]

Despite this, there are a few publications indicating that also small peptides could be directly transcribed. Matza-Porges *et al.* have previously shown that it is possible to express a functional peptide as short as 15 amino acids (aa) from a Cytomegalovirus (CMV) promoter.[28] Thus, it would be possible to create small MC vectors encoding short peptides.

Cassettes encoding a short regulatory RNA, such as small nuclear RNAs (snRNA), permits even smaller vectors, of only a few hundred bp. In paper II, we introduce the term microminicircle (miMC) for a minicircle vector encoding an U7 snRNA and thereby being only 650 bp.[29] When studying miMCs with different insert sizes, we observed that the minicircle system produced concatameric variants when the insert between the recombination sites was small. Regarding full length plasmid construct, i.e. still containing the bacterial backbone sequences, studies show that it is feasible to produce dimeric plasmids. At higher numbers, multimers of the plasmid construct became instable.[30] A higher expression per cassette copy has been reported from a dimeric plasmid construct, but the transfection efficiency was lower.[31] The multimeric MC occurs naturally in the production and would thus not suffer from instability in the same way as a plasmid constructed to be concatameric. Furthermore, a multimeric MC or miMC is a smaller construct than a multimeric plasmid and could thus be more efficiently transfected.

It has been shown that the efficiency of electroporation correlates with the size of the plasmid DNA construct,[32] and that there is a benefit of lower size also for lipofection *in vitro*[33] and *in vivo*.[34] Kreiss *et al.* discuss that the size of the pDNA may affect either the mechanism of DNA release from lipoplexes or for the intracellullar migration of DNA through the cytoplasm into the nucleus, or both.[33] McLenachan *et al.* study delivery of plasmid DNA ranging from five to 200 kilobasepairs (kbp) to mouse embryonic stem cells and report a size dependant increase of nuclear delivery when using smaller constructs.[35]

Fogg *et al*. study recombination of MC ranging from 250 to 1 000 bp.[36] At the smaller sizes, they also report that the MC tended to form concatameric constructs rather than a monomer. They propose this is due to intermolecular rather than intramolecular recombination events. Forcing a short DNA fragment into a supercoiled construct is energetically unfavourable. Their observation, of a clear inverse relationship between sequence length and efficiency of intramolecular recombination, is in accordance with our observations in paper II. Thus, size has effect on the supercoiling of the MC. In the next section, I will discuss why this is of interest.

1.2.2.2 Supercoil

Any plasmid preparation will contain pDNA with different topologies: Supercoiled material, open circular and linear. A supercoiled plasmid becomes open circular when the phosphodiester bond between bases is cut in one of the strands, and there is a release of torsion. The plasmid is linearized when both strands are cut near or at the same base pair. The amount of supercoiled material is a measure of the plasmid vector quality and regulatory authorities require the plasmid preparation intended for vaccination or gene therapy to contain a high percentage of supercoiled material. In their 'Considerations for Plasmid DNA Vaccines for Infectious Disease Indications', the FDA recommends a minimum specification for supercoiled plasmid content of >80%, whereas the Swedish Medical Products Agency only accepts a higher level of >85% and normal industry expectation for supercoiled plasmid levels are >90 or >95%.[37] There are different rationales behind this quality requirement, regarding efficiency of the vector as well as pure safety concerns.

In the paper where Chen *et al.* describe their initial study on the MC, the MC is also compared to a linear cassette excised by restriction enzymes.[10] They measure protein expression after liver transfection, and it is evident that even though a linear expression cassette devoid of bacterial sequences results in over twenty fold higher expression than a normal plasmid vector, the protein production from the MC was over tenfold higher than from the linear cassette. Cheng *et al*. delivered plasmid constructs with different topologies using a cationic polymer and observed that supercoiled plasmids yielded higher transfection efficiencies than its open circular or linearized form.[38] Remaut *et al*. studied this further using a plasmid encoding GFP, and compared nuclear and cytoplasmic injections of naked DNA as well as lipopfecion.[39] From their data, it is evident that supercoiling enhances lipofection. The microinjection experiments point towards this being due to an enhanced transport of supercoiled DNA into the nucleus, as nuclear injection of pDNA resulted in the same levels of expression independent of the pDNA topology whereas supercoiled plasmids gave a higher expression after cytoplasmic injection, both in non-dividing and dividing cells. Other studies have also found a more efficient transfection using supercoiled plasmids over open circular or linearized, and attributes this to more efficient transcription.[40] This is in accordance with studies showing that the degree of supercoil affects transcription efficiency as polymerase I prefers to initiate on negatively supercoiled templates.[41,42] Also the binding of transcription factors seems to be affected, as supercoiling promotes formation of a preinitiation complex[43]. Supercoil can affect the accessibility of promoters and genes, as negative supercoil enhances formation of transcriptionally active chromatin *in vitro*.[44]

Apart from the benefits of increased transcription, supercoiling is also a question of safety. Among the risks that WHO[45], FDA[46] and EMA[47] list for the use of DNA vaccines is the hazard of integration into the recipient's chromosomal DNA with the resulting risk of insertional mutagenesis or spreading of antibiotics resistance genes. The probability of chromosomal integration increases if the introduced pDNA has been linearized.[48]

As discussed in the previous section, a miMC can be very small, and the coiling could be affected by the short length of the DNA. The persistence length of DNA, i.e. the maximum length were the direction of one end is affected by the direction of the other end, is 50 nm or 150 bp. Bending and loops at lengths below this is associated with large energy costs, and indeed Bates *et al*. report a lower limit for supercoiling at around 170 bp.[49] They also discuss that the free energy for supercoiling is greater for circular DNA of less than 1 000 bp than for those larger than 2 000 bp. The free energy of supercoiling is length dependant at sizes below 2 000 bp. The previously mentioned Fogg *et al.* study also investigates coiling of an MC construct of 339 bp, characterizes different topoisomers, and calculates how the strain on the DNA induced by coiling is partitioned into the DNA circle.[36] However, they do not correlate their findings to any functional assays of the MC as a gene delivery vector. In paper II, we study miMCs of different sizes ranging from 650 to 1 900 bp. We study the coiling by atomic force microscopy, and connect these results to studies of transfection and expression efficiency, as well as other properties as the robustness of the vector, i.e the ability of the construct to withstand shearing forces.

1.2.2.3 Robustness

The degree of resistance to shearing forces of supercoiled MC has been investigated for a number of different construct sizes.[29,50] Cantanese *et al.* have shown that constructs below 1 200 bp were more resistant to the shearing forces associated with both nebulization and sonication whereas plasmids of conventional lengths were degraded. Nebulization to create an aerosol is routinely used to deliver drugs for the treatment of lung diseases. For plasmid delivery using nebulised aerosols, shearing of the DNA must be overcome and using MC vectors can be one step. Pneumatics is a promising needle-free method for drug delivery, especially for vaccination,[51-53] although it is also associated with the shearing of DNA.[51] It has been shown that pneumatic delivery results in 10-20% shearing for plasmids.[54] Walther *et al.* have done a careful analysis of plasmid forms,[55] but after ejection only, not through any tissue, whereas in paper II, we analyze the integrity of a conventional plasmid and miMCs of different sizes after the DNA has been pneumatically ejected through mouse skin. An interesting observation from the Catanese *et al.* report is that shearing causes predominantly

double-stranded breaks rather than nicking. This taken together with the above mentioned observation that linearization of DNA increase the risk of insertion into the genome makes the MC a safer vector than a full length plasmid when delivery methods associated with shearing are being used.

The smaller vector shows increased ability to withstand not only to mechanical stress. Zhao *et al.* have studied the fate of a miMC in serum, and compare it to longer plasmid constructs.[56] They report that the miMC is stable in serum more than ten times longer than a conventional plasmid. Survival in serum could be important for *in vivo* delivery efficiency. Schüttrumpf *et al.* reports that loss of vector is the main reason for diminishing gene expression after hydrodynamic delivery to liver,[57] so a vector with an increased stability would result in a more persistent transgenic effect. Schüttrumpf *et al.* consequently report that the expression loss over time was less when using the MC vector.

1.2.3 The MC vector for substitution treatment in muscle

In muscle, as in any target organ, most gene therapy constructs encode a protein that should either replace a nonfunctional gene or have an effect that ameliorates disease symptoms.

In paper I, we compare expression of the human vascular endothelial growth factor (hVEGF) in skeletal and heart muscle from an MC vector to the expression from a plasmid previously used in clinical trials.[58] Chang *et al*. have also studied the expression of hVEGF from a minicircle vector and compared it to a conventional plasmid. However, *in vivo* they only study skeletal muscle and compare delivery of the same weight amount of MC and plasmid two days after injection.[59] Naturally, they see a higher expression from the MC, a fold increase that fairly well corresponds to the increased dose of therapeutic DNA.

In an interesting combination of gene and cell therapy, Ong *et al*. use an MC vector to express a transcription factor in endothelial cells in the heart, and thus promote a preferable milieu for transplanted cardiac progenitor cells *in vivo*.[60] The same group earlier assayed the MC alone, and observed expression for up to 12 weeks and improvement of ventricular function as well as enhanced neoangiongenesis in a mouse model of myocardial infarction.[61]

1.2.4 The miMC for snRNA expressing cassettes in muscle

For some muscular diseases, such as Duchenne Muscular Dystrophy (DMD), the coding sequence of the disease-associated gene is very large. Even though a plasmid vector can carry far longer sequences than a viral vector, constructs of several hundred kbp can still be

problematic. The human dystrophin gene covers 2.5 megabases and the resulting messenger RNA (mRNA) is 14 kbp.

An alternative to replacing an entire gene is using much smaller cassettes encoding snRNAs aimed to alter the target gene function on an mRNA level. This can be achieved by inducing mRNA decay and thereby affecting protein levels, or by affecting the splicing pattern and thus giving rise to different spliceoforms of the protein.

1.2.4.1 Downregulation

Micro-RNAs (miRs) are small, noncoding RNAs of 20 to 22 nucleotides involved in gene regulation through translational inhibition and mRNA decay. A miMC construct encoding a precursor-miR involved in upregulation of angiogenic factors and cell survival was shown to improve heart function in a mouse model of ischemic heart disease.[62]

Another method for downregulating protein levels is to utilize a short hairpin RNA (shRNA). Like miRs, shRNA can induce mRNA degradation and thus reduces protein production. The miMC vector has been used for delivery of shRNA producing expression cassettes. Huang *et al*. designed a dual shRNA expressing MC targeting two genes involved in the degradation of a transcription factor which in turn regulates hundreds of genes and protects the myocardium from ischemic injury.[63] Injection of this vector into the myocardium leads to an enhanced stem cell mobilization and increased myocardial angiogenesis as well as improved cardiac function for eight weeks in a mouse model of myocardic infarction. The same group later used a similar strategy to improve post-ischemic neovascularization in hind limb ischemia in mouse.[64]

1.2.4.2 Splice-switching

Splice-switching aims to alter the splicing of the pre-messenger RNA (pre-mRNA). It can be used to induce inclusion of an intron which is otherwise skipped, as for the case of Spinal muscular atrophy, or to skip an intron carrying a disruptive mutation, or to rescue a frame shift caused by mutations, as is a possible treatment strategy for DMD.

Splice-switching can be induced by antisense oliogonucleotides (AON) binding to the pre-mRNA and interfering with splice sites or regulatory elements. AON therapies have been successful *in vitro* and *in vivo*, e.g. in animal models for DMD and spinal muscular atrophy, but as the effect of AONs is inherently transient, repeated treatments are needed to achieve sustained splice-switching.[65,66]

An alternative is to use a vector encoding the antisense sequence in the form of an snRNA. This enables long term expression of the splice correcting sequence. It has been shown that a modified version of the U7snRNA, normally involved in histone pre-mRNA 3' end processing, can be used as a tool for splicing modulation.[67] Also the U1 snRNA splicing factor can be modified to carry an antisense sequence targeting it to a different site. The U1 snRNA normally base-pairs with the 5' splice site and is important for splice site recognition. The single-stranded 5' end of the U1 snRNA can be altered to carry antisense sequences to target it to other sequences. As it is produced from a strong and constitutive polymerase II-dependent promoter, which works in combination with a specific termination sequence, it is suitable as a transgenic gene therapy cassette. To the best of my knowledge, we are the first to investigate the miMC as a vector for splice correction, see paper II and paper III.

1.2.5 Other applications

The vector is only the means to deliver a therapeutic agent, encoded by the DNA, to the target cell. There are many different target organs and innumerable variants for what the vector should encode. In the previous chapter, we have focused on muscle. In this chapter, we will look into some other approaches where the minicircle has been used as the delivery vector.

1.2.5.1 Therapeutic gene expression

MCs expressing VEGF has not only been used for treatment in muscle, as discussed earlier, but also as a therapy for skin wounds in diabetic mice. Proliferating cells were transfected by subcutaneous delivery of MC-VEGF vectors through sonoporation,[68] ultrasound microbubble destruction[69], or formulation with a cationic dendrimer[70]. An increased blood flow and an enhanced wound healing were seen in all studies, and for delivery with the dendrimer an increased expression of VEGF was also reported.

