Optimisation of lentiviral vectors for gene therapy of spinal muscular atrophy

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by

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Declaration of Authorship

I, Neda Ali Mohammadi Nafchi, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

Date: 04/08/2017

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease particularly characterised by degeneration of motor neurons from the ventral horn of the spinal cord. Such degeneration leads to muscle weakness and wasting (atrophy). *Survival motor neuron* (*SMN*) *1* gene is the SMA-determining gene, being absent or mutated in most people affected. *SMN2* is a highly similar gene which produces low levels of SMN protein and allows survival in the absence of *SMN1*. Full-length SMN is a ubiquitous and essential cellular protein, and low levels of it result in a wide range of systemic pathologies in affected individuals. One avenue to treat SMA is gene therapy, a technology that uses genetic material to alter gene expression and can be applied to both inherited and acquired disorders. Gene addition therapy to replace the faulty *SMN1* gene and thus increase the levels of SMN protein to a therapeutic threshold is one of the main strategies for treating SMA. Adeno-associated virus (AAV) vectors are currently being used in clinical trials to attempt such enhancement of SMN levels. A possible alternative may be provided by lentiviral vectors, which are widely used in basic research and clinical applications for gene transfer, including long-term expression. A previous study in Prof. Yáñez's lab demonstrated that lentiviral vectors were significantly more effective for *in utero* transduction of motor neurons than AAV. Therefore, this study is focused on optimising a lentiviral expression system for *SMN1* expression. A variety of integration-proficient and integration-deficient lentiviral vectors (IPLV and IDLV, respectively) with different promoters -either cytomegalovirus (CMV) or human synapsin (hSYN)- and transgenes -either wild-type or a novel codon optimised *hSMN1*- were produced to compare the efficiency of transgene expression and thus determine the lentiviral configuration resulting in the highest production of full-length SMN. Vectors were tested in a variety of proliferating and quiescent *in vitro* cell culture models for

quantitative estimation of full-length SMN production. The results of these experiments revealed that using integrating, CMV-driven, codon-optimised *hSMN1* lentivector resulted in highest production of full-length SMN protein. In general, a comparison of expression levels revealed that IPLVs produced more transgenic protein than IDLVs. However, IDLVs achieved significant transgene expression levels, particularly in quiescent or growth-arrested cells, and they are expected to be significantly safer than IPLVs. Of note, overexpression by *hSMN1* lentivectors restored gems in transduced type I SMA fibroblasts in a dose-dependent manner. Encouraged by the results of the cell culture work, we performed very preliminary *in vivo* experiments. As SMN-replacement therapy is particularly effective at early stages of post-natal development, we hypothesised that earlier treatment may be advantageous. We thus attempted to deliver control and our novel *SMN1* lentivectors to the developing embryo of wild-type and SMA mice. While it is clear that this part of the work is at a very early phase, the outcome showed encouraging viability and lack of toxicity in treated animals. In conclusion, the results of this study suggest that lentiviral vectors are potent agents for transgenic expression of codon-optimised *hSMN1*, and that *in utero* delivery has significant potential as a therapeutic strategy for SMA.

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Abbreviations

AAV: Adeno-associated viral vectors

Ad: Adenoviral vectors

ASO: Antisense oligonucleotide

a.u.: Arbitrary unit

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin

CA: Capsid

cDNA: Complementary DNA

CF: Cystic Fibrosis

ChAT: Choline Acetyltransferase

CHO: Chinese hamster ovary

CMV: Cytomegalovirus

CO$_2$: Carbon dioxide

Co-h*SMN1*: Codon optimised *human survival motor neuron 1*

DAPI: 4,6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ds: Double stranded

E: Embryonic

EDTA: Ethylenediaminetetraacetic acid

Env: Envelope

EGF: Epidermal growth factor

eGFP: Enhanced green fluorescent protein

ELISA: Enzyme-linked immunosorbent assay

EtBr: Ethidium Bromide

FACS: Fluorescence-activated cell sorting

FBS: Foetal bovine serum

FGF: Fibroblast growth factor

FL-SMN: Full length survival motor neuron

Gag: Group specific antigen

HBSS: Hank's Balanced Salt Solution

HEK: Human embryonic kidney

HIV-1: Human-immunodeficiency virus

hnRNPs: Heterologous nuclear ribonucleoproteins

HR: Homologous recombination

hSMN1: Human *survival motor neuron1*

h*SMN1*-CtF: Human *survival motor neuron*1 had a flag tag in C terminus

h*SMN1*-NtF: Human *survival motor neuron* had a flag tag in N terminus

HSV: Herpes simplex viral vectors

IDLVs: Integration-Deficient Lentiviral Vectors

IGF: Insulin-like growth factor

IN: Integrase

IPLVs: Integration-proficient lentiviral vectors

IRES: Internal ribosome entry site

ISS: Intronic splicing silencers

ITR: Inverted terminal repeat

Kb: Kilobase

kDa: Kilo Dalton

LB: Luria-Bertani

LTRs: Long terminal repeats

LRT: Late reverse transcript

LV: Lentiviral vectors

MA: Matrix protein

MN: Motor neurons

MOI: Multiplicity of infection

mRNA: Messenger RNA

mSmn: Mouse *survival motor neuron*

NC: Nucleocapsid

NeuN: Neuronal Nuclei

NGF: Nerve growth factor

NHEJ: Non-homologous end joining

NTF: Neurotrophic factor

OCT: Optimum cutting temperature

OD: Optical density

ORF: Open reading frame

PBS: Phosphate buffered saline

pbs: Primer binding site

PCR: Polymerase chain reaction

Pen & Strep: Penicillin and Streptomycin

PFA: Paraformaldehyde PFA

pol: Polymerase

PR: Protease

PS: Phosphatidylserine

qPCR: Quantitative polymerase chain reaction

qRT-PCR: quantitative RT-PCR

RCV: Replication-competent vectors

RNA: Ribonucleic acid

RRE: Rev response element

RT-PCR: Reverse transcription PCR

RT: room temperature

sc AAV: Self complementary AAV

SCID: Severe combined immunodeficiency syndrome

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIN: Self-inactivating

SMA: Spinal muscular atrophy

SMN: Survival motor neuron

ss AAV: Single stranded AAV

ssDNA: Single-stranded DNA

SU: Surface subunit

SV40: Simian virus 40

TAR: tat response element

TE: Tris-EDTA

TGF-β: Transforming growth factor-β

TM: Transmembrane protein

Tuj1: Neuronal-specific class III β-tubulin

VSV-G: Vesicular stomatitis virus glycoprotein

wt: Wild type

WPRE: Woodchuck hepatitis virus posttranscriptional element

Ψ: Packaging signal

Δ7-SMN: Delta seven survival motor neuron (lacks exon 7)

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To my beautiful angel, Diana, how fortunate I am to have such a wonderful daughter. You have been my hope and joy during this challenging time. Your little smile makes my world a more beautiful place. I want to take this moment to say I love you from the bottom of my heart and I am so sorry for all the times you needed me and I had to be in the lab or working on this thesis. Those moments broke my heart, but I did it in the hope that you would forgive me, and knowing it was to give you a better start in life, and to make the world you live in a better place.

I dedicate this thesis to my mother, Pouran Sedaghat, and my beautiful daughter, Diana Abdi.

Chapter 1 Introduction

1.1 Spinal muscular atrophy

1.1.1 Spinal muscular atrophy pathology and molecular basis

Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality. The incidence of SMA is estimated at about 1 in 6,000 to 1 in 10,000 new-borns with a carrier frequency of approximately 1/40-1/60 (D'Amico *et al.*, 2011).

The defect caused by SMA disease is attributed to a reduced level of survival motor neuron (SMN) protein. SMA is primarily characterized by the degeneration of motor neurons in the spinal cord leading to progressive atrophy of skeletal muscles and generalized weakness. Initially it was thought that motor neurons were the only affected cells, but recent emerging evidence suggests that other tissues and cells are also affected by low levels of SMN protein (Bowerman *et al.*, 2012; Gogliotti *et al.*, 2012; Hua *et al.*, 2011; Martinez *et al.*, 2012 and Park *et al.*, 2010). α-motor neurons are the cells most affected by low levels of SMN protein, and SMN restoration in these cells is sufficient for some therapeutic benefit (Finkel *et al.*, 2016 and Clinicaltrials.gov identifier NCT02122952). However an effective and successful therapy for SMA is likely to involves the consideration of SMA as a multi-system disorder (Gavrilina *et al.*, 2008; Hamilton & Gillingwater, 2013 and Park *et al.*, 2010).

The level of SMN expression is not equal in all tissues and cells. A high expression level of this protein is found in motor neurons (Monani, 2005). Ribonucleic acid (RNA) analysis from different tissues such as lung, muscle, kidney and liver showed that *survival motor neuron* (*SMN*) is widely expressed and produces a 1.7 kb messenger RNA (mRNA) transcript and moderate levels of SMN protein are found in skeletal and cardiac muscle (Lefebvre *et al.*, 1995 and Coovert *et al.*, 1997).

The full length SMN protein is 38 kDa, and is a ubiquitously expressed protein consisting of 294 amino acids, encoded by two highly similar genes called *SMN1* and

SMN2. In the human genome, these genes are located on chromosome 5q in band 13.2 (Figure 1.1). Healthy humans normally have one copy of telomeric *SMN1* and one copy of centromeric *SMN2* on chromosome 5 (Lefebvre *et al*., 1995). Heterogeneity in the number of *SMN2* copies has been reported (Mailman *et al*., 2002).
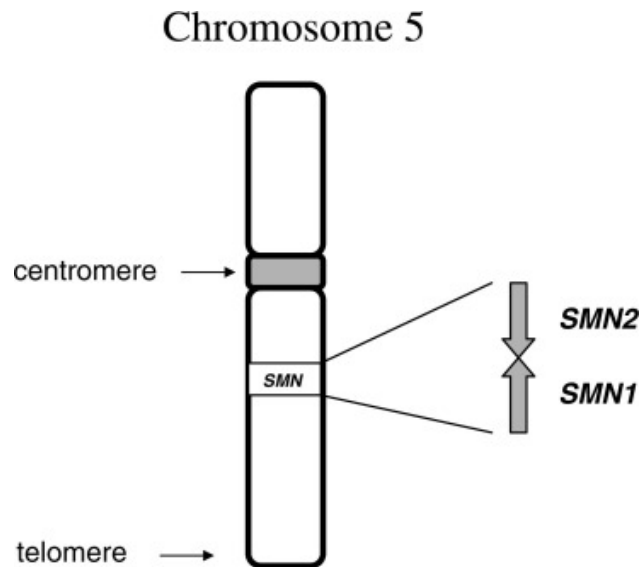
Figure 1.1: Location of *SMN* gene.

SMN gene is located on the 5th chromosome at position q13.2. This gene is responsible for the production of SMN protein. Homozygous mutations and/or deletions in the *SMN1*gene cause SMA disease and subsequent reduction of the SMN protein. Taken from (Markowitz *et al*., 2004).

SMN1 is also known as '*SMN*-determining gene'. *SMN1* is absent or truncated in 98.6% of SMA patients while mutation in *SMN2* cannot cause SMA disease (Coovert *et al*., 1997 and Lefebvre *et al*., 1995). The length of *SMN* gene is roughly 20 kb and it consists of 8 exons interrupted by 7 intron (Markowitz *et al*., 2012).

The telomeric and centromeric copies of *SMN* gene share more than 99.8% sequence homology. *SMN1* and *SMN2* can be distinguished from each other by 5 different nucleotides (Lefebvre *et al*., 1995). These nucleotides are placed in intron6, exon7,

intron7, and noncoding exon8, with only one of these five nucleotides placed in a coding region. Exon7 in *SMN* gene is a highly regulated region containing 54 nucleotides; the cytosine (C) is at position +6 of exon7 in *SMN1* is a thymidine (T) in *SMN2* (Lorson *et al*., 1999 and Christian *et al*., 2010).

SMN1 is transcribed into a full length SMN protein while *SMN2* produces only 10% of the full-length SMN protein due to C to T substitution at position 840 in exon7. This substitution does not alter an amino acid but it affects the correct splicing of exon7 and results in the skipping of exon7 during transcription (Cifuentes-Diaz *et al*., 2002). This C to T transition in *SMN2* results in a dramatic alteration in pre-mRNA splicing and causes the exon7 to be excluded from almost 90% of *SMN2* mature transcripts (Jodelka *et al*., 2010). The translation termination signal to produce a full-length protein is placed in exon7 while in the case of exon-skipped *SMN2* the translation termination region is at the 5′ end of exon 8. Lack of exon 7 decreases by ~ 2 KDa the molecular weight of SMN protein. Moreover, the truncated protein is unstable and also has reduced oligomerisation capacity, which is essential for its stability and proper function. Although *SMN2* is present in all patients, it cannot compensate for the defect in *SMN1* (Coovert *et al*., 1997 and Lefebvre *et al*., 1995). Only 10% of SMN expressed by *SMN2* is full length and identical to the protein produced by *SMN1* (Figure 1.2).
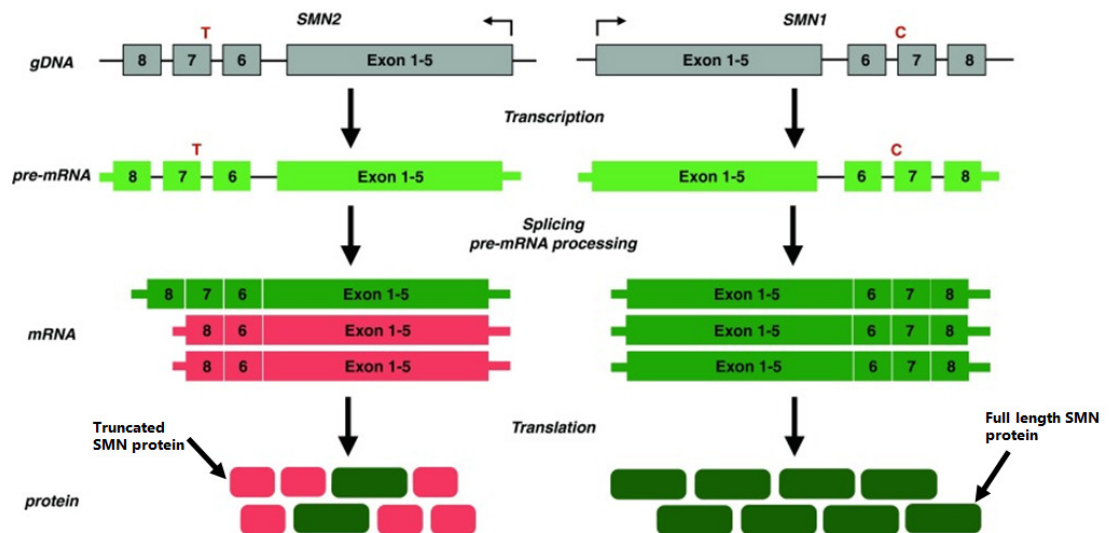
Figure 1.2: Schematic representation of differences between *SMN1* and *SMN2* gene and the effect of the C-to-T transition in exon 7 between these two genes on splicing.