To make the body produce its own protein-based drugs is an appealing thought. Yi *et al*. have assayed this possibility using an MC with the nucleotide sequence of etanercept and tocilizumab, two protein-based drugs for rheumatoid arthritis.[71] They report that these self-produced drugs were functionally active after intravenous injections of the MC in an arthritic mice model. Another study evaluates the MC in a diabetic animal model; Alam *et al.* use an MC construct for glucose-regulated expression of insulin delivered to the liver in diabetic rats.[72] They report normalized weight gain and that the treatment restores various diabetes-associated markers of metabolic dysregulation. The MC has also been used by Park *et al*. to treat insulin resistance in obese mice by delivering the gene for adiponectin.[73]

The MC has been delivered orally, formulated as chitosan nanoparticles, to induce the expression of a modified factor IX in the small intestine in haemophilia B mice.[74] Transient local transgene expression, clinically relevant levels of factor IX activity and partial phenotype correction were achieved by oral gene therapy without evidence of adverse immunological responses upon repeated administration.

Hyun *et al*. have used the MC vector to promote stem cell survival through expression of Bcl-2, a prosurvival protein that regulates the mitochondrial pathway of apoptosis. When treating stem cells with the MC, and then delivering the transfected cells to a wound, the prosurvival protein was overexpressed and bone formation was enhanced.[75] There is also a study showing the suitability of the MC for transfecting stem cells and thus transgenically modify them while retaining their ability to later differentiate, suggesting that the MC might be a suitable vector for stem cell therapies when a transient expression is enough to stimulate regeneration of a tissue.[76]

A field where the MC vector has been used in a number of studies is cancer research. Tumours have an enhanced permeability and retention, which allows both entry and sequestering of macromolecules, such as lipids, inside the tumour. Chang *et al.* exploited this property to deliver a lipoplexed MC vector to Hepatitis B Virus induced hepatocellular carcinoma in mice.[77] The MC encoded a metastasis-suppressing androgen receptor, and the transgenic protein could be detected for up to 60 days. Wu *et al.* study expression of tumour necrosis factor alpha from MC vectors *in vitro* and *in vivo* into xenographts of nasopharyngeal carcinoma in mice, and show good expression *in vitro* and reduction of xenographted tumour's growth and prolonged survival *in vivo*, although the MC only outperforms the large parental plasmid *in vivo* when using the same weight dose.[78] They show expression in tumours for 21 days after treatment from MC, albeit declining over time, whereas the expression from the large parental plasmid was all but lost after seven days. The same group later constructed an MC encoding endostatin, an angiogenesis inhibitor, and evaluated it in the same xenograph model of nasopharyngeal carcinoma.[79] They report reduced tumour growth for the full 20 days of the experiment as well as reduced vascularisation of the tumour after intratumoural injections. In an interesting cancer therapy study, Gaspar *et al.* use micelle nanocarriers to co-deliver an MC encoding the tumour necrosis factor alpha related apoptosis-inducing ligand and a chemotherapeutic drug, doxorubicin.[80] They see good uptake *in vitro* and an anti-tumoural effect in mice at relatively low concentrations. In a cancer-related study, the MC has been used to confer *in vivo* ionizing irradiation protection by expression of manganese superoxide dismutase, a radical oxygen

species neutralizer.[81] For this therapeutic approach, a transient expression is not a drawback as the overexpression is only needed during exposure to radiation, but high expression levels are needed. Therefore the MC vector was appealing. Intra-oesophageal administration or systemic delivery through intravenous injection of MC-lipoplexes both resulted in a greatly improved survival rate of the mice.

miMCs encoding shRNAs have not only been used in muscle, but also as expression vectors for inhibitory agents preventing viral replication. In a study by Yang *et al.* two viruses that are major causative agents of hand, foot and mouth disease were targeted. Expression of the shRNAs from the miMC vector blocked the replication and gene expression of these viruses *in vitro* and in a virus-infected mouse model, with an alleviation of symptoms in the infected mice.[82] In this study, Yang *et al.* designed a quite neat system for expression of two shRNAs from the same coding sequences, by flanking the sequence with two different promoters in different directions, as an snRNA is symmetric in sense and antisense.

1.2.5.2 DNA vaccine

A special case of a protein expressing vectors is a plasmid designed for DNA vaccination using plasmid based vectors. Among the advantages of using plasmids are the ease of both development and production as compared with conventional vaccine manufacturing. Moreover, DNA vaccines are known to be very stable at room temperature, which is of significance for both transport and storage.[83] Since the antigen is expressed from pDNA within the target cell, the resulting peptide is more likely to resemble the native form, of e.g. a viral protein, with all the necessary post-translational modifications.

In a report by Dietz *et al.*, the MC shows promise as a DNA vaccine vector.[84] The MC had a higher and prolonged expression *in vitro* and *in vivo* in mice and an enhanced immunogenicity *in vivo*. In a challenge experiment, the MC vector conferred better protection and elicited a stronger antigen specific CD8+ T-cell response in a mouse model of listeriosis. CD8+ T-cells are an important component of the cellular immune response against any viral infection as they can recognize and eliminate infected cells. Thus, a strong CD8+ T-cell response could be of extra importance for HIV vaccination. HIV mainly targets CD4+ T-cells and by thus affecting the function of these cells, HIV impairs the maturation of CD8+ T-cell.[85] Wang *et al.* have used an MC expressing a HIV protein for vaccination purposes.[86] In their experiments, the humoral and cellular immune response when using an MC was twice that of a conventional plasmid. Notably, in their study they also saw that intramuscular injection of an MC with *in vivo* electroporation induced the strongest humoral and cellular

immune responses as compared to intramuscular injections alone, intradermal injections with or without electroporation, and high volume perfusion of the liver. This correlates with an enhanced distribution and expression from an MC vector encoding luciferase when delivered with electroporation.[87] Wang *et al.* also report that the improvement of expression when using electroporation was four time higher for an MC vector than for a conventional plasmid.

It is worth noting that for DNA vaccine purposes, the degree of supercoiling is important, as lower degrees of coiling has been shown to result in less efficient immunization *in vivo*.[88] Also the higher effective dose when using MC vectors is an important feature for vaccine vector because, as Cai *et al.* note in their expert review, the amount of DNA required per vaccine dose is large, and this in a limited volume of a few millilitres or less.[89] Taken together, these studies and observations indicate that the MC vector is a promising tool for DNA vaccination.

1.2.5.3 Minicircles for gene editing

Gene editing aims to introduce, replace or remove a given DNA sequence by the use of sequence specific nucleases. Dad *et al.* use an MC vector *in vitro* to deliver the genes encoding the programmable nuclease, both zinc finger nucleases and transcription activator-like effector nucleases.[90] The higher vector copy number and increased expression from the MC vector results in a higher mutation frequency. Delivering the nucleases via an MC vector is also better tolerated, with higher cell viability as compared to a normal plasmid.

1.2.5.4 Minicircles for induced pluripotency

Induced pluripotent stem cells (iPS cells) are a type of stem cell that can be generated from adult cells by introducing a specific set of pluripotency-associated genes. In a brief communication, Jia *et al.* reports the use of MC vectors for iPS-cell formation from adult human adipose stem cells.[91] They clone an MC with a single cassette of four reprogramming factors, OCT4, SOX2, LIN28 and NANOG, together with a GFP reporter gene. Using three repeated nucleofections, and selection of GFP-positive cells, they obtain colonies with embryonic stem cell morphologies and can show pluripotency and expression of embryonic germ layer genes in these cells. The efficiency was low compared to viral methods, but higher than when using plasmids. Furthermore, they show that the vector did not integrate into the genome, so the MC induced iPS cells will over time be free from foreign elements.

2 AIM

This thesis aims to optimize and evaluate the MC as a nonviral vector for gene therapy, with special focus on delivery and expression in heart and skeletal muscle and on the effects of using a small expression cassette encoding an snRNA.

The specific aims were:

1. To thoroughly investigate the effect of size on coiling patterns and relate these results to studies of transfection and expression efficiency, as well as to other properties such as the robustness of the vector.
2. To evaluate the use of the MC vector for therapeutic protein expression in heart and skeletal muscle.
3. To evaluate the use of the miMC for splice-switching therapies, especially in skeletal muscle.

3 METHODOLOGY

Numerous methods where used during this work. In this chapter, many of them will be discussed with some theoretical aspects.

3.1 CONSTRUCTS AND MC PRODUCTION

For paper I, the expression cassette containing a CMV promoter, the gene for hVEGF-165 and a rabbit β-globin polyadenylation site was cloned from the phVEGF165-vector into the p2ΦC31 MC producing plasmid between the two recombination sites. This was the first generation of the ΦC3 MC producing system developed by Chen *et al.*[10,23] In this system, the inducible genes for the recombinase and endonuclease are located on the parental plasmid and production could be performed in any recombination competent *E. coli* production strain. After over-night propagation of the transduced bacteria, addition of arabinose induces the pBAD-promoter controlling the *Streptomyces* phage ΦC31 integrase gene and the I-*Sec-I* endonuclease gene. Thus, recombination of the parental plasmid into the MC and miniplasmid was induced, as well as linearization of the miniplasmid by the endonuclease at the recognition sites located outside the recombination sites. This step took place at 32 ºC at increased pH to improve the efficiency of the enzymes. After five hours, the bacteria are harvested and the MC vector purified.

In papers II and III, the improved system with the specialized ZYCY10P3S2T bacteria strain was used.[25] The expression cassettes used was U7asLuc with antisense sequences targeting the mutated β-globin intron[92] (paper II) and U7asDys with a sequence targeting across the splicing branching point in intron 22 and the U1 binding region at the donor site in intron 23 in *mdx* dystrophin pre-mRNA[93] (paper III). The modified U7 gene, along with its natural promoter and 3' elements was cloned into the pMC parental plasmid between the two recombination sites. After transduction of the ZYCY10P3S2T bacteria, carrying the inducible genes for the ΦC3 integrase and the I-*Sec-I* endonuclease, miMCs were produced according to the method published by Kay *et al*.[25], a method much similar to the one described above.

All the MCs used in the papers included in this thesis were produced in small scale fermentations in a shaking incubator. In an experiment to produce MCs on a larger scale, we utilized a pilot plant scale fermentor. A fermentor is a type of bioreactor containing and controlling the culture of microorganisms, in our case bacteria. The fermentor contains a suitable growth medium and has been inoculated with a bacteria culture to start the fermentation process. It controls the temperature, pH, ventilation and agitation of the culture. It can also be set to control the conditions of nutrients by using a fed-batch system that adds

buffers continuously or at set time points during the fermentation process, e.g. for induction of recombination by addition of arabinose. Fermentation allows for a much higher density of bacteria and thus a higher yield of plasmid or MC DNA.

MC DNA was purified using commercial kits from QIAGEN (Hilden, Germany) according to protocol, with the modification that larger buffer volumes are needed for the lysis steps. After purification, quantity and quality was assayed using UV spectrophotometry at 260 nm and agarose gel electrophoresis.

For analysis of multimers in paper II, it was important to have only one isomer present in the final product. We therefore performed an additional gel separation and purification of the isomer of interest. MC DNA was separated by size on an agarose gel. Care was taken to avoid nicking the DNA by UV light or intercalating dye: the isomer's position in the gel was marked on stained edges of the gel and the corresponding band excised from the unstained gel. The monomer was then extracted from this gel fragment by QIAquick Gel Extraction Kit (QIAGEN) according to protocol and further purified by phenol:chloroform extraction. This is a cumbersome process and has a low yield, but was necessary for the experiments in paper II.

3.2 VISUALIZATION BY ATOMIC FORCE MICROSCOPY

Atomic Force Microscopy (AFM) is a type of scanning probe microscopy which measures the force between the tip of a probe and the sample, raster-scanning over a small area of the sample. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection, which when measured, can be translated into a spatial map of the surface. The resolution of the technique is high; fractions of nm.

Advances in the methodology of AFM enable reliable imaging of DNA in various structures and topologies.[94] The nucleic acid chain is mounted on the ultraflat surface of freshly cleaved mica. Unmodified mica has a negative surface charge; however modification of a mica surface with 3-aminopropyltriethoxy silane (AP-mica) leads to a reduction in surface potential. DNA binds to AP-mica primarily due to electrostatic interactions between the protonated amino groups of the AP-mica substrate and the negatively charged DNA backbone. The low surface charge density of AP-mica allows immobilized supercoiled DNA to retain its geometry. The overall geometry of supercoiled DNA is rather labile and sensitive to environmental conditions, such as salt. Correct salt composition in the buffers enables preservation of the morphology of the supercoiled DNA molecules.[95]

In paper II, we used AFM to visualize the coiling patterns of the miMC DNA and plasmids. A volume of 30µl DNA, diluted to 0.5 µg/µl in 50 mM NaCl, was added to the AP-mica surface, incubated for five minutes and then rinsed by water. When dry, the samples were measured in air in soft tapping mode with scan rate of 1Hz. A diamond-like carbon tip cantilever with a resonant frequency of 115-190 kHz was used on a NanoScope V Atomic Force Microscopy (Veeco®, Santa Barbara, CA) equipped with J piezoelectric scanner.

3.3 TESTING OF ROBUSTNESS BY PNEUMATIC DELIVERY

In paper II, we chose to evaluate the ability of plasmid constructs and MCs of different sizes to withstand the shearing forces induced by pneumatic delivery by ejecting the DNA through mouse skin using a Biojector (Bioject Medical Technologies Inc, CA, USA) and analyzed the integrity by gel electrophoresis.

The Biojector is a needle free system for drug delivery by forcing liquid medication through a tiny orifice that is held against the skin. Pneumatics and the small diameter of the orfice create an ultra-fine stream of high-pressure fluid that penetrates the skin without using a needle. The Biojector can deliver injections to a number of depths, with intramuscular injections being the deepest injection type, delivering the medication into the muscle tissue. Most vaccines are currently delivered to the intramuscular depth. The Biojector can also deliver subcutaneously to the adipose layer below the skin, as well as intradermally.

3.4 CELL LINES AND *IN VITRO* TRANSFECTION

The transfection methods used in this thesis aims to transport plasmid based DNA constructs through the cell membrane and cytoplasm into the nucleus, where it can be transcribed.

3.4.1 Lipofection

Lipofection is a chemical transfection method utilizing the fact that cationic lipids can form complexes with DNA, condensing and shielding the negatively charged DNA. These lipoplexes can fuse with the cell membrane to deliver the enclosed DNA. In paper II, a nanoparticle tracking analyzer (NanoSight, Amesbury, UK) was used to measure the size distribution of particles of DNA after complexation with Lipofectamine 2000 (ThermoScientific, MA, USA). The particles contained in the sample are visualised by the light they scatter when illuminated by laser light and the movement of the particles is correlated to the hydrodynamic radius and Brownian motion which enables calculation of size and concentration.

In paper I, FuGENE 6 (Roche, Mannheim, Germany) was used for lipofection of MC construct into HT-1080, a human fibrosarcoma cell line, with a reagent:DNA ratio of 3:1 as per protocol. Cells were seeded one day prior to transfection in an appropriate density so that they are 70-80% confluent at the day of transfection. Cells were processed 48 h later.

The cells used in paper II, HeLa Luc/705 cells, are stably expressing a Luciferase gene in which the coding sequence is interrupted by a mutated β-globin intron.[92] The cells were transfected using Lipofectamine 2000 (ThermoScientific) according to manufacturer's protocol. Briefly, cells were seeded one day prior to transfection in an appropriate density so that they are 70-80% confluent at the day of transfection. DNA was complexed using 2.3 µl of Lipofectamine 2000 per µg of DNA in opti-MEM (ThermoScientific). Cells were processed 24 h after transfection.

The mouse H2K cell line used in paper III is a myogenic cell line derived from the *mdx* transgenic mouse which carries a point mutation in exon 23 of the dystrophin gene.[96] The cells were transfected using Lipofectamine 2000 according to manufacturer´s protocol. Briefly, 2.3 µl of Lipofectamine 2000 was used per µg of DNA. Complexes were formed in 50 µl opti-MEM and added to cells grown in 450 µl full growth media. Cells were processed 48 h later.

3.4.2 Electroporation

In paper II, the HeLa Luc/705 cells were also transfected using electroporation. Electroporation is a physical transfection method which enables direct delivery over the cell membrane and into the nucleus. In paper II, we used it for comparison of physical and chemical MC delivery methods. Electroporation occurs when an external electric field is applied to the cell and the transmembrane potential exceeds a critical threshold.[97] This leads to a transient permeabilization of the plasma membrane, possibly through the creation of nanoscale pores, that allows DNA delivery into cells, but the mechanism is not entirely known.[98] In our work, we used the 100 µl tips of Neon system (ThermoScientific) in which cells are treated in suspension in a pipette tip chamber where the electric field is generated.

3.5 *IN VIVO* DELIVERY

3.5.1 Intramuscular injection

Direct injection of pDNA into muscle results in expression of the DNA in myofiber cells. Uptake and expression of numerous transgenes have been demonstrated in various species following intramuscular injections of naked DNA.[99] The expression peaks after a few days

and then drops to a lower but steady expression, and can be detected for a very long time in some cases.[100] However, the efficiency of pDNA gene transfer into skeletal muscle is low, with around one percent of the cells being transfected after an intramuscular injection.[101] Also, expression is only seen in a very restricted area of the muscle, usually along the needle track.

In both paper I and paper III intramuscular injection was chosen as it is a straightforward delivery method suitable for the proof of principle experiments performed in these papers. Material was delivered to the *tibialis anterior* (TA) of anesthetized mice by intramuscular injection of DNA in saline. In paper I, plasmid and MC was also injected into to the heart muscle. Anesthetized mice where tracheotomized, and through a left thoracotomy, the heart was exposed and injection was performed. Ten microliters of plasmid or MC solution in physiological salt buffer was administered.

3.5.2 Hydrodynamic delivery to liver

Hydrodynamic infusion is a well-documented gene transfer technique, known to give a high transfection efficiency of the liver in mice.[102] By rapid injection of a relatively large volume of DNA solution, a controlled hydrodynamic pressure arises in the capillaries which enhance cell permeability and allows for entry into the hepatocytes. The discontinuous sinusoidal capillaries in liver are sensitive to the hydrodynamic procedure, and the high pressure is thought to induce membrane pores in the hepatocytes which are responsible for intracellular DNA transfer. When the pressure is reduced, the pores close and the material is trapped inside the cell.[103,104]

In paper II, the functionality of a miMC for splice correction was tested with co-injection of a luciferase reporter construct and a splice correcting plasmid or miMC by hydrodynamic delivery to mouse liver. Mice were treated with a two ml hydrodynamic infusion through the tail vein while anesthetized, the volume being about 10 % of the mice body weight. This allowed us to assay the long term expression *in vivo*.

3.5.3 Hydrodynamic hind limb perfusion

The use of rapid injection of a large volume of pDNA solution through a peripheral blood vessel into a limb that is temporarily isolated from normal blood flow by a clamping induces a high pressure and thus increases gene delivery also in skeletal muscle cells.[105] The high pressure allows macromolecules to pass through transient pores in the normally very impermeable endothelium of the skeletal muscle. High-pressure transvenous limb perfusion has even been tested in a clinical trial on dystrophic patients and found safe.[106]

In paper III, hydrodynamic hind limb perfusion was performed in an attempt to enhance miMC delivery to skeletal muscle. High volume perfusion of *mdx* mouse hind limb was performed under general anaesthesia and as described by Gonin *et al.*[107] The mice were injected with miMC in one ml 0.9% saline, clamping was maintained for 10 min after injection.

3.5.4 Electroporation of skeletal muscle

Electrotransfer *in vivo* is based on injection of pDNA solution into the muscle, followed by the application of as series of electric pulses over the tissue. Electroporation has been shown to increase the gene transfer not only by cell permeabilization but also by a direct active effect on the DNA molecule, promoting DNA migration and cellular uptake. But as for *in vitro* electroporation, the exact mechanism of uptake is debated.[108] Pre-treating the muscle with bovine hyaluronidase has been shown to ameliorate the cell damage associated with electroporation.[109] This method is reported to result in a higher transduction than high pressure delivery methods, resulting in expression levels comparable to those achieved with viral vectors.[110] Even though electroporation is a rather invasive method for gene delivery, studies have shown that it is feasible to deliver nonviral vectors *in vivo* using electroporation even to the diaphragm of rats.[111]

In paper III, electroporation was performed to enhance the efficiency of intramuscular injection of miMC into skeletal muscle. Hyaluronidase treatment, injection and electroporation of mdx mice was performed according to protocol by Wells *et al.*[112]

3.6 RNA ANALYSIS

Gene expression and splice-switching can readily be measured by RNA analysis.

3.6.1 Northern blot

Northern blotting is a method to detect a specific RNA by separating a total RNA population according to size by gel electrophoresis, transferring the RNA to a membrane and label the desired RNA with a detectable antisense probe. The probe can be labelled with radioactivity or chemiluminescence, which allows for quantification of the signal. For northern blotting, as opposed to reverse transcription polymerase chain reaction (RT-PCR) discussed below, there is no amplification of the sequence before detection. Therefore, it can be difficult to detect low abundant RNA, but there is no risk for amplification errors affecting the measurements.

Northern blots were used in paper II to detect expression of U7snRNAs from miMC in mouse liver samples.

3.6.2 Reverse transcription polymerase chain reaction

RT-PCR utilizes the PCR method to amplify a sequence of interest, and can thus be used to detect lower levels of RNA. The sequence is first translated from RNA to cDNA using a reverse transcriptase. The cDNA can then be used as template in a PCR reaction. As any PCR reaction, it is sensitive to contaminations being amplified. Traditional PCR runs to end point, and the sample is then analyzed by gel electrophoresis. Thus, the method is semiquantitative, even though simultaneous amplification of an endogenous control RNA, which should not be affected by the treatment, allows for normalization and comparison between samples. Designing primers that span over several exons enables detection of splice variants of mRNA.

In paper II and paper III, semiquantitative RT-PCR was used to detect splice variants of the reporter Luciferase mRNA and of *mdx* dystrophin mRNA as it enables amplification with the same primer pairs for both splice variants followed by easy visualization by gel electrophoresis.

3.6.3 Quantitative RT-PCR

Quantitative RT-PCR (q-RT-PCR) is the most sensitive method to detect RNA. As in normal RT-PCR, the RNA is first translated to cDNA using a reverse transcriptase enzyme, and the product is then used as template in the quantitative PCR reaction (qPCR). Using SYBRGreen as a fluorescent intercalator allows for quantification of the number of double-stranded molecules in the reaction. Using a probe that binds to a specific target and which is labelled with a fluorophore and a quencher that is cleaved off when the polymerase transcribes the target enables a more specific detection. The intensity of the fluorescence increases as the PCR products accumulate, until it reaches a plateau. For measurements made in the early exponential phase of the PCR, the fluorescence level can be correlated to the amount of starting material.

q-RT-PCR was used in paper II and paper III to measure the expression levels of the U7snRNAs and compare different delivery methods and vector sizes. It is evident in paper II that the increased sensitivity of the q-RT-PCR can detect differences in U7snRNA levels even where the secondary effects on mRNA correction are too small to be detected with the semiquantitative RT-PCR.

3.7 PROTEIN DETECTION

3.7.1 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a method to quantify the amount of a certain protein using antibodies targeting an epitope on that protein. The sample is immobilized on a surface and the antibody is allowed to bind to the target protein. By coupling the antibody to an enzyme, either directly or via a secondary antibody, and subsequently add a colour-changing substrate for the enzyme, it is possible to measure the colour change and thereby the amount of protein. A standard curve of known amounts of protein is used to quantify the measurements.

In paper I, ELISA was used to straightforwardly quantify the amount of hVEGF after MC delivery in cell medium and tissue samples.

3.7.2 Immunoprecipitation and western blotting

Other antibody based method for protein capture and identification is immunoprecipitation and western blotting. In immunoprecipitation, the protein in question is fished out of the crude cell lysate using an antibody directed to a suitable epitope on that specific protein, and the antibody is in turned captured e.g. by protein G coated magnetic or agarose beads or using protein G spin columns. This allows for concentration of the protein before analysis. The captured protein can then be eluted and analyzed e.g. via western blotting, if possible using a primary antibody of another species than the one used for immunoprecipitation. As the protein amount has been concentrated, quantification is more difficult than when running western blots on crude cell lysate.

In western blotting, much like northern blotting for RNA, the sample is first separated by size on a gel and then transferred to a solid membrane and probed for identification. For western blotting, the probe is an antibody with a suitable epitope on the protein in question. A measurable signal is produced using and antibody coupled to a radioactive isotope, a fluorophore or an enzyme for chemiluminescent detection. A common problem with western blotting is unspecificity of the antibodies, which leads to difficulties interpreting the results. Unusual or unexpected bands can also be due to protease degradation, which produces bands at unexpected positions. It is important that the cell lysate is prepared in the cold, and that appropriate protease inhibitors are used.

3.7.3 Luciferase assay

Luciferase is an enzyme that converts the substrate luciferin to a light in an ATP-dependant reaction. The Luciferase gene is a common reporter gene, as quantification of protein amounts is very straightforward: Cells are lysed and after addition of the substrate, the emitted light can be measured by a luminometer. Luciferase can also be measured *in vivo* by injecting the substrate into a living mouse which can be imaged and the protein levels quantified. Since light excitation is not needed for luciferase bioluminescence, there is minimal disturbance by autofluorescence.

Gaussia Luciferase was used in paper I for normalization of transfection efficiency. In paper II, the gene for firefly luciferase with a mutated 705 human β-globin mini-intron sequence inserted was used as reporter in the splice-correcting systems.[92] Expression of the gene with the mutated intron results in a truncated non-functional Luciferase protein. Masking the mutated site with an antisense sequence, however, redirects the splicing machinery that concomitantly gives rise to productive splicing of the Luciferase pre-mRNA and transcription of a functional protein. Thus, the levels of luciferase signal indicated the levels of splice-correction by the U7snRNA expressed from the miMC.

3.7.4 Histology

Histology is a method to study the morphology, architecture and composition of cells or tissues using microscopy. It allows visualization of the distribution and localization of specific cellular components. The sample is fixated either chemically or by snap freezing, sectioned and the thin sections are mounted on glass slides. Fixation preserves the structural integrity of the cells and tissue. However, chemical fixation can damage the biological functionality of proteins, particularly enzymes, and can denature them to a certain extent. It is also harmful for RNA and DNA.