There are five nucleotide differences between *SMN1* and *SMN2*. Only one of these nucleotide changes (840C→T) is in the coding sequence (exon7). This alteration, but no other variations, in the *SMN* genes affects the splicing pattern of the gene and produces truncated SMN protein (lacking exon 7) from *SMN2*. The FL-SMN protein is a 38 KDa ubiquitously expressed protein and consist of 294 amino acids. Truncated protein lacks 16 amino acids at the carboxyl terminal end generating an unstable protein, which cannot substitute for the full length SMN protein. Taken from (D'Ydewalle & Sumner, 2015).

1.1.2 Types of spinal muscular atrophy

Based on the age of onset and severity of disease, SMA can be classified into four different types: SMA type I (Werdnig-Hoffman disease), type II (intermediate), type III (mild, Kugelberg-Welander disease), and type IV (adult) (Munsat, 1991; Russman, 2007 and Wang *et al*., 2007).

SMA type I (or Werdnig-Hoffman disease) is the most severe and common type. 50% of SMA patients are diagnosed with this type. The onset of disease in this group occurs before 6 months of age and patients do not typically live longer than 2 years. Patients with SMA type I have profound hypotonia, making it difficult to control head

movements, and causing problems with breathing, swallowing and sucking. This group also suffers from increased risk of infection (Munsat, 1991 and Wang *et al*., 2007).

In children affected with SMA type II (intermediate) the onset occurs between 7 and 18 months of age, with an average survival of 2 or more years. They can sit without help but cannot walk or stand without help, and only a few are able to stand with leg braces. The respiratory process and swallowing are insufficient and weak (Munsat, 1991; Russman, 2007 and Wang *et al*., 2007).

In SMA type III (mild, Kugelberg-Welander disease) the onset is after 18 months of age and the symptoms vary largely within the group. They can walk independently, however in some cases they need to use a wheelchair. Most patients have a productive adult life with minor muscular weakness. Scoliosis often develops in these patients (Munsat, 1991 and Wang *et al*., 2007).

In patients affected with SMA type IV (adult), the onset starts during the second or third decade of their life and these patients only present mild motor impairment. They can walk without help in adult years (Russman, 2007 and Wang *et al*., 2007). Table 1.1 presents a summary of information about different type of SMA.

Table 1.1: Classification of Spinal Muscular Atrophy.

Type	Age of onset	Highest function achieved	Life span	Reference
SMA type I or Werdnig-Hoffman disease (severe)	0-6 months	Cannot sit/stand	<2 years	(Munsat, 1991 and Wang *et al.*, 2007)
SMA type II (intermediate)	7-18 months	Can sit without help but need help while standing	> 2years	(Munsat, 1991; Russman, 2007 and Wang *et al.*, 2007)
Type III or Kugelberg-Welander disease (mild)	> 18 months	Can stand and walk without help	Adult	(Munsat, 1991 and Wang *et al.*, 2007)
Type IV	Second or third decade	Walking	Adult	(Russman, 2007 and Wang *et al.*, 2007)

1.2 SMA diagnosis

Electromyography, muscle enzyme creatine kinase, nerve conduction velocities, muscle biopsy, electrodiagnostic and magnetic resonance imaging (MRI) were standard tests used to diagnose SMA disease. However, since improvements in molecular genetic testing, it has become a standard tool for diagnosing different diseases, including SMA, and the aforemetioned methods are now largely redundant. The molecular genetic methods are more accurate, reliable and timely than non-molecular genetics based methods. The molecular genetic methods have become a standard and most up to date diagnostic tools (Arnold *et al.*, 2015 Hausmanowa-Petrusewicz & Karwańska, 1986; Russman, 2007 and Wang *et al.*, 2007).

1.2.1 Molecular diagnosis

To determine whether a patient is affected with SMA it is essential to establish the deletion or mutation in *SMN1* gene. Single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) methods were the first two methods used to detect *SMN1* deletion and confirmation of SMA disease (Lefebvre *et al.*, 1995). Soon after, in 1997, the SMN gene copy number was analysed using a quantitative PCR-based method by McAndrew (McAndrew *et al.*, 1997). They diagnosed the type of SMA by determining the number of copies of *SMN2* present in the patient. Real-time PCR has also been used to analyse number of *SMN*2 gene and determine the SMA type (Cuscó *et al.*, 2002). 95% of SMA patients have homozygous *SMN1* gene deletion (Lefebvre *et al.*, 1995). The 5% remaining SMA patients are heterozygous, where one of the *SMN1* alleles has been deleted and the other has an

intragenic mutation (Prior *et al*., 2011). A guide of SMA diagnostic tests is provided in Figure 1.3.
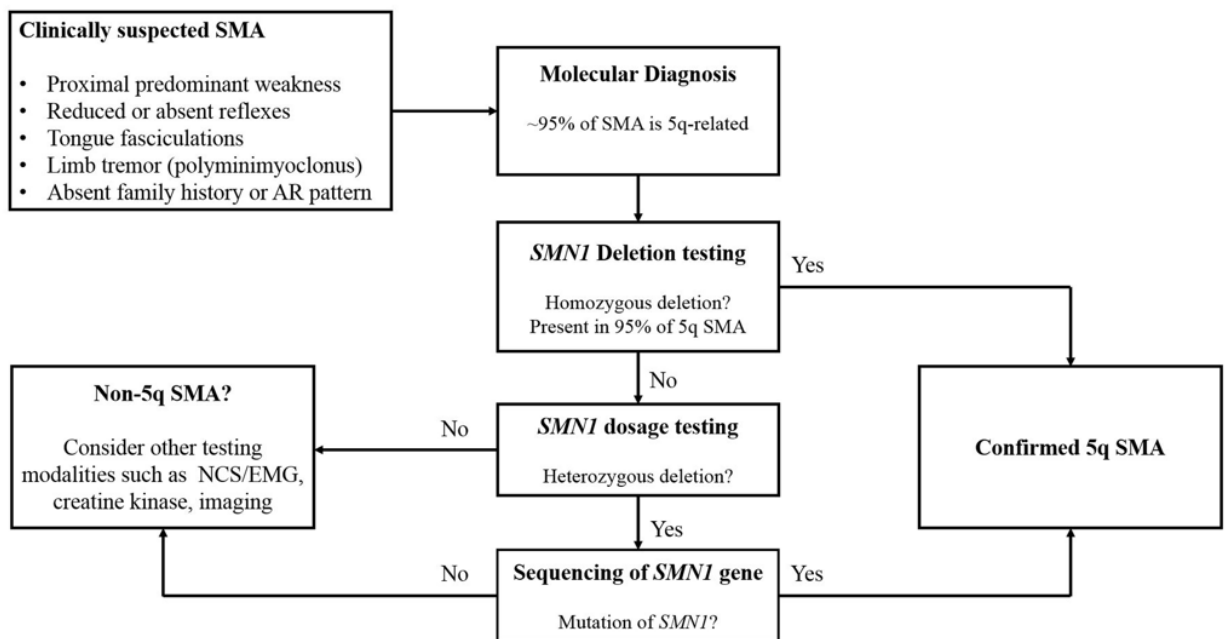
Figure 1.3: A diagram of SMA diagnostic test.

The first diagnostic test for a patient with suspected SMA should be the homozygous *SMN1* deletion. The positive result of *SMN1* deletion testing confirms SMA disease. The negative result will be used for analysing the number of *SMN1* gene and sequencing of the remaining *SMN1* gene to search for any mutation. Further diagnostic tests such as creatine kinase (CK), Electromyogram (EMG) and Nerve Conduction Studies (NCS) will be considered to detect SMA disease or any other motor neuron diseases, if the result of heterozygous deletion and mutation of *SMN1* do not confirm the SMA diseases. Taken from (Arnold *et al*., 2015).

1.2.2 Carrier Screening and prenatal carrier screening

The importance of genetic carrier screening tests is becoming clearer to the public, due to increased education. Currently over 60 laboratories worldwide carry out SMA diagnostic tests (Carré & Empey, 2016). Approximently 1 out of 47 to 1 in 72 SMA diagnostic tests are positive, with the frequency depending on the ethnicity studied (Hendrickson *et al*., 2009 and Sugarman *et a*l., 2012). Determining the number of *SMN1* alleles is essential to give a person the status of 'carrier.' A SMA carrier status is allocated to a person who has only a copy of *SMN1* gene. Dose analysis is applied to determine carrier status or not. This method is based on copy-number, thus it cannot distinguish between a carrier with "2+0" genotype and non-carriers with "1+1" genotype. In addition, this method cannot detect point mutations (Carré & Empey, 2016). Specificity approaches 100% in case of the SMA carrier screening test (Scheffer *et al*., 2000) but the sensitivity of test is almost 90% (Prior, 2008).

It is important to provide educational material to improve the public understanding of any genetic and inherited diseases such as SMA disease and to inform at-risk groups how this disease can pass to the next generation. A recommendation list was provided by the American College of Medical Genetics (ACMG) in 2008. As the SMA disease is not specific to any particular population, and SMA is a worldwide disease (Table 1.2), it is recommend that the carrier test should be made available for all couples regardless of race or ethnicity. Ideally the test should be carried out before conception or in early pregnancy. ACMG suggested formal genetic counselling services must be offered to anyone requesting this testing. They should have educational material regarding the basics of inherited genetic diseases and how they can pass between different members of a family. This is particularly important, as a carrier can have a faulty gene that does not affect them, but is at risk of passing the faulty gene on to their offspring. The

ACMG's guidlines state that all carriers should be referred for follow-up perfectional genetic counselling and offered prenatal and preimplantation diagnosis testing (Prior, 2008). In 2009, the American College of Obstetricians and Gynecologists (ACOG) published a list of recommendations. They do not recommend preconception and prenatal screening for SMA to be on offer to the general population at this time. Patients or couples with a family history of SMA or SMA- like diseases, and those who request SMA carrier screening after completing genetic counselling need to be referred to SMA carrier screening. Patients with positive results of SMA carrier screening follow up with genetic counselling to discuss prenatal and preimplantation diagnosis. An appropriate provider of prenatal genetic counseling and testing services needs to provide fetal testing for SMA for patients who request it (ACOG Committee Opinion, 2009).

Table 1.2: Carrier frequency and detection rate by ethnicity. Taken from (Sugarman *et al*., 2012).

Ethnicity	Carrier frequency	Detection rate
Pan-ethnic	1/54	91.2%
Caucasian	1/47	94.8%
Ashkenazi Jewish	1/67	90.5%
Asian Indian	1/52	90.2%
Asian	1/59	93.3%
Hispanic	1/68	90.0%
African American	1/72	70.5%
Overall	1/54	91.3%

1.2.3 Prenatal diagnosis

Prenatal diagnosis should be offered to people who have a child affected with SMA or if both parents are known to be SMA carriers. The risk of a child affected with SMA in each pregnancy is 25% when both partners carry the faulty gene.

Chorionic villus sampling (CVS) and amniocentesis are current tests which apply fetal DNA analysis to determine the copy number of *SMN1* to clarify whether the unborn child is affected with SMA or not (Carré & Empey, 2016 and D'Amico *et al*., 2011). CVS tests can be carried out by removing a cell sample from the placenta and this particular test needs to be done between the 11th and 13th weeks of pregnancy. Between weeks 15 to 20 of preganacy, an amniocentesis test can be considered if required. However, it needs to be mentioned that both of these tests increase the risk of miscarriage, and cannot identify intragenic mutations. In addition, genotypic information of the parents is required for a more informative result (Carré & Empey, 2016 and D'Amico *et al*., 2011).

1.3 Financial cost of SMA

SMA can cause tremendous physical and emotional suffering to the patient and their family. Additionally, a significant amount of funding is required to cover medical and supportive costs. This section will cover the information regarding the financial burden of the disease on affected SMA patients and their families, and the cost of prenatal screening tests. The patient and their family incur the financial burden, and insurance companies provide funds to treat the disease and cover non-medical accommodations related to the illness. A study was carried out by Lewin group to estimate the annual expenses of several muscular diseases including SMA in the United States. It was

estimated that there were 9,000 people affected with SMA, and one in every 10,000 children born in the United States were affected by this disease. The average medical expenditure for privately insured patients whose disease onset occurred prior to three years of age is 121,682 US dollars annually, whereas mean annual medical costs for patients whose have onset after three years of age is $20,085. Moreover, SMA patients may incur nonmedical costs, such as caregiver salaries, wage loss and expenses of assistive devices to enhance the patient's mobility and independence. Lewin group's study believed 81.45% of patients whose disease onset occurred prior to three years of age require at least 16 hours of attendance per day so this will affect patients and their family's wage. The cost of wage loss for these families is estimated at $35,623 per year. The estimate for families of patients with onset after three years of age is $11,110 (The Lewin Group, 2012).