After sectioning and mounting, the sample is stained. Different staining techniques are available to preferentially colour components of interest in the sample. Hematoxylin and eosin staining colours the nuclei dark blue by hematoxylin while the cytoplasm and extracellular matrix are coloured in varying degrees by eosin. It is used to visualize the general structure and integrity of the sample. Immunohistochemistry and immunofluorescence uses antibodies to identify tissue components of interest by binding of a primary antibody to the protein of interest. In direct detection methods, the primary antibody is labelled, whereas in indirect methods a secondary antibody is used for detection. Indirect detection allows for binding of several secondary antibodies to the primary, and thus a

stronger signal. The detection antibody can be coupled to an enzyme, for immunohistochemistry, or a fluorophore, in immunofluorescence. The fluorophore can be imaged directly by a fluorescent microscope, but the enzyme needs addition of its substrate to create a detectable staining of the sample. The resolution of subcellular structures is not as good using enzymatic detection as with fluorescence methods but can be combined with electron microscopy, and the shelf life of the labelled specimen is virtually unlimited.

Histology is used in paper III to visualize restoration of dystrophin expression in *mdx* mouse muscle tissue after delivery of miMC by electroporation. Although levels of U7snRNA expression and exon skipping in the mRNA are important indicators of the effect of the miMC, only actual restoration of dystrophin protein will have a physiological effect on the muscle and immunohistology allows us to visualize it in the muscle cells.

4 RESULTS AND DISCUSSION

4.1 PRODUCTION OF THE MC VECTOR

The future use of minicircles in gene therapy demands an effective production and purification system with high yield. To produce the quantities needed for clinical studies and clinical use, the system must be robust and effective.

As we experienced an impure MC product using the first generation Chen system and their protocol for MC production, some effort was made to improve the system. We analysed a number of different bacteria strains, and found that using the O17 *E. coli* strain, known for its high protein production efficiency and kindly provided by Leif Isaksson at Stockholm University, resulted in improved recombination and degradation. We also tried co-fermenting the MC plasmid with a plasmid constitutively expressing arabinose importin, but it was difficult to balance copy number rate of the two plasmids in the bacteria.

In order to produce quantities required for clinical use, the process needs to be scaled up from shaking incubators to fermentor scale. A pilot plant scale fermentation experiment on the first generation system was performed with 5 litre medium in a Novaferm (Falkenberg, Sweden) fermentor. The parental plasmid was grown over night, and then induced by injection of arabinose and continued growth for four hours. One 5 litre fermentation batch gave 65 mg plasmid product but it had a very high content of unrecombined and undigested plasmids, containing only 15% MC. This initial study of scaling up the MC production system showed that a high yield of plasmid can be obtained, but the dosing of arabinose and probably also the pH needs to be fine tuned in order to reach higher purity in the final product. Also the growth temperature could influence the yield of MC. Gaspar *et al.* evaluate different conditions and conclude that an elevated temperature gives a better yield.[113] Even if this in our hands had little effect on a shaking incubator culture, it could be a parameter to investigate for larger scale fermentation.

When Kay *et al.* published their improved system with the specialized ZYCY10P3S2T bacteria bacteria strain,[25] we were able to use that instead. However we never tried this system in a fermentor. Using this system, we have seen that we can improve the yield of small MC vectors by adding L-arabinose at several time points during the induction phase of the fermentation, to mimic a fed-batch fermentation.

4.2 EFFECTS OF SIZE

4.2.1 Characterization of small MC constructs

During production of our smallest miMC construct of 650 bp in paper II, it was evident that multimerization was more common than for the larger constructs. This could be due to steric strain on the DNA, hindering the recombinase from joining the two recombination sites on same the parental plasmid. This strain could also affect the ability to supercoil the constructs. Thus, we studied coiling of MC of different sizes and plasmids via AFM. AFM allowed us to visualize the coiling pattern and characterize the number of turns in the constructs. However, the AFM images in paper II show clear supercoiling patterns. Also the smallest construct exhibited patterns that Fogg *et al.* associate with high writhe and increasingly negative supercoiling.[36] The number of turns is evidently related to the size of the MC but not strictly correlating 1:1 as the smaller one has predominantly two to three turns, and doubling the size gives four and five turns, and three time larger construct shows five to six turns. Also, as the constructs grow larger, the topology resembles the plasmid more. Thus, the strain could be higher on the smallest constructs. The *in vitro* transfection experiments showed that the smallest construct has a lower expression than the two times larger construct, irrespectively of if the larger construct is a dimer or if it contains irrelevant DNA to simply increase the size. This could be due to the relatively high degree of supercoil of the smallest construct, which could hinder the binding of necessary protein components. It is known that the curvature of the DNA affects the accessibility to *cis*-DNA elements by inducing different chromatin formations and that bending of the DNA changes the affinity of promoter binding proteins as the energy cost of bending contributes significantly to the overall binding affinity of the transcription factors for different sequences.[114-116]

For a small construct, a certain molar dose of expression cassettes corresponds to a lower weight amount of DNA than the same number of expression cassettes in a larger construct. This evidently affects transfection efficiency when using lipofection, as we showed in paper II. It is quite logical that a lower weight amount of DNA will result in fewer lipoplexes and thus reach fewer cells, albeit with a higher molar dose for each lipoplex. Our nanoparticle analysis data shows, as expected, that the size and number of lipoplexes are in the same range for the same amount of DNA, irrespectively of the content. This should be taken into consideration when using small constructs such as MC or especially miMC. Interestingly, Wu *et al.* see the opposite trend, and strangely enough reports the highest *in vitro* expression from the lower molar dose transfected without stuffer DNA, even in comparison to a seven times larger weight comparison dose.[78] They attribute this to lower toxicity due to lower amounts of

lipofectamine, and one hopes that the toxicity at the higher doses did not occlude any other results. When using electroporation however, it is evident from the results in paper II that a larger dose of DNA is not beneficial. As discussed before, using MCs allows for using a lower amount of DNA while still delivering a high number of expression cassettes. When Madeira *et al.* use electroporation to transfect neural stem cells, they see a higher amount of MC in the cells as compared to a plasmid, and attribute this partly to the smaller size of the MC vector.[76] The same group also reports a size effect for different plasmid constructs when electroporating stem cells, and discusses that this could be due to better uptake of the smaller construct.[117]

When using hydrodynamic delivery to liver, as in the *in vivo* studies in paper II, the material is forcibly pushed into the cells as the organ expands causing enlargement of liver fenestrae and generation of transient pores in the plasma membrane of hepatocytes of the liver. This method is thereby not dependant on formulation or active uptake. Thus, as expected, there was no effect of adding stuffer DNA to keep the total amount of DNA equal. In these *in vivo* experiments, there is a higher and more sustained expression from the miMC as compared to the plasmid. We used co-delivery of a reporter plasmid carrying the mutated luciferase gene for the splice correction, and assayed two different reporter constructs. In the first experiment, the reporter gene expression was driven by a CMV promoter, and there was a drop in luciferase signal after one week. When re-administrating the reporter construct, it was evident that the miMC still expressed the splice-correcting snRNA, as the luciferase signal returned, but the expression from the splice-correcting plasmid had been lost. In a follow up experiment, we used a reporter construct designed for a long term expression in liver. Throughout this *in vivo* experiment, all mice treated with splice-correcting constructs had a higher expression than the control mice, which only received the reporter plasmid together with an empty plasmid. One day after infusion, mice treated with miMC had about 20% higher luminescent signal as compared to the mice treated with splice-correcting plasmid. Over time, the signal from the plasmid declined more rapidly compared to the miMC and at the final time point the miMC treated mice had a several hundred percent higher signal than the plasmid group. Even here there is a gradual drop in luciferase signal over time also for the miMC treated mice, but it matches the curve from the control mice.

It is possible that an increased robustness of the miMC contributes to the higher expression and effect that we and others have seen repeatedly using the hydrodynamic delivery method.[10,15,57,118] In paper II, we demonstrate a higher robustness of our small miMC constructs as compared to the larger and to a conventional plasmid. The fate of the miMC or

pDNA after delivery through mouse skin using pneumatics was assayed. As discussed earlier, pneumatics is known to cause shearing of DNA. The miMC, however, seems to be able to withstand these shearing forces to a much greater extent than the plasmid. What is perhaps most noteworthy is that the plasmid DNA is partially destroyed and does no longer meet the FDA requirements of an 80% supercoiled fraction. The MC construct fared much better, with the nicked fraction being ten times lower than for the full length plasmid construct. This shearing of the plasmid may not only increase the risk of insertional mutagenesis but also results in a lower effective dose, since linearized and open circular topologies have been shown to have lower transfection efficiencies and expression.[88,119] Our results are corroborated by the experiments on serum stability and ability to withstand shearing forces associated with sonication and nebulization discussed earlier.

4.2.2 Construction of a small MC for vaccine purposes

The increased robustness has implications for DNA vaccination, since pneumatics is a method used for delivery of DNA vaccine. As it could be beneficial to use a small expression cassette, short immunogenic peptides are of interest. A candidate short peptide is the T20 peptide, also known as enfuvirtide. It is the first HIV-1 fusion inhibitor peptide-based drug to be approved for treatment of AIDS patients, under the brand name Fuzeon.[120,121] T20 consists of 36 aa mimicking the C-helix sequence of the HIV-1 envelope transmembrane domain gp41. T20 blocks the early step in the virus life cycle prior to reverse transcription and can prevent *de novo* infection and cell-to-cell virus transmission. Although it has been previously shown that gp41 antibodies do not impair T20 antiviral effect in the treatment of HIV, little is known about the immunoprotective properties of T20 itself.[122] The T20 peptide can be recognized as an HIV antigen since it contains a broadly reactive epitope, the so called 2F5 epitope.[123] This immunoreactive activity of the T20 peptide would results from the fact that the peptide mimics residues 643-678 of the HIV-1 glycoprotein gp41. Thus, any antibodies or CTL reactivity formed against the T20 peptide will also find epitopes of this critical region of the HIV-1 transmembrane protein. Natural antibody responses to gp41 and the entire gp120 occur soon after HIV-infection, and can result in neutralizing antibodies.

In an ongoing study by Hinkula *et al.*, the T20 peptide has been evaluated as a part of an HIV protein/DNA vaccine. Figure 3 shows that administering the T20 peptide together with the HIV-1 DNA vaccine constructs enhances anti-Env immunogenicity.

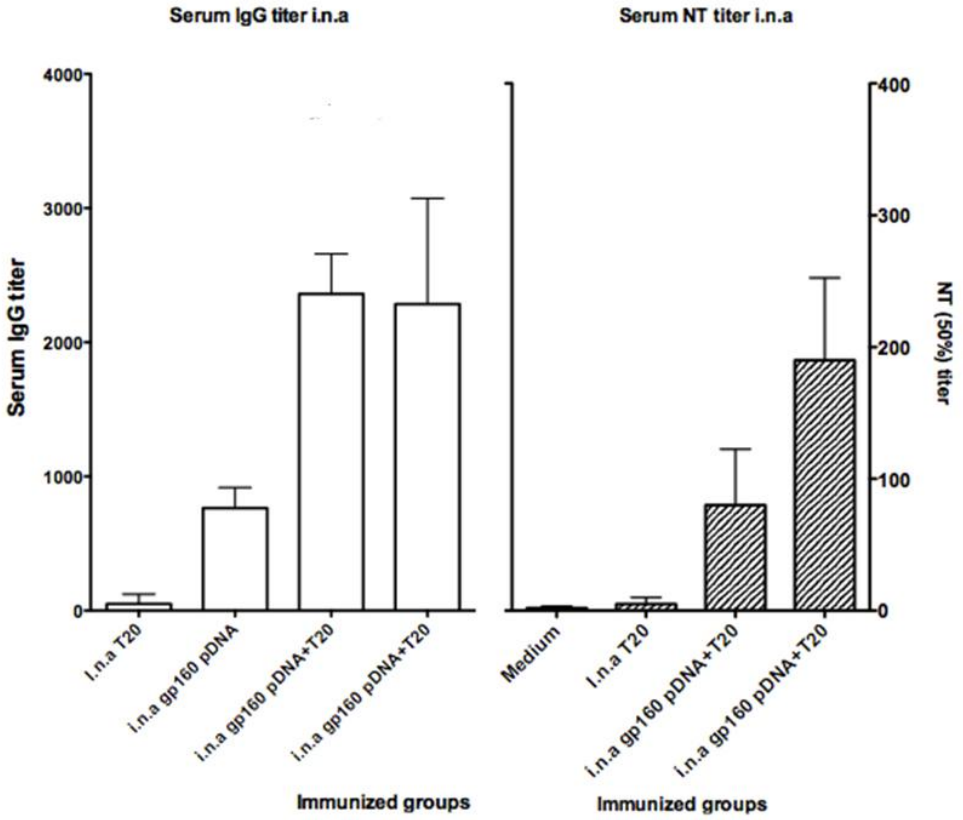

Figure 3. A comparison of intranasal (i.n.a) deposition of medium, T20 peptide alone, DNA vaccine plasmid gp160 alone or T20 mixed with plasmid gp160. T20 peptide mixed with the plasmid appears to enhance both serum IgG titres of binding to gp160 as well as neutralizing antibodies (NT) to HIV-1 subtype B. Hinkula *et al*, manuscript.

Therefore, we have cloned the T20 coding sequence and an expression cassette previously used in HIV inhibition studies[124] into pMC-vector for MC-production. The construct containing the T20 coding sequence was donated by Drs K. Cheng and D. Goldenberg, Immunomedics, see also Chang *et al.*[125] The resulting MC was 1.1 kbp, i.e. less than one third of a corresponding conventional plasmid.

The MC was transfected into HeLa, Hek and U2OS cells using Lipofectamine 2000 according to manufacturer's protocol and 24 µg to a 10 cm plate. Figure 4 clearly shows the expression of ssT20 *in vitro* 48 hours after transfection, both in an immunoprecipitated sample and in crude cell lysate. The prominent bands of larger molecular weight are the heavy and light chain of the antibody used in immunoprecipitation. As the same antibody was used for the western blot, also these were detected by the secondary antibody. Evidently, it thus is feasible to produce immunogenic peptides as small as 36 aa transgenically in human cells. T20 has previously been expressed as a multimeric fusion protein, which was shown to

have an antiviral effect *in vitro*.[126] Dervillez *et al.* also expressed the C46 peptide, a T20 derived peptide, alone and showed expression but no secretion of the peptide. However, they did not study the immunogenicity of their peptide construct. For immunostimulation, secretion is not crucial, since the transfected cells will present the antigen on MHC molecules. T20 can thereby be recognized as foreign and trigger an immune response. Here, we show that the MC vector can express the monomeric T20 peptide in human cell lines, and the protein was detectable by western blotting using the neutralizing human antibody 2F5. Further studies will show the immunogenic properties of the T20 peptide expressed from an MC vector *in vivo*.

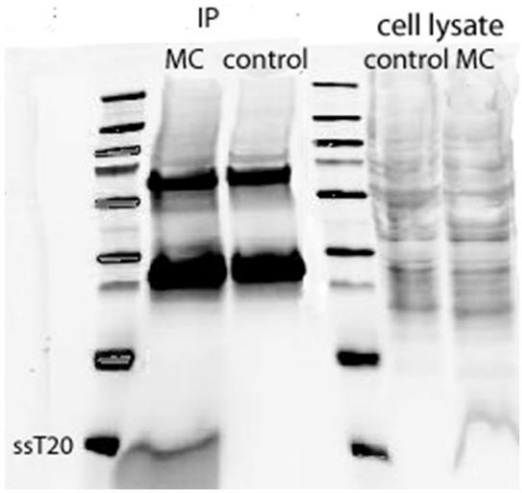

Figure 4. Expression of T20 peptide from MC vector *in vitro*. A western blot on immunoprecipitated (IP) peptide and cell lysate from HeLa cells transfected with MC or untransfected (control).

4.3 PROTEIN EXPRESSION FROM MC AND PLASMID VECTOR IN MUSCLE

Treatment of ischemic tissue with growth factors such as VEGF had shown promising results in animal experiments but clinical trials were disappointing.[127] This was in part attributed to the short half-life of the growth factor *in vivo*. Transgenic expression in the tissue is a possible and interesting solution to this problem. However, problems preventing successful clinical application of gene therapy for cardiac diseases can primarily be related to inefficient gene transfer, host immune responses, and the lack of sustainable therapeutic transgene expression. As the MC vector addresses two of these problems, we designed a study in paper I where we compare the expression of hVEGF protein from an MC vector to plasmid constructs. In this study, three different full-length plasmid constructs were used in parallel to

the MC vector. A plasmid vector, which had been previously used in clinical studies, phVEGF165,[128,129] was compared to the commercially available pVAX1 vector (Invitrogen, CA, USA). The third full-length plasmid construct investigated was the MC-parental plasmid. As this study was performed using the first generation Chen system, the parental plasmid was rather large, over 9 kbp. It was evident from the *in vitro* studies in a human cell line that the large size of the parental plasmid impairs the efficiency of the vector. However, the phVEGF165 vector was much more efficient than the pVAX, despite being almost twice the size. The MC vector, containing the same cassette as the phVEGF165 construct but encompassing only 2.4 kbp, surpassed even the highly expressing phVEGF165 construct. Thus, for the *in vivo* experiments, only these two constructs where used. The constructs were compared in heart and in skeletal muscle in equimolar and equal weight doses, delivered by direct intramuscular injection of naked DNA. In heart, the expression is equal when the same molar dose is used, both after 24 hours and after seven days. When injecting the same weight dose of MC and plasmid, the hVEGF expression is higher in the MC treated hearts due to the increased dose of therapeutic DNA. In skeletal muscle, the MC vector has a slightly elevated expression when compared to an equimolar dose of plasmid, but only an equal weight dose gives a significant difference after seven days. It is possible that the difference in expression would become more pronounced over time. However, as the method used to detect hVEGF *in vivo* demands sacrifice of the animals, hVEGF is not the optimal reporter to characterize expression of MC as compared to plasmid over time. Lijkwan *et al.* use luciferase as a reporter gene to compare expression from equimolar treatment with MC and a plasmid construct in mouse skeletal muscle.[64] They report significantly higher expression from the MC for up to 28 days. However, there is barely any expression from the plasmid at the dose given. Huang *et al.* do a similar comparison in murine heart.[61] In heart, the MC had a higher expression than the plasmid and although the expression did drop, luciferase was detectable for 90 days. When using the therapeutically more relevant HIF-1α protein, Huang *et al.* were able to detect significant expression from the MC vector 14 days after treatment, with levels more than twice those of the plasmid construct.

A difference between the studies in paper I and those of Lijkwan *et al.* and Huang *et al.*, apart from different reporter genes, is that they both utilized the ubiquitin promoter to drive the protein expression, whereas our constructs contain the CMV promoter. The importance of promoter is highlighted in another paper comparing MC and plasmid for hVEGF expression, where Chang *et al.* studies three different promoters, among them CMV, and report quite different effects *in vitro* and *in vivo* two days after treatment.[59] Gill *et al.* investigate different viral promoters, such as CMV, and compare their gene expression profile to the human

ubiquitin C and elongation factor 1alpha promoters in lung.[130] The ubiquitin promoter had a far more long-term expression than the CMV promoter, which drops to 10% of initial expression level after two weeks. This drop in expression was not correlated with any loss of vector. Riu *et al.* aim to shed light on the mechanism of plasmid silencing by studying histone modification in plasmids and MC vectors, and also compare different promoters in liver.[19] When studying the Rous sarcoma virus promoter, a prominent difference in the patterns between the MC and the full-length plasmid was observed, indicating a formation and spreading of heterochromatin in the plasmid. The pattern of histone modifications on the MC DNA was consistent with euchromatin, which correlates with the prolonged transgene expression in liver. These differences in histone modifications between MC and plasmid were not seen after 24 hours or seven days, only after several weeks. This is of interest when looking at paper I and the Chang *et al.* results; chromatin changes would not yet affect the expression levels at the time points studied. When Riu *et al.* characterize the ubiquitin C promoter, the chromatin patterns in the plasmid and MC vectors are more alike also over time, and so is the expression profile. They hypothesize that this promoter contains several recognition sites associated with transcription factors that are able to maintain the original euchromatin histone modification patterns. That the design of the gene expression cassette highly influences the levels of gene expression is also evident in paper I, in the comparison between the pVAX and the phVEGFI165 vectors which both contain the CMV promoter but with different enhancer elements. Regarding vector type, the smaller size, the absence of antibiotics resistance gene and a lower CpG content make the MC a useful and appealing alternative for future cardiovascular gene therapy treatments even when the expression levels per construct is comparable to that of the plasmid.

4.4 SNRNA EXPRESSION FROM MC IN MUSCLE

In paper II, we demonstrated that the MC can be used as a vector for delivery of a splice correcting cassette, and that it had a higher and more robust effect than a plasmid construct in liver. However, the study utilized an artificial luciferase system and an experimental setup using co-injections of reporter plasmid and splice-correcting construct *in vivo.* DMD is a more clinically relevant system, as many mutations causing the loss of dystrophin in DMD can potentially be rescued by inducing skipping of mutated exons in the pre-mRNA to restore the reading frame. The induced exon-skipping leads to production of a shortened, but functional, protein and the phenotype will be similar to Becker Muscular Dystrophy – a much less severe illness than DMD.[131]

In paper III, the miMC was assayed as vector for a splice-switching snRNA targeting the in the *mdx* mouse model. The *mdx* mouse has a point mutation in exon 23 of the mouse dystrophin gene, which introduces a premature stop codon that causes the absence of full-length dystrophin. A miMC containing a U7 splice correcting cassette previously used by Goyenvalle *et al.*[93], and which induces skipping of exon 23 was cloned. The miMC was tested *in vitro* in mouse H2K *mdx* muscle cells as well as in the live animal. *In vitro*, the miMC outperformed a plasmid construct carrying the same cassette by far. Already at the lowest dose, 0.5 µg miMC, the miMC induced splice-switching, whereas using the same molar dose of plasmid had no effect. In a comparison using 4 µg miMC and the same weight amount of plasmid, the miMC induced ten times higher correction than the plasmid. It is possible that a plasmid dose corresponding to the molar amount of cassettes in 4 µg miMC could induce decent splicing, but the levels would most certainly be toxic for the cells, as the plasmid is nearly six times larger than the miMC. In the *in vivo* experiments, the miMC was first delivered by intramuscular injections into the TA of the *mdx* mice. After two weeks, the miMC had induced a significant increase in dystrophin levels in the treated muscle. These experiments show that it is feasible to use the miMC as a vector for inducing splice-switching of dystrophin mRNA and restoration of protein production in muscle.

However, as the levels of dystrophin were modest after naked injection, we also assayed two different physical methods to increase miMC delivery. High volume perfusion of *mdx* mouse hind limb did not induce any splice-switching that was detectable with end point RT-PCR four weeks after treatment. Electroporation of the muscle after injection of the miMC did induce expression of the splice-switching snRNA quantifiable by qRT-PCR, and rescue of protein production detectable by immunohistology two weeks after treatment. However, the protein levels are in pair with the amounts achieved by intramuscular injection alone. These two results both highlight the question of timing of the analysis. Tennyson *et al*. report that the dystrophin is a low abundance transcript with 5-10 mRNA copies per cell, and that the ratio of nascent to mature dystrophin transcript indicates that dystrophin synthesis may not be at steady state in the adult skeletal muscle.[132] Spitali *et al.* describe that the mRNA transcript levels are not constant across the gene, perhaps because of incomplete transcription.[133] This can influence the amount of mRNA available for splice-switching and subsequent protein production. It is thus possible that two weeks is too short to see the effect of an increased delivery. This could explain our data as well as the observation from Goyenvalle *et al.* when they study AAV-vectors for delivery of the U7 construct inducing splice-switching of dystrophin. They describe little effect on dystrophin levels after two weeks, but a pronounced skipping and protein restoration after four to eight weeks.[93] However, they also assay the

expression of U7snRNA by end point PCR, and although no quantitative values are reported they show expression from the constructs already after two weeks. This is in accordance with our results, and indicates that two weeks is not enough time for the splice-switched mRNA and rescued dystrophin protein to accumulate. On the other hand, the same group show in another study that if the dystrophin rescue is suboptimal, a splice-switching AAV vector can be lost already after three weeks.[134] They attribute this to the cycles of degeneration and regeneration of muscles associated with DMD. This suggests that even if the miMC was functional and had a slight effect it could have been lost at the time of assay four weeks after treatment if the delivery using high volume perfusion was poor. These results highlight the need for efficient vector delivery. Future experiments will tell if delivery miMC with electroporation will result in high enough expression and protein rescue to overcome the loss of transfected cells through degeneration.

The MC could be a more optimal vector than the conventional plasmid for delivery with electroporation. As discussed before it seems that the CpG content of the vector affects the tissue response to the electroporation in muscle.[18] As the MC is devoid of bacterial sequences, the CpG content will be reduced compared to a full length plasmid carrying the same cassette. A lower amount of CpGs would lead to a lower inflammatory response to the electroporation treatment, and could thus result in a more stable gene expression. The reduced size resulting from the removal of bacterial sequences could also enhance the delivery, as studies have shown that the efficiency of electroporation correlates with the size of the plasmid DNA constructs, with large size hampering effective gene transfer.[32] Chabot *et al.* have studied the mechanism of electroporation of MC *in vitro* using a GFP reporter gene, and note that the MC had an increased interaction with the cell membrane, as compared to a conventional plasmid vector, and hypothesises that this could be due to the smaller size of the MC vector. [87] Their study also reports a higher and more sustained expression *in vivo* with the MC vector after electroporation to muscle. Thus, as it is even smaller than the MC vector used by Chabot *et al.*, there is hope for the miMC.

5 CONCLUSION AND FUTURE PERSPECTIVES

The work presented in this thesis has aimed to investigate the possibilities to use the MC as a vector for gene transfer into muscle. I have studied how the size of the MC vector affects transfer and expression efficiency, as well as other aspects such as robustness. The vector has been used for expression of both a therapeutic protein and a splice-switching snRNA in muscle.