SMA patients and their family also need funding nonmedical expenditures, estimated at 51,665 US dollars per year (onset before three years). This is needed to cover other nonmedical expenditures, such as home and vehicle modifications, and non-medical professional care. This cost comes down to $14,295 for patients whose onset occurred after three years of life. Altogether, the total annual cost for early-onset (before three years of life) SMA patients is estimated at $684 million and $273 million for SMA patients with an onset after three years of age (The Lewin Group, 2012).

Little *et al*., 2010 investigated the cost-effectiveness of prenatal screening. They estimated $4.9 million for universal prenatal screening for SMA per quality-adjusted life year. Based on their study, prenatal screening for SMA is not cost-effective if offered to the public, and it might only be cost-effective in cases of high risk, such as those with a family history.

Overall, this information underscores the need for effective therapeutics and early intervention for SMA disease (The Lewin Group, 2012).

1.4 Regulation of *SMN* gene

Germain-Desprez *et al*., 2001 demonstrated that the two *SMN* genes (*SMN1* and *SMN2*) are regulated transcriptionally during cell growth and differentiation stage. *SMN* promoter activity depends on the cell types at different developmental stages. For the first time ever, they showed that *SMN* promoter activity is reduced during cellular differentiation and the level of this reduction is four times greater than undifferentiated cells. Analysis of promoter region of *SMN1* and *SMN2* genes demonstrates identical promoter sequences for both genes and *cis*-regulatory elements located within the regulatory promoter. These elements are required for initiating and controlling transcription. There are two strong transcription initiation sites indicated in *SMN* gene: the first transcription initiation site is placed 163 base pair (+1) and the second site is located 246 base pair (-79) upstream of the transcription initiation site in exon 1 (Germain-Desprez *et al*., 2001). The analysis of different tissues show the same *SMN* gene transcription initiation sites for different type tissues of the same developmental stage, while the initiate transcription sites are different depending on developmental stage: fetal stage (_79) or adulate (+1). Germain-Desprez *et al*., 2001, demonstrated RNA from *SMN* during fetal period have a longer 5′ untranslated region (5′UTR) which seems to be the primary product of the *SMN1* gene. Therefore, *SMN1* and *SMN2* are subject to transcriptional regulation during cellular differentiation (Germain-Desprez *et al*., 2001).

1.5 Survival motor neuron protein

As previously mentioned, the full-length SMN protein is a 294 amino acid protein with a molecular weight of 38kDa. SMN protein can be detected in all tissues and cell types but the level of *SMN* expression is not equivalent (Gabanella *et al.*, 2005). High levels of *SMN* expression are detected in the brain, liver, spinal cord, kidneys, and moderate expression in cardiac and skeletal muscle (Coovert *et al.*, 1997). A comparison between the level of SMN protein in the spinal cord of a SMA type I patient and healthy person of same age showed the level of SMN protein was 100-fold less in spinal cord of SMA I patient (Lefebvre *et al.*, 1997).

SMN protein has been suggested to have a variety of biological activities, with roles proposed in transcription, pre-mRNA splicing, stress responses, RNA stability, snRNA biogenesis, axonal RNA trafficking (Burghes & Beattie, 2009) and apoptosis (Parker *et al.*, 2008). The role of SMN protein in these biological activities is described in the following section.

1.6 Biological properties of survival motor neuron

Self-association is a key factor of the importing process of SMN protein into the nucleus (Morse *et al.*, 2007). Morse *et al.*, 2007 demonstrated that the inhibition of SMN self-association can result in the accumulation of SMN protein in cytoplasm, which suggests the presence of oligomeric SMN in the active import complex. The mechanism of SMN nuclear import is still unknown, however, in 2004, Narayanan *et al.*, reported that the SMN nuclear import depends on the presence of Sm snRNPs and that deficiency in self-association of SMN protein can prevent the binding of Sm proteins.

Moreover, self-association domains of SMN protein play an essential role for all SMN functions including RNA splicing, because most SMN binding partners will not interact with monomeric SMN (Lorson *et al*., 1998 and Young *et al*., 2000). There are two separate self-association domains in exon6 and 2b of SMN protein (Lorson *et al*., 1998 and Young *et al*., 2000). Exon 6 contains an evolutionarily highly conserved YG box domain at C terminal which mediates self-association of SMN protein (Lorson *et al*., 1998; Morse *et al*., 2007 and Talbot *et al*., 1997). 'PAKKNKSQK' is a nine amino acid motif within exon 2b that encodes the self-association domain. In addition to coding the self-association domain, this sequence ('PAKKNKSQK') also has an integral role in Cajal body targeting (Morse *et al*., 2007 and Young *et al*., 2000).

Exon7 is responsible for the stability of SMN protein and *in vitro* experiments, demonstrating that the lack of exon7 results in a two-fold shorter half-life for SMN protein (Burnett *et al*., 2009). Exon7 also contains a five amino acid sequence: Gln-Asn-Gln-Lys-Glu (QNQKE). It has been suggested by Zhang *et al*., 2007 that this sequence (QNQKE) plays a role in cytoplasmic localisation of SMN protein and also has axonal function.

1.6.1 SMN complex

The SMN complex consists of at least six other proteins called Gemins. This complex is large, 60 S. It remains very stable for many hours, and is resistant to a high concentration of salt and detergents (Paushkin *et al*., 2002 and Pellizzoni *et al*., 2002). This macromolecular complex appears to have a stabilising effect on SMN protein, whereas the failure of truncated SMN protein to form this complex could explain the shorter half-life of truncated SMN protein compared to the full-length SMN protein. Self-

oligomerisation of SMN protein is necessary to form this macromolecular complex while truncated SMN proteins produced from the *SMN2* gene show a greatly reduced ability to self-associate. SMN protein is placed at a lynchpin position at the heart of this large complex that forms through interaction with all Gemins and SMN protein (Battle *et al*., 2007).

Gemin2 with 32 kDa molecular weight and no homology to any other proteins is one of the proteins essential for forming SMN complex (Liu *et al*., 1997). Gemin2 is involved in two different SMN complexes: a SMN complex forms by binding all of the Gemins and SMN protein and the other complex contains only SMN protein and Gemin2 (Battle *et al*., 2007). Battle *et al*., 2007 suggested the interaction of SMN protein and Gemin2 protein forms a stable core for SMN complex and these two proteins regulate each other's cellular stability. Direct interaction of Gemin2 with SMN protein formed by overlapping of an amino-terminal nucleic acid-binding domain in exon 2a 2b of SMN protein with interaction region of Gemin2 (Bertrandy *et al*., 1999 and Young *et al*., 2000).

Gemin3 is a DEAD-box RNA helicase with 92 kDa molecular weight, and sharing homology with DEXDc superfamily. Gemin3 binds with full length SMN protein directly via its non-conserved COOH-terminal domain. In addition to interaction between Gemin3 and SMN protein, Gemin3 protein also interacts with SmB, SmD2, and SmD3. The DEAD box serves as catalytic activity which has a critical role in the function of the SMN complex in RNA metabolism (Charroux *et al*., 1999). In 2000 Charroux and his colleague introduced Gemin4 as a novel component of the SMN complex. Gemin4 is a 120 kDa protein with no homology with any other proteins. Gemin4 interacts with SMN protein indirectly via its interaction with the DEAD box protein Gemin3.

The other component of the SMN complex is Gemin5 protein, which interacts with SMN protein and several Sm core proteins directly. Gemin5 is a RNA-binding protein (RBP) and a large multi-domain protein with 168 kDa molecular weight containing 13 WD repeats, which are placed at the amino terminal end of this protein, this protein shares homology with WD40 protein superfamily. The Gemin5 protein recognizes the small nuclear RNAs (snRNA) through its fifth WD repeat domains and derive from them a SMN complex, allowing the assembly of the small nuclear ribonucleoproteins (snRNPs) (Piñeiro *et al*., 2015).

Gemin6 is another protein component of the SMN complex with 19 kDa molecular weight which interacts with SMN protein indirectly and via Gemin7 (Pellizzoni *et al*., 2002). In 2002 Baccon *et al*., introduced Gemin7 as a novel protein component of the SMN complex. Gemin7, with a 15 kDa molecular weight, interacts with SMN complex and Gemin6 directly. Gemin6 and 7 interact with several Sm proteins of spliceosomal small nuclear ribonucleoproteins (Baccon *et al*., 2002 and Pellizzoni *et al*., 2002). The structure of both Gemin6 and Gemin7 contain folds that are similar to those observed in the spliceosomal Sm proteins.

Gemin8 was described by Carissimi *et al*., 2006. The molecular weight of this protein is 29 kDa and this protein has no homology with any other proteins. Gemin8 interacts with SMN complex directly as well as a heterodimer of Gemin6 and Gemin7. Direct interaction of Gemin8 with Gemin6 and Gemin7 heterodimer and, together with unrip to form a heteromeric subunit of the SMN complex. Gemin8 interact with Sm proteins, and Gemin8 containing SMN complexes are an essential component for snRNP assembly (Carissimi *et al*., 2006).

1.6.2 Roles of SMN protein

SMN protein serve many functions, such as R-loop resolution (Zhao *et al.*, 2016), translational regulation (Sanchez *et al.*, 2013), roles in small nuclear ribonucleoprotein (snRNP) assembly and Precursor mRNA (pre-mRNA) splicing (Zhang *et al.*, 2008), regulation of actin dynamics and transport of β-actin mRNA (Mélissa Bowerman *et al.*, 2009 and Mélissa Bowerman *et al.*, 2010) and secretion vesicles (Rossoll *et al.*, 2003 and Ting *et al.*, 2012).

mRNA is synthesized as a precursor, pre-mRNA. Pre-mRNA is an immature single strand of mRNA, which after further processing, results in the mature mRNA (Campbell *et al.*, 2008). Initially, it was expected that the SMN protein would have a direct role in pre-mRNA splicing, but it has since been described as the SMN playing an indirect role in pre-mRNA splicing process through its involvement in the formation and maturation of snRNP particles (Coady & Lorson, 2011). RNA polymerase II transcribe both introns and exons from the DNA molecule, therefore the pre-mRNA contains translated (exons) and non-translated (introns) regions, while the mRNA molecule that enters cytoplasm is an abridged version (Campbell *et al.*, 2008). In the RNA splicing process, the introns (non-translated region) are cut out from the pre-mRNA molecule and the exons joined together, forming a mRNA molecule, thus the mRNA synthesis of fully translated regions (Campbell *et al.*, 2008).

The signal for RNA splicing is a short nucleotide sequence at each end of an intron recognised by particles called snRNPs (Campbell *et al.*, 2008). snRNPs are located in the cell nucleus and are composed of protein molecules and one or two small nuclear RNA (snRNA); each molecule is about 150 nucleotides long and are uridine-rich U1, U2, U4, U5 and U6. There are two sets of proteins molecules that bind to snRNPs: one set are common proteins to all snRNPs and the other set is protein specific to each snRNP

(Will & Lührmann, 2001). The common proteins to all snRNPs are called Sm proteins (SmB/B′, SmD1, SmD2, SmD3, SmE, SmF and SmG) within the cytoplasm. The Sm proteins are expressed by seven genes: *SM B/B′, D1, D2, D3, E, F and G F and G* and bind into a conserved uridine-rich sequence on the snRNAs called Sm site to form a heptameric ring (Battle *et al*., 2006 and Will & Lührmann, 2001). The SMN/ Gemin complex interacts with Sm protein and mediates the formation of a ring-like structure around the Sm site of snRNAs. In the cytoplasm, the SMN complex binds directly to both snRNAs and Sm protein. The SMN protein binds to symmetrical dimethyl-arginine residues in SmB, SmD1 and SmD3 (Battle *et al*., 2007). Except that Gemin2, the other component of the SMN complex, will bind directly to Sm proteins. In addition to binding SMN complex with Sm site, the SMN complex binds to snRNAs itself through Gemin5 at the 3′-end of snRNAs (Battle *et al*., 2006 and Pellizzoni *et al.*, 2002). In an ATP-dependent reaction, there is interaction between the Sm protein, Gemins proteins and the SMN proteins, thereby the SMN complex assembles all seven Sm proteins into a heptameric Sm site onto the Sm site within the snRNA (Meister *et al*., 2002). Forming Sm core act as a nuclear imported signal which interact with β nuclear import receptor (Palacios *et al*., 1997). Then SMN/ Gemin/ Sm core complex interact with other part of nuclear import complex which is sunrportin-1. This combination resulting to transport the entire complex into the nucleus. U-snRNP releases the imported complex into the nucleus and then sunrportin-1 molecule is shuttled back into the cytoplasm. U-snRNPs require further modifications and additional subdomains to be able to associate with pre-mRNA splicing.

The SMN complex has a critical role in the maturation process of snRNPs that are involved in general cellular function and survival of any cells and tissues (Narayanan *et al*., 2002 and Rexach & Blobel, 1995) . The low level of snRNP detected in cell line

derived from SMA patients is a consequence of the low level of SMN protein (Wan *et al*., 2005). The key role of SMN protein in the RNA splicing process is essential for all cell types.