From the experimental data which are the basis for this thesis, as well as from the related literature, it is evident that the MC has certain advantages as well as limitations. Table 2, adapted from Stenler *et al.*,[135] summarizes some of the published advantages of the MC vector, with exemplifying references. Due to its smaller size and lack of untranscribed bacterial sequences, it has a prolonged and more stable expression than the conventional plasmid. Furthermore, the vector does not contain any antibiotics resistance genes, which limits the risk of spreading of these genes. Optimizing the regulatory sequences in the expression cassette will affect the expression profile and can increase the effect even further. Any further loss of expression over time can be overcome by repeated treatment, which has been shown to be quite well tolerated when using the MC in mouse skeletal muscle.[61] The smaller size also allows for a higher effective dose, and has been shown to be beneficial in gene transfer, both when using chemical and physical methods.

Effect of MC	Assay	Reference
Efficient immune response	HIV-1 DNA vaccination in mouse muscle	Wang et al. 2014
Prolonged expression	Human factor IX and alpha1-antitrypsin in mouse liver	Chen et al. 2003
Enhanced serum stability	Incubation in human serum	Zhao et al. 2010
Increased shearing resistance	Sonication and nebulization	Cantanese et al. 2012
Robust supercoiled fraction	Biojection through mouse hide	Stenler et al. 2014

Table 2. MC versus plasmid in biological assays. Comparison of different properties of the MC and plasmid, with selected references. Modified from Stenler *et al.*[135]

5.1 DELIVERY METHODS

Efficient delivery remains one of the main difficulties when using nonviral vectors, and the MC is no exception. Our work and others indicate that electroporation can be a suitable method to enhance delivery of the MC into muscle. The vector itself could be further optimized for muscular delivery by electroporation. A sequence that has been shown to increase gene expression in smooth muscle after electroporation is a region of the smooth muscle γ-actin promoter.[136] It contains a tissue specific transcription factor binding site which drives nuclear accumulation of the vector. Hydrodynamic perfusion of hind limb is another method that has been used successfully by others for delivery to muscle of viral vectors and

ASO as well as for plasmids, although it did not show increased gene delivery in our hands. However, neither hydrodynamics nor electroporation would be a realistic tool for delivery to every muscle in the body, as would be needed for efficient treatment of many muscular diseases, such as DMD. These methods would be more suitable for delivery of vectors for treatment of e.g. cardiovascular diseases, such as cardiac or peripheral limb ischemia, where it would be possible to target the affected muscles or limbs only. An efficiently transfected muscle could in principle also be used as production site for a secreted therapeutic protein that could have its target elsewhere in the body. As the skeletal muscle cells are not dividing, the vector would be present in the tissue for a long time.

Hydrodynamics has also been used in several studies for delivery of MC to the liver of mice, and has shown a higher and more robust expression as compared to plasmid. However, for the method to be safe for humans there is a need for optimization of e.g. injection volumes and techniques for clamping of vessels to affect only parts of the liver. Ultrasound is another physical method that has been used for gene delivery of MC vectors, targeting the salivary glands of mice.[137] The study reports that the MC vector had an increased expression of luciferase as compared to a conventional plasmid. Furthermore, the authors analyze the proteomic profile of the gland and see that plasmid caused a change of protein content, especially in pathways associated with immunity, cellular stress, and morphogenesis. This was seen to a much smaller extent when using the MC as gene delivery vector. The plasmid backbone induces an immunological response similar to that seen when using viral vectors. Thus the MC vector does not only enable a higher expression, but is also less toxic than a full-length plasmid vector.

Chemical delivery is another approach to achieve more efficient delivery, which is sorely needed for full body muscular treatments using nonviral vectors. Chemical delivery refers to the use of different carrier lipids or peptides to condense and protect the DNA and enhance the cellular uptake. There are numerous studies on optimizing the chemistry of the carrier, as well as coupling it to signal moieties for improved uptake and intracellular traffic. These can be nuclear localization signals or cell binding ligands. Such ligands can also promote targeting of the vector to a given organ. The use of cell penetrating peptides, covalently or non-covalently attached to the cargo, can facilitate entry into cells. As an example of how these methods can be combined, Ko *et al.* report that targeted gene delivery to ischemic myocardium by intravenous injection in rats is enhanced when pDNA and lipid complexes are modified with cell penetrating peptides and a monoclonal antibody specific for cardiac myosin.[138]

5.2 DNA VACCINATION

With the limitations of delivery efficiency, the MC might be more suitable for use when only local expression is needed. One such situation is DNA vaccination, where the antigen is commonly delivered to a small area intradermally or intramusculary. Electroporation has been shown to be an efficient delivery method for eliciting strong immune responses in humans.[139] Both the increased DNA uptake and the local tissue damage acting as an adjuvant is believed to improve the immunization. As discussed previously, the MC could be a more optimal vector for delivery with electroporation than a conventional plasmid due to its smaller size. Another benefit of the smaller size is the increased therapeutic dose per µg DNA when using MCs as compared to normal plasmids. This is of importance as high local transgene expression is crucial for a good immune response. However, one feature of plasmid DNA itself that is thought to induce an immune response is the unmetylated CpGs discussed earlier. It has been shown that a CpG rich sequence can act as adjuvant for vaccines. An MC vector for DNA vaccination will have less CpGs than a conventional plasmid, as it is shorter. However, optimized CpG sequences could be cloned into to the MC cassette. Some studies, e.g. Coban *et al*, have shown that inclusion of certain optimized CpG sequences in a plasmid enhances the immune response in mice.[140] These sequences are only between 30 and 50 bp so the resulting MC would still be considerably smaller than a conventional plasmid. Alternatively, the MC could be delivered together with synthetic CpG oligonucleotides. Co-administering CpG oligonucleotides with a variety of vaccine agents has improved the humoral and cellular immune responses.[141] For DNA vaccine purposes, it could very well be an advantage to not have the adjuvant covalently attached to the DNA sequence from which the antigen is to be expressed.

5.3 *EX VIVO* THERAPIES

Ex vivo therapy can be seen as an extreme example of local therapeutic gene expression. *Ex vivo* treatment aims to remove cells from a patient, modify them in the laboratory and then return the modified cells to the patient where they affect the disease. In this case, it could be beneficial to be able to use a high therapeutic dose of a non-integrating vector devoid of any resistance genes, which could be transferred back to the patient. Evans and Hyde review several gene therapy approaches to regenerate the musculoskeletal system, among them *ex vivo* strategies mainly using viral vectors.[142] Usas *et al.*[143] study muscle derived stem cells and the possibility to modify them *ex vivo*, e.g. using retroviral vectors to enhance bone formation; this naturally with a risk of introducing cells harbouring an insertional mutation. They also modified the stem cells with a combination of growth factors, such as VEGF,

which enhanced bone generation. When considering using growth factors, the prolonged, but still in the end transient, expression associated with episomal vectors such as the MC could be beneficial, as growth factors are known to be involved in several steps of cancer development.[144] Indeed, Sheyn *et al.* use adipose tissue-derived stem cells modified *ex vivo* with a plasmid vector to enhance bone formation and spinal fusion.[145] They conclude that the nonviral gene delivery method used to genetically engineer the stem cells *ex vivo* is safe and transient, limiting overexpression of the osteogenic gene to a period of a few weeks. Also the MC vector has been used *ex* vivo in human adipose-derived stromal cells, to induce a transient overexpression of the Bcl-2 prosurvival protein.[75] This enhanced cell survival and bone formation upon implantation into a mouse model, and expression was seen for up to four weeks.

5.4 SUMMARY

The MC is one of the safest vectors available for gene therapy. It demonstrates a more stable expression as compared to conventional plasmids, and its smaller size allows for a high dose and possibly more efficient transfection. It can be used in muscle both to express therapeutic proteins and for RNA therapeutics. Even though the expression is prolonged as compared to conventional plasmids, and can be further improved by designing the regulatory elements in the vector, the expression will be less stable than for integrating vectors. For some uses, transient expression is not necessarily a disadvantage. However, for use where systemic treatment is need, delivery must be enhanced by the use of chemical or physical transfection methods. Thus, the vector might be more suitable for treatments where only local expression is needed, such as single organ treatment, DNA vaccination or *ex vivo* treatment.

6 ACKNOWLEDGEMENTS

The work for this thesis has been a long, winding and enlightening road, and I would like to express my sincere thanks to all the people who have walked alongside me for a while and supported me on my journey towards the PhD. Here I will try to credit some of you by name.

My main supervisor Dr **Pontus Blomberg** who once upon a time left his contact information on the handouts for a lecture for master students at Uppsala University, and thus enabled one of the students to email him for a master thesis project, and there began the journey. Thank you for your never-ending support and belief in me and in the project.

My main co-supervisor Dr **Karin Lundin**, for helping a new student find her way in the lab and her feet in a new environment. You are always ready to drop whatever you are doing to give a helping hand or a piece of advice to your students. Thank you also for allowing your students more freedom as they need it to grow.

My other co-supervisor Professor **Edvard Smith**, for giving me a place in your research group and continuously advising me on how to design studies and perform experiments and develop into an independent researcher. I have learnt much about science and have had a very inspiring time in your group.

Co-authors in the group, **Joel Nordin**, **Oscar Wiklander** and Dr **Samir EL Andaloussi**, it has been a privilege to work with you. Thank you for all the effort you have put into the miMC experiments, and for fun company both in the lab, over lunch and in the gym.

Co-authors in the heart lab, **Agneta Nilsson,** Professor **Christer Sylvén** and Dr **Oscar Simonsson**, thank you for all your help on designing and performing the MC-VEGF experiments.

Professor **Britta Wahren** at the Department of Microbiology, Tumor and Cell Biology, thank you for always taking an interest in the potential of the MC as a vaccine construct and for supporting me in that line of research. Thank you also for yours and Doctor **David Hallengärd's** work on the robustness of small MC constructs.

The people at the Department of Surface Chemistry, Royal Institute of Technology: Professor **Mark Rutland** and **Maria Badal-Tejedor**, thank you for introducing me to the possibilities of AFM and providing me with stunning images of my small DNA circles, one of them illustrating the front page of this very thesis.

I also want to thank the other members of the MCG group, old and new. Thank you for all the inspiriting discussions, for sharing your knowledge, for a helping hand when needed as well as for laughs and lunches. Dear fellow spring term 2015 defendants, **Burcu**, thanks for your easy smile and fun times and **Dara**, thanks for your kindness and readily sharing your western blot expertise. Best of luck with your impending defences and the life thereafter! **Janne**, thanks for enjoyable coffee talks and good discussions on splice switching and your help with understanding the magic of northern blots. Good luck on your new endeavours.

Many thanks to **Emelie** and **Lotta** for teaching me much about PCR and how to keep a lab in order. Lotta, may your new career as a massage therapist be as rewarding as your old one in the lab. Western blot team **Abdulrahman**, **Beston** and **Manuela**, thanks for readily sharing your expertise and experience on immunoprecipitation and protein detection. **Anna,** thanks for your company and direct humor. **Eman**, it has been a real pleasure sharing the lab bench with you. Fellow PhD students and master students: **Dhanu**, **Cristina**, **Giulia**, **Helena**, **Niels**, **Olof**, **Quin**, **Sophie**, **Sylvain**, **Vladimir** and **Yue**, thank you for your friendship and help, and best of luck with all your projects.

Colleagues at Vecura, **Charlotte**, **Inger**, **Jenny**, **Heidi**, **Katrin**, **Kristina** and **Mevlida**. Thank you for housing me and sharing your fikas, lab space and knowledge, and for generally being such nice persons to be around.

Thanks to the KFC personnel, **Hanna**, **Kathrin** and **Merja**, for always being so forthcoming and supporting in all administrative matters.

I wish to express my sincere gratitude to Dr **Mark Kay** at Stanford and Dr **Zhi-Ying Cheng** now at Shenzhen Advanced Technology Institute for allowing me the opportunity to work on their minicircle system, both the first and the second generation, and especially to Dr Cheng for his patient advice when I needed it. It was a pleasure to publish this thesis' first paper with you.

I am very happy that I, thanks to the **European Consortium in Science and Technology**, was given the opportunity to visit Professor **Matthew Wood** at Oxford University and Professor **Aurélie Goyenvalle** in her lab in Versailles, as well as working some weeks in Professor **Dominic Wells'** group at the RVC in London. I am also very grateful for you having so many lovely and helpful members in your research groups. Among them I want to specially thank **Susan Hammond** for her work on the dystrophin miMC and for housing me when I was visiting.

My sincere thanks to Drs **Ken Cheng** and **David Goldenberg** at Immunomedics, for their kind donation of the T20 coding sequence and thus enabling my research on the MC-T20 for DNA vaccination.

Furthermore, I would not be where I am today without my dear friends outside the KI. **Annica**, **Annika**, **Emma**, **Frida**, **Ingela**, **Johan**, **Johan**, **Magnus**, **Mathias**, **Lotta**, **Sara** and **Ylva**, thank you for all the adventures of many different kinds that we have had together. I sincerely hope there will be many more!

Finally, my most heartfelt thanks to my parents, **Anita Stenler** and **Claes Pagoldh**, for bringing me up in an inquisitive and ambitious atmosphere, while constantly being there with your unquestioning support and encouragement. And for providing me with the best sister one could ever ask for, **Teresa Stenler**. Thanks, sis, for always being fun and/or serious depending on what I need. And last, but first in my heart, thank you **Jon Back**, for being the companion of my days. To great things to come.