Many systems and organs are affected by SMA, such as the autonomic nervous system (Araujo *et al*., 2009), the skeletal muscle system (Hayhurst *et al*., 2012), the heart (Bevan *et al*., 2010), liver (Hua *et al*., 2011), and pancreas (Bowerman *et al*., 2012). This is due to SMN protein deficiency. However, motor neuron cells are the most sensitive and the most strongly affected by the defect of SMN protein (Burns *et al*., 2016). Motor neurons have a temporal requirement for SMN protein in order to be fully functional and to survive (Kariya *et al*., 2014). Many studies have focused on why selective motor neurons degenerate due to low level of SMN protein which is a ubiquitously expressed protein. However, our knowledge of the causes of the motor neuron pathology in SMA is still limited. It is not clear why motor neuron cells are highly sensitive to the level of SMN protein and why a low level of SMN protein results in the degeneration of motor neuron cells (Rossoll *et al*., 2002 and Rossoll *et al*., 2003). Studies suggested that the low level of SMN protein was not sufficient to support the crucial cellular mechanisms required for the survival and maintanace of motor neuron cells (Kariya *et al*., 2014 and Lefebvre *et al*., 1995). Additionally, the level of SMN expression in motor neurons is not equivalent in different developmental stages. As described above, SMN complex is critical and links with snRNP biogenesis. When the snRNP level was found to be high at the embryonic stage, so was the level of SMN protein (d'Ydewalle & Sumner, 2015). The highest level of SMN protein manifested in the gestational and early neonatal stages, however the expression level reduced to basal levels (d'Ydewalle & Sumner, 2015 and Soler-Botija *et al*., 2005). In prenatal and perinatal stages, the level of SMN protein is high. In these stages, the diameter of developing motor axons are small but they reach

to their target muscles. As a result, neuro muscular junctions are not developed completely. It has been demonstrated that the high level expression of SMN protein decreased sharply at postnatal day 17. It is during this developmental stage that the mature neuron muscular junctions are fully formed. The low level of SMN protein associated with SMA disease causes the incomplete maturation of the motor unit and degeneration of the motor neuron and axon (Kariya *et al.*, 2014). Thus, this finding stresses the crucial role of SMN protein in the maturation and maintenance of neuron muscular junction.
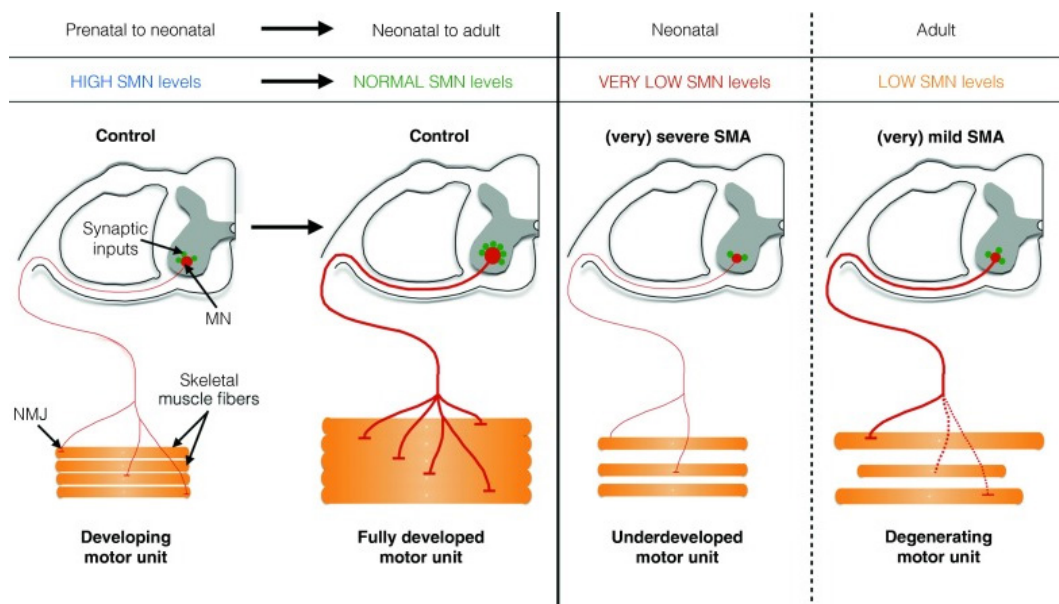
Figure 1.4: SMN is indispensable for development and maintenance of the motor unit.

The graphic above shows the expression of SMN gene as it changes in different developmental stages. There is a decrease in levels of SMN protein between prenatal and early postnatal periods in the spinal cord. The highest level of SMN protein is in the prenatal and perinatal stages. High levels of SMM protein are required for developing motor axons in these two developmental stages of life. In these stages, the diameter of axons is small but they reach to their targeted muscles, and a low level of SMN protein causes immature synaptic inputs and neuromuscular junctions. The level of SMN protein is reduced to a normal level once the motor unit is fully developed and this level of SMN protein is essential for the maintenance of motor unit function. Incomplete maturation of the motor unit, immuration development of synaptic inputs and partial development of neuromuscular junctions occurred in the SMA affected patient, due to low levels of SMN protein. Taken from (d'Ydewalle & Sumner, 2015).

As the SMN protein is an essential molecule in the RNA splicing process, it has been suggested that defects in mRNA biogenesis due to low level of SMN protein might activate intracellular stress signaling pathways, which might lead motor neurons to degenerate in SMA disease (Ahmad *et al*., 2016). It is not clear what intracellular pathways are and how they are involved in the degeneration of motor neurons, however, it needs to be mentioned that by using *in vivo* and *in vitro* model it has recently been established that low levels of SMN protein activate Rho-kinase (ROCK) and the c-Jun NH(2)-terminal kinase (JNK) signaling pathway (Ahmad *et al*., 2016). JNK signaling pathway is found to be active in the motor neurons of SMA patients and SMA mice model (Genabai *et al*., 2015). Sunayama *et al*., 2005 demonstrated knockdown of SMN will activate the JNK signaling pathway in motor neuron culture. JNKs belong to the MAPK family, JNKs play a role in neuronal cell growth, apoptosis, synaptic plasticity, on brain development, the adult brain, memory and brain morphology. Cellular stress will strongly activate the JNK pathway (Coffey, 2014). ROCK belongs to the AGC family of kinases, which play a role in the regulation of the cytoskeleton, neuronal growth, degeneration, differentiation and path finding in motor neurons. *In vitro* experiments showed the low level of SMN results in the activation of ROCK pathway (Bowerman *et al*., 2007 and Nölle *et al*., 2011).

In 2002, Rossoll *et al*., described a novel binding partner for SMN protein. SMN protein interacts with the highly related RNA-binding heterogeneous nuclear ribonucleoprotein (hnRNP-R). A high level of hnRNP-R protein was detected in motor neuron axons and a lower level of hnRNP-R expression observed in sensory axons (Rossoll *et al*., 2002). The high level of SMN protein is localised in axon and growth cones motor neuron cells. The SMN protein is not colocalised with Gemin2 in axons. Considering the interaction

of SMN protein and Gemin2 is an indispensable complex's components for assembling snRNPs, but the fact the SMN protein is not binding with Gemin2 suggests that SMN performs a different and specific function in in axons of motor neurons (Jablonka *et al*., 2001 and Rossoll *et al*., 2003).

In 2003, Rossoll and his colleague demonstrated that only full length SMN protein and its binding partner hnRNP-R could form a complex. This complex interacts with 3′ UTR of β-actin mRNA and translocates to axon and growth cones of motor neuron cells. Therefore, low levels of SMN protein cause the alteration of β-actin protein and mRNA localisation in axon and growth cones of motor neurons. Binding of SMN with hnRNP-R is essential for association of hnRNP-R with β-actin mRNA. The effect of SMN protein levels in the interaction of hnRNP-R with β-actin mRNA could explain the relatively high sensitivity of motor neurons in SMA disease (Rossoll *et al*., 2003). In addition to axon growth, developing motor neuron junctions require a high level accumulation of β-actin mRNA, and the interaction of SMN with hnRNP-R is key for this β-actin accumulation. Thus, a low level of SMN protein has a direct effect on the accumulation of β-actin. It reduces the level of β-actin accumulation and causes poorly developed motor neuron junctions (Coady & Lorson, 2011).

In addition to SMN function in RNA metabolism, it has been suggested that SMN protein plays a role in the regulation of gene expression through an interaction of SMN protein with a nuclear transcription factor. Abnormal gene expression has been observed in SMA disease (Strasswimmer *et al*., 1999).

1.7 SMN is not only a motor neuron disease

Recent studies have proven that SMN is not only a disease of the lower motor neuron and that motor neurons are not the only cells and tissues effected by the low level of SMN protein in SMA disease (Hamilton & Gillingwater, 2013 and Simone *et al*., 2016). Park *et al*., 2010 introduced mice models that expressed low levels of SMN protein specifically in motor neuron cells only. Their novel mouse model demonstrated that depleting the SMN protein in motor neuron cells results in a SMA-like phenotype rather than full-blown SMA disease and low levels of SMN protein in other cells and tissues are necessary to create SMA disease phenotype. The result of these studies clearly emphasized that in addition to motor neuron other cells and tissues contribute to SMA pathology, so SMA is a multi-system disorder and is not only a motor neuron disease (Hamilton & Gillingwater, 2013 and Park *et al*., 2010). Several different researchers presented the finding that SMN protein is critical to the normal function of organs such as muscles (Martinez *et al*., 2012), heart (Gogliotti *et al*., 2012) and pancreas development (Bowerman *et al*., 2012). Table 1.3 and Figure 1.5 represent a number of organs and cells affected by a low level of SMN protein in SMA pathogenesis.

Even when it comes to the nervous system, motor neurons are not the only population affected by SMA disease in this system. Wishart and colleagues stressed the role of SMN in the development of the brain, and for the first time they demonstrated that perinatal growth and normal brain development require high levels of SMN protein (Wishart *et al*., 2010). Not all brain regions require the same level of SMN protein. Regionally selective modifications in brain morphology were apparent particularly in areas normally associated with higher SMN levels such as the hippocampus. Low levels of SMN protein cause modifications in morphology, modified expression levels of proteins regulating proliferation, reduced levels of cellular proliferation and postnatal

hippocampal neurogenesis, migration and developmental processes in hippocampus (Wishart *et al*., 2010).

Hunter *et al*., 2014 claimed that low levels of SMN protein also affect Schwann cells. Schwann cells require a high level of SMN protein for functioning and normal development. Low levels of SMN in Schwann cells results in the failure of these cells to produce normal levels of key extracellular matrix proteins, including laminina2. This failure causes delayed maturation of axo–glial interactions, myelination defects and abnormal composition of extracellular matrix in peripheral nerve. In the light of the combined evidence, the development of a therapeutic strategy that targets the entire nervous system is required to treat SMA disease.

Shanmugarajan *et al*., 2007 suggested lower motor neuron degeneration is not the only cause of bone disease in SMA patients and SMN protein has particular roles in bone cell function. Study of SMA mice skeletal phenotype showed abnormalities in the lower body, bone quality and poorly developed caudal vertebra in comparison to wild type mice. The level of bone area, bone mineral density and bone mineral content is substantially low in effected mice with SMA. Pelvic bone fracture was observed in SMA mice as well as other abnormalities. Moreover, the level of osteoblast differentiation marker, osteopontin, osteocalcin and osterix mRNA expression was lower in SMA mice than non-affected mice (Shanmugarajan e*t al*., 2007, 2009).

Another organ affected in SMA disease is the liver (Hua *et al*., 2011 and Vitte *et al*., 2004). A low level of SMN protein has a significant effect on the liver, which is not surprising as the level of SMN protein is high in the healthy liver. Investigation of SMA mice liver showed striking liver dysfunction, iron overloaded (Vitte *et al*., 2004). SMA mice are small in size, which could indicate growth retardation, and liver-derived insulin-like growth factor 1 (IGF-1) which is required to support both normal growth

postnatal and cardiac development and function. The enzyme-linked immunosorbent assay (ELISA) showed low levels of IGF-1 in SMA mice model. Low levels of IGF1 in SMA mice is likely because of low expression of I*gfals* gene expression. I*gfals* gene expresses IGF-binding-protein acid labile subunit (IGFALS), which is a hepatic protein that stabilises IGF-1. Low level of I*gfals* gene expression correlates with low levels of SMN protein in SMA disease (Hua *et al*., 2011).

Thus, there is strong evidence to support the idea that SMA disease is not solely a result of motor neuron degeneration. Multiple systems become vulnerable in SMA disease because of a low level of SMN, so SMA is a multi-system disorder disease. In conclusion, a good understanding of different phenotypes subsequent to the low expression of *SMN* gene is crucial to develop a fully functional therapeutic strategy (Hamilton & Gillingwater, 2013).

Table1.3: Summary of peripheral organ defects in SMA pathogenesis.