7 REFERENCES

1. Vectors used in Gene Therapy. Gene therapy Clinical Trials Worldwide 2015; http://www.abedia.com/wiley/vectors.php. Accessed 19 March, 2015.

2. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003;302(5644):415-419.

3. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. The Journal of clinical investigation. 2008;118(9):3132-3142.

4. Howe SJ, Mansour MR, Schwarzwaelder K, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. The Journal of clinical investigation. 2008;118(9):3143-3150.

5. Gill DR, Pringle IA, Hyde SC. Progress and prospects: the design and production of plasmid vectors. Gene Ther. 2009;16(2):165-171.

6. Wooddell CI, Reppen T, Wolff JA, Herweijer H. Sustained liver-specific transgene expression from the albumin promoter in mice following hydrodynamic plasmid DNA delivery. J Gene Med. 2008;10(5):551-563.

7. Brown BD, Gentner B, Cantore A, et al. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol. 2007;25(12):1457-1467.

8. Jenke AC, Stehle IM, Herrmann F, et al. Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome. Proc Natl Acad Sci U S A. 2004;101(31):11322-11327.

9. Wong SP, Argyros O, Harbottle RP. Sustained expression from DNA vectors. Advances in genetics. 2015;89:113-152.

10. Chen ZY, He CY, Ehrhardt A, Kay MA. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. Mol Ther. 2003;8(3):495-500.

11. Chen ZY, He CY, Meuse L, Kay MA. Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo. Gene Ther. 2004;11(10):856-864.

12. Tan Y, Li S, Pitt BR, Huang L. The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression in vivo. Hum Gene Ther. 1999;10(13):2153-2161.

13. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. Hum Gene Ther. 1997;8(17):2019-2029.

14. Hyde SC, Pringle IA, Abdullah S, et al. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. Nat Biotechnol. 2008;26(5):549-551.

15. Chen ZY, Riu E, He CY, Xu H, Kay MA. Silencing of episomal transgene expression in liver by plasmid bacterial backbone DNA is independent of CpG methylation. Mol Ther. 2008;16(3):548-556.

16. Kosovac D, Wild J, Ludwig C, Meissner S, Bauer AP, Wagner R. Minimal doses of a sequence-optimized transgene mediate high-level and long-term EPO expression in vivo: challenging CpG-free gene design. Gene Ther. 2011;18(2):189-198.

17. Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacological reviews. 2006;58(1):32-45.

18. Mann CJ, Anguela XM, Montane J, et al. Molecular signature of the immune and tissue response to non-coding plasmid DNA in skeletal muscle after electrotransfer. Gene Ther. 2012;19(12):1177-1186.

19. Riu E, Chen ZY, Xu H, He CY, Kay MA. Histone modifications are associated with the persistence or silencing of vector-mediated transgene expression in vivo. Mol Ther. 2007;15(7):1348-1355.

20. Darquet AM, Cameron B, Wils P, Scherman D, Crouzet J. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. Gene Ther. 1997;4(12):1341-1349.

21. Bigger BW, Tolmachov O, Collombet JM, Fragkos M, Palaszewski I, Coutelle C. An araC-controlled bacterial cre expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy. J Biol Chem. 2001;276(25):23018-23027.

22. Mayrhofer P, Blaesen M, Schleef M, Jechlinger W. Minicircle-DNA production by site specific recombination and protein-DNA interaction chromatography. J Gene Med. 2008;10(11):1253-1269.

23. Chen ZY, He CY, Kay MA. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. Hum Gene Ther. 2005;16(1):126-131.

24. Siegele DA, Hu JC. Gene expression from plasmids containing the araBAD promoter at subsaturating inducer concentrations represents mixed populations. Proc Natl Acad Sci U S A. 1997;94(15):8168-8172.

25. Kay MA HC, Chen ZY. A robust system for production of minicircle DNA vectors. Nat Biotechnol. . 2010;28(12):1287-1289.

26. Lukacs GL, Haggie P, Seksek O, Lechardeur D, Freedman N, Verkman AS. Size-dependent DNA mobility in cytoplasm and nucleus. J Biol Chem. 2000;275(3):1625-1629.

27. Kozak M. Initiation of translation in prokaryotes and eukaryotes. Gene. 1999;234(2):187-208.

28. Matza-Porges S, Horresh I, Tavor E, Panet A, Honigman A. Expression of an anti apoptotic recombinant short peptide in mammalian cells. Apoptosis : an international journal on programmed cell death. 2005;10(5):987-996.

29. Stenler S, Wiklander OP, Badal-Tejedor M, et al. Micro-minicircle Gene Therapy: Implications of Size on Fermentation, Complexation, Shearing Resistance, and Expression. Molecular therapy. Nucleic acids. 2014;2:e140.

30. Voss C, Schmidt T, Schleef M, Friehs K, Flaschel E. Production of supercoiled multimeric plasmid DNA for biopharmaceutical application. J Biotechnol. 2003;105(3):205-213.

31. Maucksch C, Bohla A, Hoffmann F, et al. Transgene expression of transfected supercoiled plasmid DNA concatemers in mammalian cells. J Gene Med. 2009;11(5):444-453.

32. Molnar MJ, Gilbert R, Lu Y, et al. Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles. Mol Ther. 2004;10(3):447-455.

33. Kreiss P, Cameron B, Rangara R, et al. Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. Nucleic Acids Res. 1999;27(19):3792-3798.

34. Loisel S, Floch V, Le Gall C, Ferec C. Factors influencing the efficiency of lipoplexes mediated gene transfer in lung after intravenous administration 1 *. Journal of liposome research. 2001;11(2-3):127-138.

35. McLenachan S, Sarsero JP, Ioannou PA. Flow-cytometric analysis of mouse embryonic stem cell lipofection using small and large DNA constructs. Genomics. 2007;89(6):708-720.

36. Fogg JM, Kolmakova N, Rees I, et al. Exploring writhe in supercoiled minicircle DNA. J Phys Condens Matter. 2006;18(14):S145-S159.

37. Hitchcock AG, Sergeant JA, Rahman SF, Tharia HA, Blom H. Scale-Up of a Plasmid DNA Purification Process. BioProcess International. 2010;8(11):46–54.

38. Cherng JY, Schuurmans-Nieuwenbroek NM, Jiskoot W, et al. Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. J Control Release. 1999;60(2-3):343-353.

39. Remaut K, Sanders NN, Fayazpour F, Demeester J, De Smedt SC. Influence of plasmid DNA topology on the transfection properties of DOTAP/DOPE lipoplexes. J Control Release. 2006;115(3):335-343.

40. Weintraub H, Cheng PF, Conrad K. Expression of transfected DNA depends on DNA topology. Cell. 1986;46(1):115-122.

41. Tabuchi H, Hirose S. DNA supercoiling facilitates formation of the transcription initiation complex on the fibroin gene promoter. J Biol Chem. 1988;263(30):15282-15287.

42. Schultz MC, Brill SJ, Ju Q, Sternglanz R, Reeder RH. Topoisomerases and yeast rRNA transcription: negative supercoiling stimulates initiation and topoisomerase activity is required for elongation. Genes & development. 1992;6(7):1332-1341.

43. Mizutani M, Ohta T, Watanabe H, Handa H, Hirose S. Negative supercoiling of DNA facilitates an interaction between transcription factor IID and the fibroin gene promoter. Proc Natl Acad Sci U S A. 1991;88(3):718-722.

44. Sekiguchi JM, Kmiec EB. DNA superhelicity enhances the assembly of transcriptionally active chromatin in vitro. Mol Gen Genet. 1989;220(1):73-80.

45. WHO. The WHO Expert Committee on Biological Standardization 56th report: Number 941. Geneva: World Health Organization; 2007.

46. FDA. U.S. Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research: Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications2007.

47. EMEA. Comittee for Veterinary medicinal products: Notes for Guidance: DNA-vaccines non-amplifiable in eukaryotic cells for veterinary use. In: Products TEAftEfM2000.

48. Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc Natl Acad Sci U S A. 1985;82(13):4438-4442.

49. Bates AD, Maxwell A. DNA gyrase can supercoil DNA circles as small as 174 base pairs. EMBO J. 1989;8(6):1861-1866.

50. Catanese DJ, Jr., Fogg JM, Schrock DE, 2nd, Gilbert BE, Zechiedrich L. Supercoiled Minivector DNA resists shear forces associated with gene therapy delivery. Gene Ther. 2012;19(1):94-100.

51. Vahlsing HL, Yankauckas MA, Sawdey M, Gromkowski SH, Manthorpe M. Immunization with plasmid DNA using a pneumatic gun. J Immunol Methods. 1994;175(1):11-22.

52. Wang R, Epstein J, Baraceros FM, et al. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. Proc Natl Acad Sci U S A. 2001;98(19):10817-10822.

53. Hallengard D, Brave A, Isaguliants M, et al. A combination of intradermal jet-injection and electroporation overcomes in vivo dose restriction of DNA vaccines. Genetic vaccines and therapy. 2012;10(1):5.

54. Walther W, Stein U, Fichtner I, Schlag PM. Low-volume jet injection for efficient nonviral in vivo gene transfer. Molecular biotechnology. 2004;28(2):121-128.

55. Walther W, Stein U, Fichtner I, et al. Intratumoral low-volume jet-injection for efficient nonviral gene transfer. Molecular biotechnology. 2002;21(2):105-115.

56. Zhao N, Fogg JM, Zechiedrich L, Zu Y. Transfection of shRNA-encoding Minivector DNA of a few hundred base pairs to regulate gene expression in lymphoma cells. Gene Ther. 2011;18(3):220-224.

57. Schuttrumpf J, Milanov P, Abriss D, Roth S, Tonn T, Seifried E. Transgene loss and changes in the promoter methylation status as determinants for expression duration in nonviral gene transfer for factor IX. Hum Gene Ther. 2011;22(1):101-106.

58. Stenler S, Andersson A, Simonson OE, et al. Gene transfer to mouse heart and skeletal muscles using a minicircle expressing human vascular endothelial growth factor. J Cardiovasc Pharmacol. 2009;53(1):18-23.

59. Chang CW, Christensen LV, Lee M, Kim SW. Efficient expression of vascular endothelial growth factor using minicircle DNA for angiogenic gene therapy. J Control Release. 2008;125(2):155-163.

60. Ong SG, Lee WH, Huang M, et al. Cross Talk of Combined Gene and Cell Therapy in Ischemic Heart Disease: Role of Exosomal MicroRNA Transfer. Circulation. 2014;130(11 Suppl 1):S60-69.

61. Huang M, Chen Z, Hu S, et al. Novel minicircle vector for gene therapy in murine myocardial infarction. Circulation. 2009;120(11 Suppl):S230-237.

62. Hu S, Huang M, Li Z, et al. MicroRNA-210 as a novel therapy for treatment of ischemic heart disease. Circulation. 2010;122(11 Suppl):S124-131.

63. Huang M, Nguyen P, Jia F, et al. Double knockdown of prolyl hydroxylase and factor-inhibiting hypoxia-inducible factor with nonviral minicircle gene therapy enhances stem cell mobilization and angiogenesis after myocardial infarction. Circulation. 2011;124(11 Suppl):S46-54.

64. Lijkwan MA, Hellingman AA, Bos EJ, et al. Short hairpin RNA gene silencing of prolyl hydroxylase-2 with a minicircle vector improves neovascularization of hindlimb ischemia. Hum Gene Ther. 2014;25(1):41-49.

65. Douglas AG, Wood MJ. Splicing therapy for neuromuscular disease. Molecular and cellular neurosciences. 2013;56:169-185.

66. Goyenvalle A, Griffith G, Babbs A, et al. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. 2015.

67. Schumperli D, Pillai RS. The special Sm core structure of the U7 snRNP: far-reaching significance of a small nuclear ribonucleoprotein. Cell Mol Life Sci. 2004;61(19-20):2560-2570.

68. Yoon CS, Jung HS, Kwon MJ, et al. Sonoporation of the Minicircle-VEGF(165) for Wound Healing of Diabetic Mice. Pharm Res. 2009;26(4):794-801.

69. Ko J, Jun H, Chung H, et al. Comparison of EGF with VEGF non-viral gene therapy for cutaneous wound healing of streptozotocin diabetic mice. Diabetes & metabolism journal. 2011;35(3):226-235.

70. Kwon MJ, An S, Choi S, et al. Effective healing of diabetic skin wounds by using nonviral gene therapy based on minicircle vascular endothelial growth factor DNA and a cationic dendrimer. J Gene Med. 2012;14(4):272-278.

71. Yi H, Kim Y, Kim J, et al. A new strategy to deliver synthetic protein drugs: self-reproducible biologics using minicircles. Scientific reports. 2014;4:5961.

72. Alam T, Wai P, Held D, Vakili ST, Forsberg E, Sollinger H. Correction of Diabetic Hyperglycemia and Amelioration of Metabolic Anomalies by Minicircle DNA Mediated Glucose-Dependent Hepatic Insulin Production. PloS one. 2013;8(6):e67515.

73. Park JH, Lee M, Kim SW. Non-viral adiponectin gene therapy into obese type 2 diabetic mice ameliorates insulin resistance. J Control Release. 2006;114(1):118-125.