Biological function	SMN-dependent intrinsic defects	References
Autonomic nervous system	Cardiac dysfunction, bradycardia, abnormal fluctuation of blood pressure, high plasma concentration, vasculitis, coagulopathy and vascular abnormality.	(Araujo *et al*., 2009; Bevan *et al*., 2010 and Hachiya *et al*., 2005)
Liver function	Reduced hepatic *Igfals* expression, leading to a pronounced reduction in circulating insulin-like growth factor 1 (IGF1) and stunted growth	(Hua *et al*., 2011)
Muscle development	Impaired satellite cell differentiation muscle weakness and delay in expression of mature isoforms of proteins important for muscle function.	(Boyer *et al*., 2013 and Hayhurst *et al*., 2012)
Pancreas development	Abnormalities in pancreatic islet cells with dramatic increase in glucagon-producing α-cells than of insulin-producing β-cells, fasting hyperglycemia, hyperglucagonemia, glucose resistance	(Bowerman *et al*., 2012, 2014)
Neuromuscular junction function and maturation	Abnormal neurofilament accumulation in the nerve terminals and poor terminal arborization, impaired maturation of the NMJ, defect post-natal development of the neuromuscular synapse, disrupted astrocyte functions and impaired normal development and function of Schwann cells.	(Hunter *et al*., 2014; Kariya *et al*., 2008 and Rindt *et al*., 2015)
Brain	Impaired hippocampal neurogenesis and brain development	(Wishart *et al*., 2010)
Lung	Discolorations of the lungs suggestive of atelectasis or pulmonary infarctions	(Schreml *et al*., 2013)
Intestine	Intestinal edema	(Schreml *et al*., 2013)
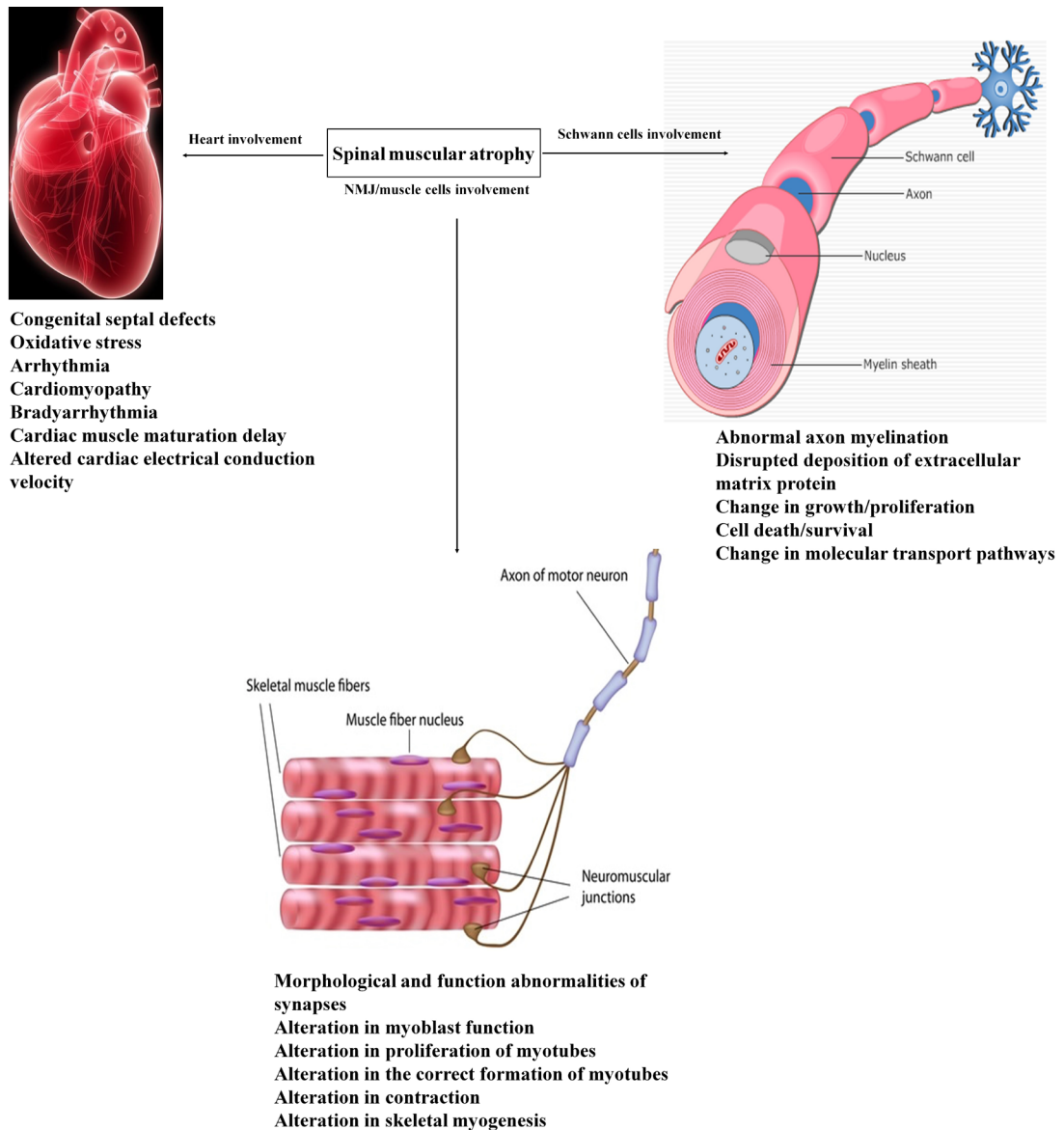
Figure 1.5: Example of non-motor neuronal tissue pathology in SMA: cardiac defects.

SMA is caused due to loss of the *SMN1* gene and results in low levels of full length SMN protein in all different tissue and cell types. Motor neurons require high levels of full length SMN protein for survival and normal development and function. But SMA is not only a motor neuron disease, and other cells and tissues contribute to SMA pathology. Taken and modified from (Simone *et al*., 2016).

1.8 Current progress towards SMA therapy

The most common and readily available treatment is the use of drugs or small molecules, which focus on increasing *SMN2* gene expression, or transcription to increase the level of full length SMN protein. Histone deacetylase inhibitors (HDACis) were the first drugs used for treatment of SMA (Hahnen *et al*., 2006 and Lunke & El-Osta, 2013). HDACis increase endogenous *SMN2* promoter activity, thus increasing the level of SMN protein in the cytoplasm and nuclei of cells. The result of testing this drug in SMA mice model showed improvement of life span for up to 38 days compared to untreated mice with an average survival of 13 days (Chang *et al*., 2001 and Narver *et al*., 2008). Moreover, this drug improved muscle pathology, cardiac phenotypes, motor performance and weight (Avila *et al*., 2007 and Heier *et al*., 2012).

Valproic acid and phenylbutyrate are two other drugs that have undergone clinical trials but did not show very promising results (Mercuri *et al*., 2004 and Weihl *et al*., 2006). However, testing of valproic acid on patients affected with SMA III and IV showed some improvement in muscle strength (Weihl *et al*., 2006).

Suberoylanilide hydroxamic acid (Hahnen *et al*., 2006) and Trichostatin A (Avila *et al*., 2007) are two other drugs that have been considered for treatment of SMA and are undergoing clinical trials.

Another therapeutic approach is the use of antisense oligonucleotides (ASOs). ASOs can be used to correct splicing of *SMN2* transcripts and increase the level of SMN protein. These act by binding to *SMN2* intronic splicing silencer, which increases SMN levels by avoiding skipping of exon 7. ASOs10-27 was tested in SMA mice model and showed promising results in improving neuromuscular pathology, weight, muscular fibro size and necrotic phenotypes. Furthermore, it increased the life span by up to 25-fold as compared to untreated mice (Hua *et al*., 2010, 2011; Passini *et al*., 2011and Williams *et*

al., 2009). This drug is under phase I clinical trials in individuals with SMA type I-III and it has been established that this drug can be directly injected into the cerebral spinal fluid via lumbar puncture.

On December 2016, the U.S. Food and Drug Administration approved Spinraza (nusinersen). This is the first drug that has been approved to treat children and adults affected with SMA. Spinraza is an ASO, this drug increases the level of full length SMN protein by binding and altering the splicing of a single RNA from the SMN2 gene and enhancing the inclusion of exon 7 into the SMN protein. Spinraza is delivered directly into the cerebrospinal fluid via intrathecal injection (Finkel *et al*., 2016). The efficacy of Spinraza was studied in a clinical trial, with a participation group of 121 SMA patients. The results of the clinical trial demonstrated improvement in motor function. In addition, a greater number of patients treated with Spinraza survived, compared to untreated patients. The most common side effects found in treated patients were upper respiratory infection, lower respiratory infection, constipation, renal toxicity, neurotoxicity, coagulation abnormalities and thrombocytopenia (Finkel *et al*., 2016). This drug is available in the US and a wider distribution into more countries in 2017 is in progress.

Naryshkin *et al*., 2014 identified three small molecules, SMN_C1, SMN_C2 and SMN_C3, that have therapeutic advantages for SMA disease and can be considered as a therapeutic strategy to treat SMA patients. These small molecules selectively modulate the inclusion of exon 7 in the SMN2 mRNA. These compounds can be administered orally and are able to cross the blood-brain barrier. The test for these compounds were performed on both *in vitro* and *in vivo* models. For *in vitro* expriments, the SMA I patient fibroblast cells were used and the treat the cells were treated with SMN_C1, SMN_C2 and SMN_C3. The theraputic effect of these compounds was investigated 24 hours after the treatment and it demonstrated that the level of Δ7 mRNA decreased and the level of

FL *SMN2* mRNA increased, thus the level of full length SMN protein increased in SMA I patient fibroblast cells. Moreover, these compounds showed positive results when they were tested whether they are capable of increasing SMN protein level in disease-relevant cells which were motor neurons generated from SMA patients's iPSCs. Analysis of result of Δ7 mices established that these three compounds increase SMN protein level, survival time, body weight and improved motor neuron function. These compounds increased the SMN protein level *in vivo* and *vitro* experiment in different cell types while they do not have major effect on the expression of genes other than *SMN2* (Naryshkin *et al*., 2014).

Roche (Basel, Switzerland), PTC Therapeutics (South Plainfield, New Jersey, United States) and SMA Foundation started a clinical trial to test a small molecule called RG7800. RG7800 acts as the first SMN2 splicing modifer in clinical trial phase (1b/2a). The clinical trial started in November 2014 but was halted in May 2015 due to safety concerns. The safety issue arose when unexpected side-effects were observed in the eyes of treated mice using RG7800, and the clinical trial was suspended immediately as a precautionary measure. It needs to be mentioned that no safety issue has been reported by SMA patients who were involved in this clinical trial. The SMA patients in this clinical trial were treated with a lower concentration than the dose that was used to treat animals. This study remains on hold and the safety issue is under investigation (Calder *et al*., 2016).

A second SMN2 splicing modifer which encourages the production of more SMN protein called RG7916 was introduced soon after holding a clinical trial for RG7800. The small molecule was tested in healthy volunteers in a Phase I clinical trial in January 2016. The purpose of this clinical trial is to evaluate the safety, pharmacokinetics and effect of this molecule in healthy volunteers. This clinical trial is ongoing and it is a

collaboration between Roche (Basel, Switzerland), PTC Therapeutics (South Plainfield, New Jersey, United States) and SMA Foundation (Calder *et al*., 2016).

There are advantages associated with the use of small molecules for therapy purposes, namely that they are simply producable and orally available. However it is important to note the main limitaion of using small molecules, which is the risk of unexpected side effect and/or affecting non-targeted genes (Faravelli *et al*., 2015).

Gene therapy is highlighted as a potential option for the treatment of SMA. As previously explained, SMA is caused by insufficient levels of SMN protein, which occurs as a response to mutation/deletion in *SMN1*, and although functional *SMN2* is present, it cannot code for a sufficient amount of protein to maintain normal phenotype. The first study to use, a viral vector to deliver *SMN* gene into patient cells demonstrated that the adenoviral vector encoding the *SMN* gene could increase the level of SMN protein in SMAI fibroblast (DiDonato *et al*., 2003). This study also confirmed that the SMN protein expressed using *SMN* gene delivered by adenoviral vector is fully functional and interacts with its normal cellular binding pattern. Study of the number of gems in transduced SMA I fibroblast showed the number of gems restored in transduced SMA I fibroblast to the same or more than the number of gems in control fibroblast.

Recent research in SMNΔ7 mouse fibroblast shows that SMA can be rescued by increasing the expression of *SMN1* (Valori *et al*., 2010). Valori *et al*., 2010 demonstrated that level of SMN expression in fibroblast cells from type-1 SMA patients transduced with codon optimised-*SMN1* is about 2-fold higher than the level of expression obtained from fibroblasts transduced with wild type-*SMN1*. Previously it has been shown that incubating SMA type-1 fibroblast with lentivectors containing human *SMN1* gene can lead to an increase in the level of SMN protein (Azzouz et al., 2004). This extended the life span of mice by 3-5 days and decreased the death of MNs in SMA mice.

Interestingly, only a minimal immune response was noted in these mice at 7 days after injection. Hence, this provides a proof of principle for the use of lentiviral vectors to deliver *SMN1* gene as a probable gene therapy option for treating SMA.

Injected SMA mice with one dose of self-complementary adeno-associated virus 9 (scAAV9-*SMN*) demonstrated very promising results in terms of rescuing neuromuscular physiology and motor neuron function and extend SMA mice life span (Foust *et al*., 2010). As one of the great approaches of this study is related to time point injection, this study will be explained in more details in the following section (Foust *et al*., 2010).

Single intracerebroventricular injection of scAAV9 delivering human *SMN* gene under the control of the chicken β-actin promoter (CBA) carried out at postnatal day one SMA mice. The outcome of this study demonstrated the highest increase in survival time (282 days) of SMA mice reported at the date of this publication. The improvement in survival correlated with an increase in body weight, motor neuron performance and behavioural improvement (Meyer *et al*., 2015). Meyer *et al*., 2015 demonstrated at least 20 to 40 percent of motor neurons throughout the spinal cord have to be transduced for a considerable therapeutical effect. A maximum therapeutic effect will be achieved when half of the motor neurons in the spinal cord are transduced.

In addition, pre-clinical experiments have been carried out on SMA non-human primate models. (Duque *et al*., 2015) established a large SMA animal model which could potentially be a useful model for the pre-clinical stage. To generate a SMA pig model the level of SMN protein in pig motor neuron cells was reduced efficiently by knockdown. In this study, *SMN1* gene was knocked down in motor neuron five-day old pigs using intrathecal delivery of scAAV9-shRN. To our knowledge, this study is the first report of the large SMA model in the domestic pigs. Duque *et al*., 2015

demonstrated that a 73% knockdown in motor neurons postnatally is sufficient to generate an SMA-like phenotype. Key SMA symptoms appeared, such as muscle weakness, loss of motor neurons and abnormal posture appeared three or four weeks post injection.

SMA pig model received scAAV9_*hSMN* intracisternally when onset of symptoms was determined in animals whose *SMN* gene was knowkdown. Delivery of human *SMN* had a marked impact on SMA disease progression. Therefore, their result indicated gene therapy was useful in SMA pig model at post-symptoms.

A Phase I/II gene transfer clinical trial on affected children with SMA Type I is in progress. This study started in April 2014 in Nationwide Children's Hospital in Columbus, Ohio. AAV9 carrying the *SMN* gene under the control of a hybrid CMV enhancer/ CBA promoter injected intravenously through a peripheral limb vein. Dr. Jerry R. Mendell, the leader of this study, presented data from this ongoing Phase I/II trial of AVXS-101 at the World Muscle Congress Brighton, United Kingdom – October 5, 2015. The preliminary observations in terms of safety and tolerability of the injected vectors in the nine patients studied to date are promising. A test designed to measure motor skills of patients affected with SMA Type I by The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND). All patients in this study are subjected to this test to determine whether there is any improvement in their motor function. The outcome of this test demonstrated that patients injected with 6.7 X 10^{13} vg/kg of vectors have modest improvement in their motor function and greater improvement observed in the group of patients who received 2.0 X 10^{14} vg/kg of vector. This clinical trial is ongoing and estimated completion date for this study is December 2017

(https://clinicaltrials.gov/ct2/show/NCT02122952?term=NCT02122952&rank

=1 and http://investors.avexis.com/phoenix.zhtml?c=254285&p=irol-newsArticle&ID=2166123, on 11.08.2016).

Although the field of gene therapy has some promising approaches, there are some complications associated with the use of vectors: (i) vectors can lead to insertional mutagenesis if they integrate into the host chromosome, (ii) virus-based vectors can cause an immune response which reacts against the vector or in some cases neutralizes the delivered vectors; (iii) chance of the viral reactivation in the vector due to recombination events (Braun, 2013).

1.9 Therapeutic time window

Another subject which is under discussion in the field of SMA therapy is the best time to deliver SMN-targeted therapies. Intravenously injected scAAV9-*SMN* into SMA mice model at postnatal day 2 and 5 (P2 and P5) demonstrated different mean survival in treated animals (Foust *et al*., 2010). Animals which received injection at P2 increased mean survival from 14 days to 250 days. However, P5-injected animals survived 15 days longer than mean survival.

In contrast, two separate studies by Foust *et al*., 2010 and Hua *et al*., 2011 demonstrated injected SMA mice model at P8 (onset of obvious symptoms) provide very little amelioration of disease symptoms.

Furthermore, in 2014 Robbins *et al*., injected SMA mice with scAAV9-*SMN1* vector via intracerebroventricular (ICV) at different early time points. In this study SMN mice were administered a single injection of 1×10^{11}, on a single day from P2 to P8. Injection at earlier time points demonstrated a greater therapeutic benefit than the injection at later time point. In terms of survival time, the SMA mice that received injection at P2 survived

more than 130 days. It needs to be mentioned that the therapeutic effect of viral vector injection steadily declined at late time point injection, however they lived significantly longer than non-transduced SMA mice. In terms of weight gain, the injected SMA mice showed higher increased weight compared with non-injected SMN mouse. On the other hand, comparing how injection at different time points is relevant with body weight showed injected animals can be divided into two group based on similar weight gain from birth to their peak. Animals injected at P2-P4 were placed into one group and animals injected at P5-P7 were placed into the other. Study of motor neuron function confirmed that injection at the early time point produced stronger motor function.

Thus, taken together, it is clear that maximal therapeutic benefit will be achieved when treatment is applied at very early stage of postnatal. This therapeutic time window leaves one question unanswered: what happens at the early stage of SMA disease which makes it necessary to have a time limit for treatment. Therefore, diagnosis of SMA disease in newborn babies is critical, in order to start treatment as soon as possible to achieve robust therapeutic benefits.

1.10 Gene therapy

Gene therapy is defined as the use of genetic material to prevent / to correct disease. This can be achieved by the introduction of genetic material into host cells (Verma & Weitzman, 2005). The basis of molecular genetics and gene transfer in bacteria was established in the 1960s, and was soon followed by the transfer of genes of interest into animals and humans using genetically modified cultured cells and virus-based vectors (Wolff & Lederberg, 1994). Delivering genes to mammalian cells offers great possibility to the treatment of human disease. By the end of 1990, gene therapy began to make serious progress with two *ex vivo* gene therapy trials approved in the US. However, in 1999, a patient who suffered from ornithine transcarbamylase deficiency and participated in the trial died within 98 hours. The patient was injected with an adenoviral vector carrying corrected copy of the gene. The patient died due to systemic inflammation, intravascular coagulation, and multi-organ failure (Raper *et al*., 2003 and Wilson, 2009). Several other cases were reported around the first clinical trials due to insertional mutagenesis of viral vectors used (Browning & Trobridge, 2016). This hampered the success of gene therapy clinically, but the field is continually improving for effective clinical application (Scollay, 2001).

Gene therapy can be broadly classified into: (1) Somatic cell gene therapy, which involves modification of the somatic cells or tissue, and (2) germ-line gene therapy, in which genetic changes are employed into a gamete, zygote, or an early embryo. Modifications in germ-line therapy are permanent and are passed on to the next generation. However, germ-line gene therapy is illegal due to ethical considerations (Nielsen, 1997). Various gene therapy approaches have been employed for the treatments of different diseases, such as gene addition, gene correction, gene silencing, reprogramming and cell elimination. Gene addition involves transferring the relevant

genes into cells using a vector at non-homologous chromosomal sites. The gene of interest and its control signals are packaged into either non-viral or viral vectors and delivered into the host cell. The new gene has its own control signals including start and stop codons. This strategy is most used in diseases arising due to the loss of function of genes. In contrast, in gene correction, defective genes or gene fragments are exchanged with a corrected portion of gene with or without supplemental recombinant machinery that efficiently recombines with the defective gene at its chromosomal location (Mulligan, 2011 and Verma & Weitzman, 2005).

Gene silencing involves interruption or suppression of gene expression at either transcriptional or translational levels. This method is usually used to turn-off specific genes in diseases in which, for example, tissues produce too much protein from a specific gene. Cell elimination strategies involve the use of suicide genes, oncolytic viruses, anti-angiogenesis agents, or toxic proteins that can mount an immune response to the unwanted cells. This strategy is widely used for treatment of cancer and overgrowth of certain cell types (Bast, 2004; Dykxhoorn & Lieberman, 2005 and Seilicovich *et al*., 2005).

Transfer of genetic material of interest in a cell can take place either *in vivo* or *ex vivo*. In *ex vivo* models, the gene transfer involves genetic modification of target cells in the laboratory, which are then returned to the patients. This method can be applied to a defined cell population such as T-lymphocytes, hematopoietic stem cells, or tumour cells. In *in vivo* models, the gene is transferred to the recipient cells directly, Figure 1.6. In both strategies, a vector delivers the genetic material to the intracellular site where it can function (Verma & Weitzman, 2005 and Wolff & Lederberg, 1994).
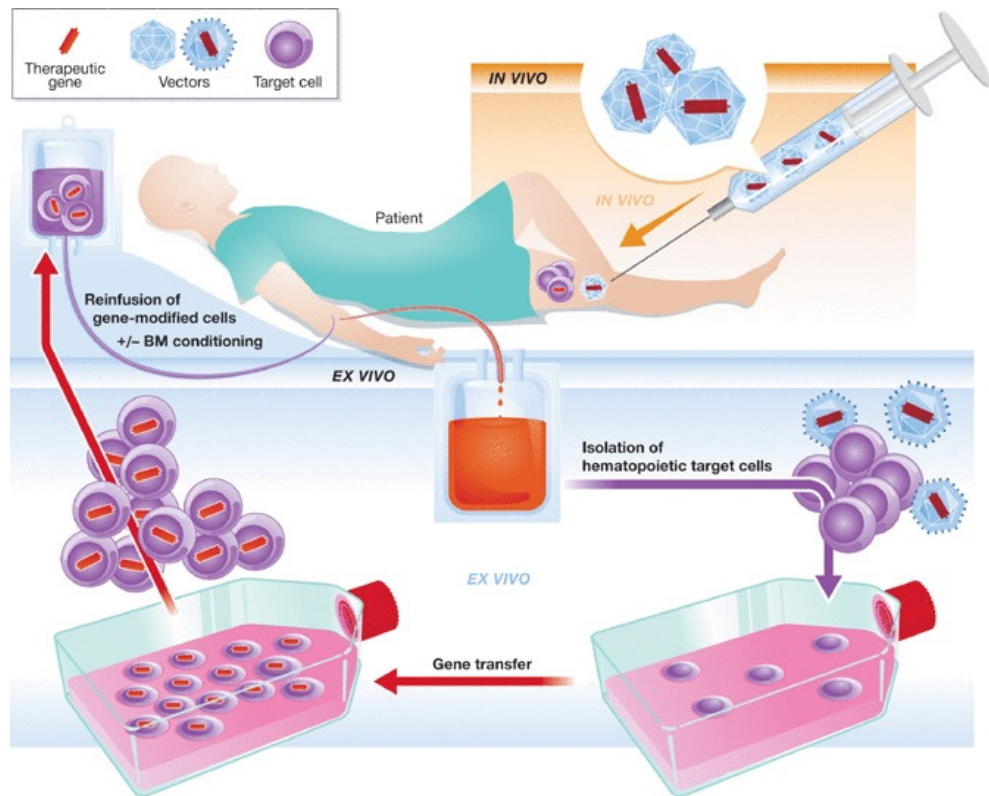
Figure 1.6: *Ex vivo* and *in vivo* gene transfer.

Ex vivo gene therapy involves the harvesting of a patient's cells. The collected cells are maintained in the laboratory. The cells are modified using the viral vectors carrying the therapeutic gene. The transduced cells are transplanted back into the patient's body. Conversely, *in vivo* gene therapy can be carried out by direct injection of a virus carrying the gene of interest into the patient's body. Taken from (Kaufmann *et al*., 2013).

1.10.1 Gene therapy vectors

An effective gene delivery system should be capable of efficient, stable gene transfer into a wide verity of cells, tissues and organs without causing any associated pathogenic effect (Verma & Weitzman, 2005). Carriers in gene therapy are classified into two major types: viral and non-viral vectors. Viral vectors are derived from either RNA or DNA viruses. The non-viral delivery system vectors consist of naked DNA delivered to cells *ex vivo* either via electroporation or by intravascular delivery. Mostly naked DNA methods have been used for genetic immunization studies (Verma & Weitzman, 2005).

Naked or plasmid DNA are used only for 17.9% of gene therapy clinical trials while the majority (45%) of trials use viral vectors (http:// www.wiley.co.uk/genmed/clinical/ on 21.03.2016).

Viral vectors can be divided into two categories, based on their genetic material. The most commonly administered RNA viral vectors are derived from retroviruses and these were among the very first viral delivery systems to be developed for gene therapy purposes. Adenovirus, the adeno-associated virus, Poxviruses, Baculovirus and Herpes Simplex are DNA viral systems, Table1.4 provides key information about different types of viral vector.

Table1.4: viral vectors for gene therapy.

Viral system	Particle size	Genetic material	Size of genome	Maximum transgene capacity	References
Adenovirus	70-100 nm	dsDNA	26-40 kb	Up to 36 kb	(Choudhury *et al.*, 2016 and Warnock *et al.*, 2011)
Retrovirus	80-100 nm	ssRNA	7-12 kb	7-8 kb	(Choudhury *et al.*, 2016 and Warnock *et al.*, 2011)
Lentivirus	100 nm	ssRNA	7-12 kb	7-9 kb	(Choudhury *et al.*, 2016 and Warnock *et al.*, 2011)
Adeno-associated virus	20-25 nm	ssDNA	4.7 kb	4.7 kb	(Choudhury *et al.*, 2016)
Baculovirus	40-50 nm	dsDNA	80 to 180 kb	Up to 134kb	(Sung *et al.*, 2014)
Herpes simplex virus	120-300 nm	dsDNA	152 kb	Up to 150	(Choudhury *et al.*, 2016 and Warnock *et al.*, 2011)
Poxvirus	200-300 nm	dsDNA	190kb	25kb	(Vannucci *et al.*, 2013)

An ideal vector to deliver genes of interest should be safe, easy to produce at high concentration and to purify using simple techniques, able to transduce dividing and non-dividing cells, be stable and provide high levels of therapeutic genes in target tissue without any immunological response, toxicity, or damage to the surrounding matrix and tissue (Mohan *et al*., 2013). The advantages and disadvantages of different viral delivery systems for gene therapy are summarised in Table 1.5.

Table 1.5: Advantages and disadvantages of most common viral vectors.

Vectors	Advantages	Disadvantages	References
Adenovirus	•Ability to transduce both dividing and quiescent cells • Large insert capacity • Non-oncogenic • Stability of recombinant vectors • High-level transgene expression • Easy production with ready to use packaging cell • Vector particles produced at high titer	• High level of pre-existing immunity • Highly immunogenic • The vector genome does not integrate into the host genome • Transient expression of the transgene	(Sung *et al.*, 2014; Vannucci *et al.*, 2013 and Warnock *et al.*, 2011)
Retrovirus	• The vector genome integrates into host cell genome • Engineering fairly simple • Wild cellular tropism • Low immunogenicity • No (or very low) pre-existing immunity • Theoretically stable transgene expression	• Inability to transduce non-dividing cells • Cellular targeting difficult to achieve • High risk of insertional mutagenesis • Random integration of the retroviral genome • Limited insert size • Require transfection of multiple plasmids into packaging cells for vector production • Transgene prone to silencing	(Sung *et al.*, 2014 and Vannucci *et al.*, 2013)
Lentivirus	• Ability to transduce both dividing and quiescent cells • The vector genome integrates into host genome • Integration-defective vectors available • Stable transgene expression • Absence of pre-existing immunity	• Possible insertional mutagenesis • Transient expression of the transgene with integration-defective vectors • Limited insert size • Requiring transfection of multiple plasmids into packaging cells for vector production	(Sung *et al.*, 2014 and Vannucci *et al.*, 2013)
Adeno-associated virus	• Ability to transduce both dividing and quiescent cells • Low immunogenicity • Broad host cell type tropism range • Non-pathogenic	• Pre-existing immunity • Smaller size limits the amount of foreign genes that can be inserted • Possible transgene integration	Sung *et al.*, 2014; Vannucci *et al.*, 2013 and Warnock *et al.*, 2011

	• Capable of long term transgene expression	• Requiring transfection of multiple plasmids into packaging cells for vector production	
Poxvirus	• Multiple sites of transgene insertion • Particularly apt as attenuated recombinant vaccine • Well suited as oncolytic vectors •low level of pre-existing immunity	• Potentially cytotoxic • Highly immunogenic • Heterologous promoter difficult to use • Generation of recombinants complicated •Transient expression of the transgene	(Vannucci *et al.*, 2013)
Herpes simplex virus	• Wild-type cellular tropism • Large insert capacity • Natural tropism for neuronal (HSV-vectors) or B lymphoid cells (EBV) • Well suited as oncolytic vector	• Possible residual cytotoxicity • The vector genome does not integrate into the host genome • Transient expression of the transgene • Risk of recombination in latently herpes simplex virus-infected cells • High level of pre-existing immunity	Vannucci *et al.*, 2013)
Baculovirus	• Non-pathogenic to human • DNA degradation and lack of integration that improve safety • No-pre-existing immunity • Easy production by infection in BSL-1 facilities • Large cloning capacity	• Transient transgene expression • Vulnerable to mechanical force and loss of virus titer during virus concentration and purification • Inactivated by serum complement	(Sung *et al.*, 2014)|

Each viral vector has an inherent set of attributes that affects its suitability for a specific form of gene therapy. For instance, in some forms of gene therapy long term expression from a relatively small number of cells is enough but in other cases it might need expression in a large number of cells for effective treatment (Kay *et al.*, 2001). Thus, it is unlikely that only one viral vector will emerge as a suitable vector to treat all diseases, as they have different abilities and structures. Therefore, a range of vectors will be

required to fulfill the objective of each disease; Table 1.6 lists the gene therapy clinical trials that used different type of viral vectors to deliver gene of interest.