74. Quade-Lyssy P, Milanov P, Abriss D, et al. Oral gene therapy for hemophilia B using chitosan-formulated FIX mutants. Journal of thrombosis and haemostasis : JTH. 2014;12(6):932-942.

75. Hyun J, Grova M, Nejadnik H, et al. Enhancing in vivo survival of adipose-derived stromal cells through Bcl-2 overexpression using a minicircle vector. Stem cells translational medicine. 2013;2(9):690-702.

76. Madeira C, Rodrigues CA, Reis MS, et al. Nonviral gene delivery to neural stem cells with minicircles by microporation. Biomacromolecules. 2013;14(5):1379-1387.

77. Chang TY, Chung CY, Chuang WM, Li LY, Jeng LB, Ma WL. Durable expression of minicircle DNA-liposome-delivered androgen receptor cDNA in mice with hepatocellular carcinoma. BioMed research international. 2014;2014:156356.

78. Wu J, Xiao X, Zhao P, et al. Minicircle-IFNgamma induces antiproliferative and antitumoral effects in human nasopharyngeal carcinoma. Clinical cancer research : an

official journal of the American Association for Cancer Research. 2006;12(15):4702-4713.

79. Xu BL, Yuan L, Wu JX, et al. Mc-hES, a novel plasmid carrying human endostatin gene, inhibits nasopharyngeal carcinoma growth. Cancer gene therapy. 2012;19(2):110-117.

80. Gaspar VM, Goncalves C, de Melo-Diogo D, et al. Poly(2-ethyl-2-oxazoline)-PLA-g-PEI amphiphilic triblock micelles for co-delivery of minicircle DNA and chemotherapeutics. J Control Release. 2014;189:90-104.

81. Zhang X, Epperly MW, Kay MA, et al. Radioprotection in vitro and in vivo by minicircle plasmid carrying the human manganese superoxide dismutase transgene. Hum Gene Ther. 2008;19(8):820-826.

82. Yang Z, Li G, Zhang Y, Liu X, Tien P. A novel minicircle vector based system for inhibting the replication and gene expression of enterovirus 71 and coxsackievirus A16. Antiviral research. 2012;96(2):234-244.

83. Quaak SG, Haanen JB, Beijnen JH, Nuijen B. Naked plasmid DNA formulation: effect of different disaccharides on stability after lyophilisation. AAPS PharmSciTech. 2010;11(1):344-350.

84. Dietz WM, Skinner NE, Hamilton SE, et al. Minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses. Mol Ther. 2013;21(8):1526-1535.

85. Gulzar N, Copeland KF. CD8+ T-cells: function and response to HIV infection. Current HIV research. 2004;2(1):23-37.

86. Wang Q, Jiang W, Chen Y, et al. In vivo electroporation of minicircle DNA as a novel method of vaccine delivery to enhance HIV-1-specific immune responses. Journal of virology. 2014;88(4):1924-1934.

87. Chabot S, Orio J, Schmeer M, Schleef M, Golzio M, Teissie J. Minicircle DNA electrotransfer for efficient tissue-targeted gene delivery. Gene Ther. 2012.

88. Cupillard L, Juillard V, Latour S, et al. Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats. Vaccine. 2005;23(16):1910-1916.

89. Cai Y, Rodriguez S, Hebel H. DNA vaccine manufacture: scale and quality. Expert review of vaccines. 2009;8(9):1277-1291.

90. Dad AB, Ramakrishna S, Song M, Kim H. Enhanced gene disruption by programmable nucleases delivered by a minicircle vector. Gene Ther. 2014.

91. Jia F, Wilson KD, Sun N, et al. A nonviral minicircle vector for deriving human iPS cells. Nature methods. 2010;7(3):197-199.

92. Kang SH, Cho MJ, Kole R. Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry. 1998;37(18):6235-6239.

93. Goyenvalle A, Vulin A, Fougerousse F, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science. 2004;306(5702):1796-1799.

94. Lyubchenko YL, Shlyakhtenko LS, Ando T. Imaging of nucleic acids with atomic force microscopy. Methods. 2011;54(2):274-283.

95. Cherny DI, Jovin TM. Electron and scanning force microscopy studies of alterations in supercoiled DNA tertiary structure. Journal of molecular biology. 2001;313(2):295-307.

96. Morgan JE, Beauchamp JR, Pagel CN, et al. Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. Developmental biology. 1994;162(2):486-498.

97. Golzio M, Rols MP, Teissie J. In vitro and in vivo electric field-mediated permeabilization, gene transfer, and expression. Methods. 2004;33(2):126-135.

98. Teissie J, Golzio M, Rols MP. Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge. Biochimica et biophysica acta. 2005;1724(3):270-280.

99. Braun S. Muscular gene transfer using nonviral vectors. Current gene therapy. 2008;8(5):391-405.

100. Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Human molecular genetics. 1992;1(6):363-369.

101. Jiao S, Williams P, Berg RK, et al. Direct gene transfer into nonhuman primate myofibers in vivo. Hum Gene Ther. 1992;3(1):21-33.

102. Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. Mol Ther. 2007;15(12):2063-2069.

103. Zhang G, Gao X, Song YK, et al. Hydroporation as the mechanism of hydrodynamic delivery. Gene Ther. 2004;11(8):675-682.

104. Al-Dosari MS, Knapp JE, Liu D. Hydrodynamic delivery. Advances in genetics. 2005;54:65-82.

105. Budker V, Zhang G, Danko I, Williams P, Wolff J. The efficient expression of intravascularly delivered DNA in rat muscle. Gene Ther. 1998;5(2):272-276.

106. Fan Z, Kocis K, Valley R, et al. Safety and feasibility of high-pressure transvenous limb perfusion with 0.9% saline in human muscular dystrophy. Mol Ther. 2012;20(2):456-461.

107. Gonin P, Arandel L, Van Wittenberghe L, Marais T, Perez N, Danos O. Femoral intra-arterial injection: a tool to deliver and assess recombinant AAV constructs in rodents whole hind limb. J Gene Med. 2005;7(6):782-791.

108. Trollet C, Bloquel C, Scherman D, Bigey P. Electrotransfer into skeletal muscle for protein expression. Current gene therapy. 2006;6(5):561-578.

109. Gollins H, McMahon J, Wells KE, Wells DJ. High-efficiency plasmid gene transfer into dystrophic muscle. Gene Ther. 2003;10(6):504-512.

110. Konieczny P, Swiderski K, Chamberlain JS. Gene and cell-mediated therapies for muscular dystrophy. Muscle & nerve. 2013;47(5):649-663.

111. Beshay M, Gazdhar A, Gugger M, Reymond M, Schmid RA. The feasibility of non-viral gene transfer to the diaphragm in vivo. Development, growth & differentiation. 2009;51(6):547-553.

112. Wells KE, McMahon J, Foster H, Ferrer A, Wells DJ. Gene delivery to dystrophic muscle. Methods Mol Biol. 2008;423:421-431.

113. Gaspar VM, Maia CJ, Queiroz JA, Pichon C, Correia IJ, Sousa F. Improved minicircle DNA biosynthesis for gene therapy applications. Human gene therapy methods. 2014;25(2):93-105.

114. Nishikawa J, Amano M, Fukue Y, et al. Left-handedly curved DNA regulates accessibility to cis-DNA elements in chromatin. Nucleic Acids Res. 2003;31(22):6651-6662.

115. Parvin JD, McCormick RJ, Sharp PA, Fisher DE. Pre-bending of a promoter sequence enhances affinity for the TATA-binding factor. Nature. 1995;373(6516):724-727.

116. Starr DB, Hoopes BC, Hawley DK. DNA bending is an important component of site-specific recognition by the TATA binding protein. Journal of molecular biology. 1995;250(4):434-446.

117. Ribeiro S, Mairhofer J, Madeira C, et al. Plasmid DNA size does affect nonviral gene delivery efficiency in stem cells. Cellular reprogramming. 2012;14(2):130-137.

118. Viecelli HM, Harbottle RP, Wong SP, et al. Treatment of phenylketonuria using minicircle-based naked-DNA gene transfer to murine liver. Hepatology (Baltimore, Md.). 2014;60(3):1035-1043.

119. Kano Y, Miyashita T, Nakamura H, Kuroki K, Nagata A, Imamoto F. In vivo correlation between DNA supercoiling and transcription. Gene. 1981;13(2):173-184.

120. Makinson A, Reynes J. The fusion inhibitor enfuvirtide in recent antiretroviral strategies. Current opinion in HIV and AIDS. 2009;4(2):150-158.

121. Matthews T, Salgo M, Greenberg M, Chung J, DeMasi R, Bolognesi D. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nature reviews. Drug discovery. 2004;3(3):215-225.

122. Walmsley S, Henry K, Katlama C, et al. Enfuvirtide (T-20) cross-reactive glycoprotein 41 antibody does not impair the efficacy or safety of enfuvirtide. The Journal of infectious diseases. 2003;188(12):1827-1833.

123. Muster T, Steindl F, Purtscher M, et al. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. Journal of virology. 1993;67(11):6642-6647.

124. Calarota S, Bratt G, Nordlund S, et al. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. Lancet. 1998;351(9112):1320-1325.

125. Chang CH, Hinkula J, Loo M, et al. A novel class of anti-HIV agents with multiple copies of enfuvirtide enhances inhibition of viral replication and cellular transmission in vitro. PloS one. 2012;7(7):e41235.

126. Dervillez X, Huther A, Schuhmacher J, et al. Stable expression of soluble therapeutic peptides in eukaryotic cells by multimerisation: application to the HIV-1 fusion inhibitory peptide C46. ChemMedChem. 2006;1(3):330-339.

127. Henry TD, Annex BH, McKendall GR, et al. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation. 2003;107(10):1359-1365.

128. Simovic D, Isner JM, Ropper AH, Pieczek A, Weinberg DH. Improvement in chronic ischemic neuropathy after intramuscular phVEGF165 gene transfer in patients with critical limb ischemia. Archives of neurology. 2001;58(5):761-768.

129. Gyongyosi M, Khorsand A, Zamini S, et al. NOGA-guided analysis of regional myocardial perfusion abnormalities treated with intramyocardial injections of plasmid encoding vascular endothelial growth factor A-165 in patients with chronic myocardial ischemia: subanalysis of the EUROINJECT-ONE multicenter double-blind randomized study. Circulation. 2005;112(9 Suppl):I157-165.

130. Gill DR, Smyth SE, Goddard CA, et al. Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. Gene Ther. 2001;8(20):1539-1546.

131. van den Bergen JC, Schade van Westrum SM, Dekker L, et al. Clinical characterisation of Becker muscular dystrophy patients predicts favourable outcome in exon-skipping therapy. Journal of neurology, neurosurgery, and psychiatry. 2014;85(1):92-98.

132. Tennyson CN, Shi Q, Worton RG. Stability of the human dystrophin transcript in muscle. Nucleic Acids Res. 1996;24(15):3059-3064.

133. Spitali P, van den Bergen JC, Verhaart IE, et al. DMD transcript imbalance determines dystrophin levels. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013;27(12):4909-4916.

134. Le Hir M, Goyenvalle A, Peccate C, et al. AAV genome loss from dystrophic mouse muscles during AAV-U7 snRNA-mediated exon-skipping therapy. Mol Ther. 2013;21(8):1551-1558.

135. Stenler S, Blomberg P, Smith CE. Safety and efficacy of DNA vaccines: Plasmids vs. minicircles. Human vaccines & immunotherapeutics. 2014;10(5):1306-1308.

136. Young JL, Zimmer WE, Dean DA. Smooth muscle-specific gene delivery in the vasculature based on restriction of DNA nuclear import. Experimental biology and medicine (Maywood, N.J.). 2008;233(7):840-848.

137. Geguchadze R, Wang Z, Zourelias L, et al. Proteomic profiling of salivary gland after nonviral gene transfer mediated by conventional plasmids and minicircles. Molecular therapy. Methods & clinical development. 2014;1:14007.

138. Ko YT, Hartner WC, Kale A, Torchilin VP. Gene delivery into ischemic myocardium by double-targeted lipoplexes with anti-myosin antibody and TAT peptide. Gene Ther. 2009;16(1):52-59.

139. Sallberg M, Frelin L, Ahlen G, Sallberg-Chen M. Electroporation for therapeutic DNA vaccination in patients. Medical microbiology and immunology. 2015;204(1):131-135.

140. Coban C, Ishii KJ, Gursel M, Klinman DM, Kumar N. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. Journal of leukocyte biology. 2005;78(3):647-655.

141. Klinman DM, Klaschik S, Sato T, Tross D. CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. Advanced drug delivery reviews. 2009;61(3):248-255.

142. Evans CH, Huard J. Gene therapy approaches to regenerating the musculoskeletal system. Nature reviews. Rheumatology. 2015;11(4):234-242.

143. Usas A, Huard J. Muscle-derived stem cells for tissue engineering and regenerative therapy. Biomaterials. 2007;28(36):5401-5406.

144. Witsch E, Sela M, Yarden Y. Roles for growth factors in cancer progression. Physiology (Bethesda, Md.). 2010;25(2):85-101.

145. Sheyn D, Pelled G, Zilberman Y, et al. Nonvirally engineered porcine adipose tissue-derived stem cells: use in posterior spinal fusion. Stem cells (Dayton, Ohio). 2008;26(4):1056-1064.