The life cycle and structure of viruses represent highly evolved natural tools that could be exploited to engineer recombinant vectors to deliver therapeutic genes into a range of hosts. The life cycle of a virus can be divided into two-phases, infection and replication, which leads to the introduction of viral genes into host genetic material and resulting into pathogenesis. Converting a virus into a vector is based on the ability to separate the components that are necessary for causing disease from those that are involved in replication (Verma & Weitzman, 2005).

Problems that might arise when using vectors for gene therapy include toxicity from the infusion materials, humoral immune responses against the therapeutic gene product, cellular immune responses directed against the transduced cells and possibility of insertional mutagenesis by certain integration vectors (Kay *et al*., 2001). But in recent years, remarkable progress has been made in developing and improving gene therapy into an applicable treatment. The number of candidate diseases to be targeted with gene therapy has increased significantly (Walther & Schlag, 2013). As only lentiviral vectors were employed in this thesis, the following sections focus mainly on them.

Table 1.6: Examples of viral vectors in gene therapy trials

Disease	Vector	Transgene	Phase	Trial code
Alzheimer	AAV	Nerve Growth Factor	I/II	NCT00087789, NCT00876863
Spinal Muscular Atrophy Type 1	AAV	Survival Motor Neuron	I	NCT02122952
Leber Congenital Amaurosis	AAV	Retinoid isomerohydrolase	I/II	NCT02781480
Pleural Malignancies	Adenoviral	Interferon-beta	I	NCT00299962
Severe Combined Immunodeficiency	Retrovirus	Adenosine deaminase	II	NCT00794508
Beta-thalassemia	Lentivirus	Human Beta-globin	I/II	NCT02453477
Parkinson	Lentivirus	Aromatic amino acid decarboxylase, Tyrosine hydroxylase, GTP-cyclohydrolase 1	I/II	NCT00627588
Glioblastoma multiforme (GBM), other gliomas	Oncolytic poliovirus		I	NCT00805376, NCT01956734
Cancer Pain	Herpes Simplex Virus	Preproenkephalin	I	NCT00804076

1.10.1.1 Retroviral vectors

The most commonly used viral vectors employed in gene therapy are derived from the retrovirus family. Retrovirus-based vectors are used to deliver various genes of interest into humans and animals *in vitro* or *in vivo* because they are unique in their properties to integrate their genome into the host genome. This family of viruses is equipped with an enzyme called reverse transcriptase, which transcribes a RNA template into DNA, providing RNA to DNA information flow, which is the reverse flow of genetic information. The unusual phenomenon is the source of the name retroviruses (retro meaning backward) (Hacein-Bey-Abina *et al*., 2003 and Hendrie & Russell, 2005). Retroviruses are lipid-enveloped particles comprising a homodimer of linear, signal strand RNA genome of 7 to 11 kilobases and positive-sense. The retrovirus family includes several subfamilies such as oncovirinae, lentivirinae (such as human immunodeficiency virus (HIV) and other immunodeficiency viruses), and spumavirinae. The viral envelope glycoprotein dictates the variety of host range via their interaction with receptor on the host cells. In other word, viruses identify host cells by a "lock and key" fit between viral surface protein and specific receptor molecules located externally to the cell surface (Kay *et al*., 2001and Mohan *et al*., 2013).

1.10.1.2 Lentiviral vectors

Lentiviruses are a genus within the retrovirus family, and are known as complex retroviruses based on the details of the viral genome. The genome structure of lentiviral vector consists of two identical single-strand RNA molecules (Frankel & Young, 1998). Lentiviruses have potential to transduce both dividing and non-dividing cells, thus making them an attractive tool for gene therapy. Vectors derived from lentiviruses have

been proven efficient gene delivery vehicles as they integrate into the host's chromosomes and show continued expression for a long time (Blömer *et al*., 1997). They also have a relatively large cloning capacity for forging genomic material, which is sufficient for most clinical purposes. Lentiviral vectors are able to be pseudotyped with different viral envelope proteins that influence tropism and transduction efficiency of this viral vector (Frankel & Young, 1998 and Picanco-Castro *et al*., 2012).

Lentiviral vectors are based on HIV-1 and have interesting properties to be used as an effective vector system. The outermost envelope of the HIV consists of two envelope glycoproteins: gp120 and gp41: these envelop protein mediated entry of HIV virus into host cells, Figure 1.7. Inside the viral envelopes is a bullet-shaped core called (CA), with each CA containing roughly 2,000 molecules per virion. HIV's genome is roughly 10 kb long and contains two single strands of RNA capped by long terminal repeats (LTRs) at both ends, Figure 1.8. LTRs play an important role in reverse transcription of viral genome, control production of new viruses, and integration into host (Frankel & Young, 1998).
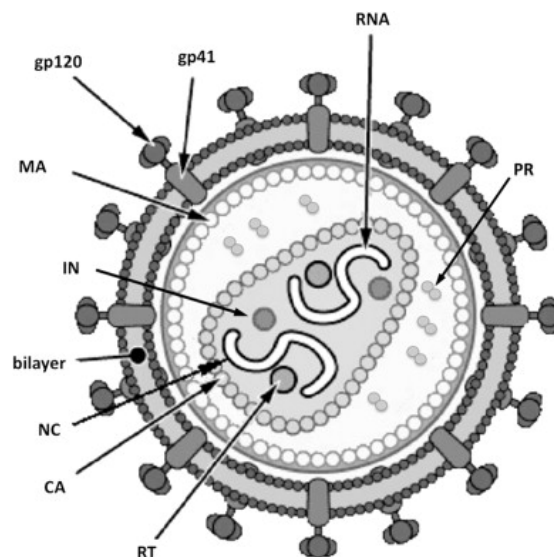
Figure 1.7: Structure of mature HIV virion

Structural elements of HIV-1 virus are MA: matrix protein; NC: nucleocapsid protein; CA: structural capsid protein; RT: reverse transcriptase; IN: integrase; PR: protease, glycoprotein gp120; glycoprotein gp41; protease (PR). Taken from (Teixeira *et al*., 2011).

HIV genome consists of three open reading frames containing the structural genes *gag*, *pol* and *env*. These genes are common to all retroviruses. *Gag* encodes a polyprotein that is cleaved into matrix (MA), CA and nucleocapsid (NC) protein. *Env* gene cods for glycoprotein gp160, which is cleaved to gp120 and gp41, the outer membrane proteins of HIV virions. These two glycoprotein (gp120 and gp41) are essential for normal infection of CD4 cells by wild type HIV virus. *Pol* gene encodes for various viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) that are essential for viral replication and survival (Frankel & Young, 1998).

In addition to the structural proteins, HIV also codes for 6 other genes that regulate replication and viral pathogenesis. These include *tat*, *rev*, *nef*, *vpr* and *vpu,* Figure 1.8. *Rev* and *tat* genes express regulatory proteins that activate viral transcription and regulate the splicing and nuclear exports of viral transcripts respectively. *Vif*, *Vpr*, *Vpu* and *Nef* genes encode for accessory proteins. *Vpr* plays a pivotal role in viral life cycle and transport of viral genome to the host nucleus. *Vpu* and *Nef* have a similar function and they also increase virus budding. The viral genome is flanked by LTRs. The flanking LTRs are required for viral transcription, reverse transcription and integration processes, Figure 1.8. The genome dimerisation and packaging signal 'Ψ' is placed between the *gag* gene and 5´-LTR (Frankel & Young, 1998 and Sakuma *et al*., 2012).
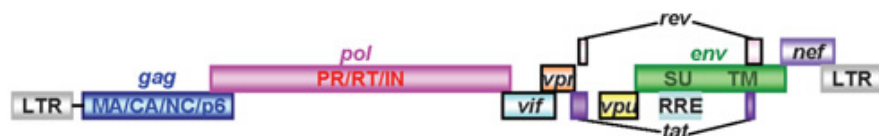
Figure 1.8: Schematic representation of the HIV genome.

The viral RNA contains three essential genes (*gag, pol* and *env*), regulatory (*rev* and *tat*) and accessory (*vif, vpr, vpu* and *nef*) genes flanked by LTRs. The *Gag* gene encodes for matrix protein (MA), capsid protein (CA), nucleocapsid protein (NC) and P6. The *pol* gene encodes three essential replication enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) while the *env* gene encodes for transmembrane (TM) and surface subunit (SU) parts of the envelope glycoprotein. Taken from (*Sakuma* et al.*, 2012)*.

It is clear that lentiviral vectors are important and useful in gene therapy, but lentiviral vectors are derived from the HIV virus. This virus can produce very serious diseases that are almost untreatable. Therefore, the biosafety of HIV-based vectors is a major consideration for use in a clinical environment (Zufferey *et al*., 1998). Also, there is a possible risk of insertional mutagenesis and position effect variegation for the host because of the integration of a viral vector into host's gene. Research reports insertional mutagenesis and high level of aberrantly spliced chimeric transcript in the gene of interest due to severe side effects of using lentiviral vector (Hacein-Bey-Abina *et al*., 2003 and Moiani *et al*., 2012). Hacein-Bey-Abina *et al*., 2003 reported insertional mutagenesis in two patients almost three years after gene therapy that resulted in aberrant transcription and expression. Moiani *et al*., 2012 demonstrated unexpected high level of abnormally spliced transcripts upon integration in target genes result in side effect of using a lentiviral vectors. Using a stable non-integrating vector system can minimize these risks, as described in detail below. Also, several other different structure modifications in the HIV-1 have been made to improve the safety and to promote the efficiency of vectors (Picanco-Castro *et al*., 2012). These improvements are explained in following sections.

The most common method of lentiviral vector production is based on transient transfection of three or four plasmids into a cell line, Figure 1.9 (Verma & Weitzman, 2005). Human embryonic kidney cells (HEK293T), is the cell line mostly used for viral vector production because it is easily and highly transfectable. Additionally, these cell lines normally grow adherent (monolayer culture) in the presence of 10% fetal bovine serum but these cells can easily be adapted to serum-free suspension culture. These features make this cell line a very promising candidate for lentiviral vectors production on a large scale. Essential genes of lentivirus must be expressed in HEK293T to allow

the generation of lentiviral particles, thus the HEK293T cells are transfected with certain amount of transgene, packaging, REV and vesicular stomatitis virus glycoprotein envelope (VSV-G) plasmids (Picanco-Castro *et al*., 2012). The transgene is placed between the lentiviral long terminal repeats (LTRs) to allow for target cell integration. To improve vector biosafety and avoid risk of viral genome integration into host genome, LTRs are replaced with a strong promoter such as cytomegalovirus (CMV) promoter and self-inactivation LTR hybrids. Using VSV-G envelope allows the production of high titer lentiviral vectors, more details provided on their development in lentiviral section. The most common chemical compound to transfect the virus production cells is calcium phosphate, which offers fine transfection efficiency and it is very cost-effective method to produce lentiviral vectors. Recombinant lentiviral vectors were harvested from the cell supernatant three days after transfection of HEK 293T cells. The concentration of viral vectors can be achieved by precipitation or ultracentrifugation (Picanco-Castro *et al*., 2012).
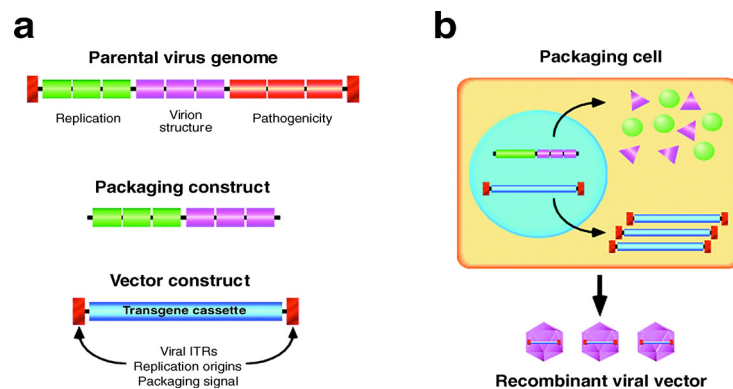
Figure 1.9: Strategy to engineer a virus into a vector.

The principle of engineering a virus into safe delivery system relies on the identification of the viral sequences required for replication, assembly of viral particles, packaging of the viral genome and delivery of the transgene into the target cell, and pathogenicity. The latter must be removed to make the vector non-pathogenic. The viral genetic material required in *cis* is inserted into the vector construct, together with the gene of interest. The packaging cassette encodes for genes essential for replication and production of structural proteins. The packaging and vector constructs are delivered into the packaging cells, where the packaging construct produces proteins and particles required for replication and assembly viral vector. Taken from (Verma & Weitzman, 2005).

Lentivirus infects host cells by associating with a surface receptor/s on the host cell. The glycoproteins of the viral envelope are attracted to a specific cellular receptor/s. Following binding, the viral envelope fuses with the host membrane leading to the release of the virus core into the cytoplasm (Sakuma *et al*., 2012). Soon after this internalization, the viral RNA is then reverse transcribed into DNA, which is incorporated into the host genome by the enzyme reverse transcriptase. The viral DNA is then imported into the host cell's nucleus where it is inserted to the host genome by the viral enzyme integrase. In this stage, the infection is in the targeted cells and is, for all intents and purposes, permanent and the expression of the recombinant protein can be detected one or two days after the integration of the proviral DNA (Teixeira *et al*., 2011). At this stage, the virus is known as provirus. During the next stage, the viral genome synthesis begins with the transcription, by RNA polymerase of the proviral DNA into RNA. The LTRs flanked at the ends of the viral genome regulate transcription and polyadenylation of viral mRNAs. The LTR located at the 5′-end of genome acts as a combined enhancer and promoter for transcription by infected host cell RNA polymerase while the other LTR end at the 3′-end stabilised these transcripts by mediating their polyadenylation (Sakuma *et al*., 2012). The transcribed RNA from proviral DNA which contains the code to produce the capsid proteins, other proteins needed for viral assembly, envelope proteins and other auxiliary proteins are transported outside the nucleus for translation by ribosome. Two exported copies of the viral genomic RNA and newly produced viral proteins are assembled together at the plasma membrane to form a new virus. After release from the host cell membrane, the virion is initially non-infectious and is known as an immature virion. The immature virions convert into mature infection viruses by activation of PR to cleave the Gag and Gag-Pol polyproteins into their four structural proteins and the PR, RT and IN functional enzymes

(Teixeira *et al*., 2011). A viral vector does not have capabilities for replication and infection because most of the viral genes are excluded from the packaged genome (Cockrell & Kafri, 2007).

1.10.1.3 Developments in lentiviral vector production

Many structural modifications in the HIV-1 have been made in order to improve the efficiency and the safety of the HIV-1 based vectors. Since HIV-1 Env glycoprotein has a highly restricted host range and it recognizes cells containing CD4 and co-receptors, the development of retrovirus vectors pseudotyped with a variety of envelope proteins such as VSV-G was a significant improvement (Burns *et al*., 1993). VSV-G interacts with a ubiquitous cellular "receptor" on cells and by substituting HIV-1 envelope with VSV-G, offers transduction of several tissues and cells type of different hosts, even including non-mammalian hosts (fish), both *in vivo* and *in vitro* experiments. Infections in the HIV-1 pseudotyped with VSV-G were increased by 20- to 130-fold more than wild type virus (no VSV-G pseudotyped) (Aiken, 1997). It also reduces the requirements for viral accessory proteins for full infectivity by directing lentiviral vector entry to an endocytic pathway (Cronin *et al* 2005). The other advantage of VSV-G is that this envelope is more stable than a retroviral or lentiviral envelope and it can withstand the shear forces that allow for concentration of viruses by ultracentrifugation to a higher titer than before. In addition, it makes viruses resistant to freeze/thaw cycling and they can be stored for several years at -80°C (Burns *et al*., 993).

However, there are disadvantages to using VSV-G pseudotyped lentivirus vectors for clinical application, because there is complement and antibody in human serum which mediates an immune response against VSV-G envelopes, thus inactivating the vector

(Croyle *et al*., 2004). In 2004, Croyle *et al*., demonstrated conjugation of monomethoxypoly (ethylene) glycol to VSV-G envelope protein of a VSV-G pseudotyped HIV-based vector, to protect the vector from inactivation in complement-active human and mouse sera. This modification does not affect transduction efficiency. This approach was established to extend the rate of vector half-life (by 5-fold) and reduced the vector inactivation in the serum by a factor of 1,000 *in vivo*. In order to increase safety, optimise transduction and limit vector entry to target cell and tissue, using various envelope proteins is a potential tool and continues to be a significant challenge in gene therapy area.

Vector mobilization, insertional mutagenesis of replication competent lentiviral vectors (RCLs), and germ-line transmission of vector sequences are all possible hazards that need to be considered during generating and production of lentiviral vectors (Manilla *et al*., 2005).

1.10.1.3.1 First-generation lentiviral vector

The first-generation of HIV-1 packaging cassette was introduced by Naldini *et al*., 1996. In this generation, three separate plasmids are used to produce a lentiviral vector, Figure 1.10. This expressing system allows the delivery of the genes of interest into target cells without expressing viral proteins. Also, using three different plasmids means reducing the chance of generating RCLs because at least two recombination events are required to yield a RCL. The expression system for vector preparations was composed by a packaging construct, a transfer vector genome construct and an Env plasmid encoding a viral glycoprotein. The packaging plasmid expresses *tat* and *rev* for regulatory protein, *vif*, *vpr*, *vpu* and *nef* for accessory protein and *gag* and *pol* protein from a strong

mammalian promoter (usually the CMV promoter) to express viral particles in the host cell. In the envelope plasmid the native HIV-1 envelope was replaced with a viral glycoprotein, such as VSV-G, to provide a receptor binding protein to the vector particles. These two plasmids have been modified and all the *cis*-elements removed, thus they do not have a packaging signal, LTRs or a primer binding site. This specific modification is to avoid their transmission into vector particles and also to reduce chances of generating RCLs in vector production. Removing the *cis*-elements from delivery package curtails the generation of RCLs by avoiding the packaging of the full-length mRNA encoding trans-elements into the vector.

The transgene plasmid contains a gene of interest and all of the *cis*-acting elements (LRT, Ψ and Rev-response element (RRE)), which are required for efficient packaging, reverse transcription, nuclear import and integration but expresses no HIV protein. The transgene cassette expresses the full length vector RNA and the gene of interest under the control of either the viral LTR or an internal promoter in transduced cells (Naldini *et al*., 1996; Naldini *et al*., 2012 and Sakuma *et al*., 2012).

1.10.1.3.2 Second generation lentiviral vectors

The biosafety of lentiviral vector increased by deletion of all accessory genes from the original plasmid, Figure 1.10. *Nef*, *vif*, *vpr* and *vpu* are called accessory genes because they are dispensable for virus replication in cell culture. However, the proteins encoded by accessory genes are essential for the HIV propagation/virulence in primary cells or *in vivo*. These deletion in the packaging system are indispensable for the efficient gene transduction by a lentiviral vector and for avoiding HIV-1 propagation and virulence. In

the second generation, lentiviral vectors contain only four of the nine HIV genes. The new packaging plasmid only expresses *gag*, *pol*, *tat* and *rev* genes (Zufferey *et al*., 1997).

1.10.1.3.3 Third-generation lentiviral vectors

The safety of lentiviral vectors significantly improved in the third generation. *Tat* and *rev* are essential for HIV-1 replication, and replacing the U3 promoter region in 5′ LTR of the transfer vector with a strong viral promoter such as CMV can offset the requirement for *Tat*. In 1998, Dull *et al*., established that the deletion of *REV* gene is indispensable for the expression of *gag* and *pol* and cannot be eliminated from the system. Thus, the system is dependent on *REV* while independent of *Tat.* These results introduced the third generation system. In this system, the lentiviral vector is based on four non-overlapping expression cassettes,: (i) *gag* and *pol* genes are placed on one expression cassette, (ii) a cassette expressing *rev* gene, (iii) an Env (VSV-G) cassette, and (iv) an expression construct which contains the gene of interest under control of a heterologous strong promoter, Figure 1.10. The new packaging system has only three of the nine genes of HIV, which are *gag*, *pol* and *rev*, and lacks all the accessory proteins that are necessary for the pathological abilities of HIV-1. Using four separate plasmids in this generation minimizes the chance of RCLs generation. In this case, at least three recombination events are required to generate a RCLs (Dull *et al*., 1998 and Sakuma *et al*., 2012). Escarpe *et al*., 2003 designed a sensitive assay for detection of RCLs in large-scale preparations of HIV-based lentiviral vectors. They ran a test and scanned a total of 1.4×10^{10} transducing units of vector from 10 independent 14-litre production lots to detect RCLs. Their result demonstrated there is no RCLs in scanned samples. Therefore,

this result strongly supports the significant safety improvements of lentiviral vectors in the third generation.

1.10.1.3.4 Self-inactivating vectors

The presence of LTRs on both sides of the HIV-1 vector genome allows the possibility of transcriptional activity from the LTRs, as they include viral enhancers and promoter regions, and was used in early vectors. In addition, the virus requires the LTRs for reverse transcription and integration into the host genome. The LTRs have an enhancer-promoter in U3, and a polyadenylation signal. The U3 region includes TATA-box-, Sp1-, NF-κB- and NFAT (nuclear factor of activated T-cells)-binding sites, which were removed to produce self-inactivating vectors (Iwakuma *et al.*, 1999 and Miyoshi *et al.*,1998), Figure 1.10. Through the process of reverse transcription of the lentiviral vector system, the deletion in the U3 region of the 3′ LTR is transposed to the U3 region of 5′ LTR in the provirus, resulting in the transcriptional inactivation of the LTR promoter, which no longer expresses a full-length vector RNA. This deletion improves the biosafety of the lentiviral vector by resulting in transcriptional inactivation of potentially homologous to wild type virus in transduced host cell. It also reduces insertional activation of cellular oncogenes by residual promoter activities of integrated LTRs (Zufferey *et al.*, 1998).

1.10.1.3.5 Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)

Lentiviral vectors can efficiently transduce a variety of cells, but transgene expression can be improved by further engineering. The Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is a *cis*-acting RNA element that has been used to improve lentiviral vector expression. Insertion of WPRE in sense orientation in the 3' untranslated region (3´ UTR) of a transgene significantly improves level of expression (Zufferey *et al*., 1999) . Zufferey *et al*., 1999 demonstrated using WPRE in the construction of vectors will improve their functionality in terms of expression of gene of interest, which is delivered by vectors. Moreover, WPRE activity is not cell type or species dependent and can be used to stimulate transgene expression in several cell lines, primary cells and *in vivo*.
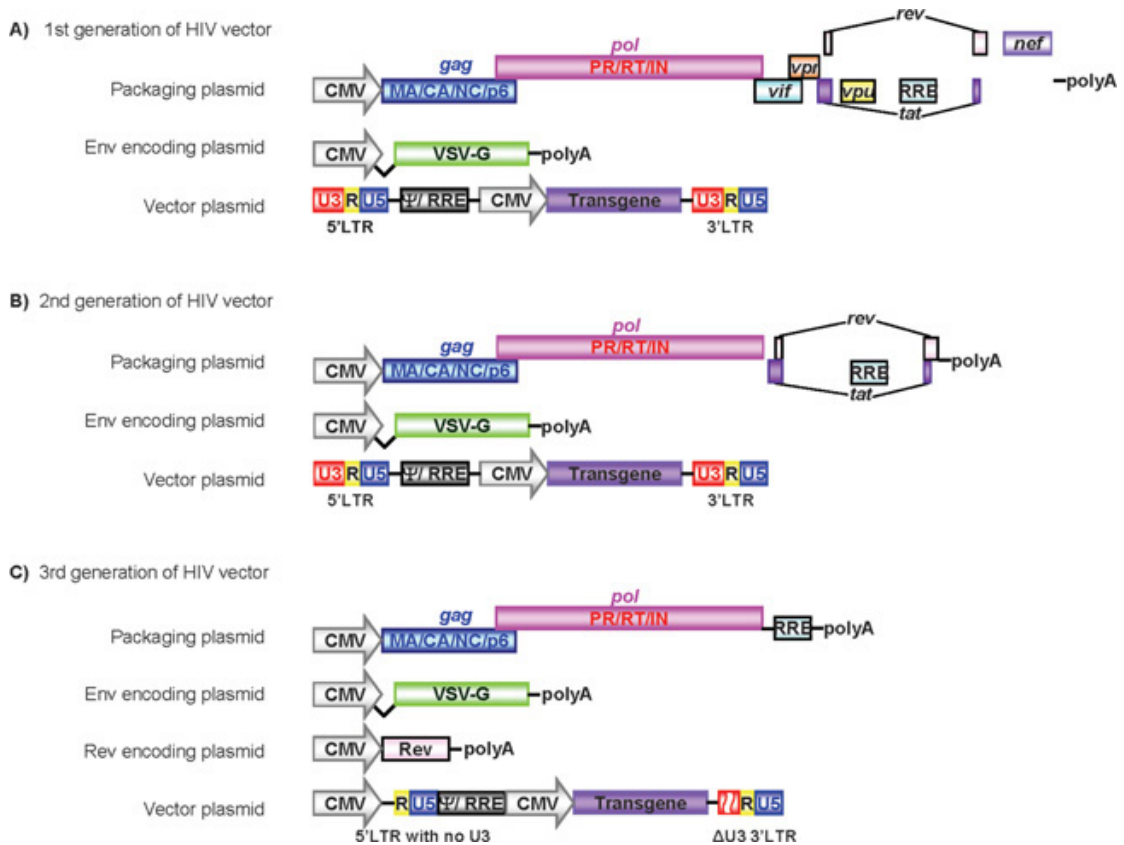
Figure 1.10: Schematic of constructs required in the production of different generations of lentiviral vectors.

(A) In the first generation lentiviral vector, the packaging plasmid expresses all viral proteins except *Env*. VSV-G is an envelope glycoprotein, expressed by *Env* encoding plasmid. The vector plasmid flanked by LTRs expresses the transgene. (B) The different between first and second generation is the elimination of all accessory genes except *Tat* and *Rev* from packaging plasmid. (C) In third generation, *Rev* was placed in a separate expression cassette and *tat* was eliminated. The other strategy to improve biosafety in third generation vectors was deletion of most of the U3 region from the 3′-LTR to make the vector self-inactivating and minimise the likelihood of generating RCLs. Moreover, a strong promoter such as CMV is placed in the vector plasmid for expression of the RNA to be packaged. Taken from (Sakuma *et al*., 2012).

1.10.1.4 Integration-deficient lentiviral vectors

Genetic insertion of lentiviral DNA into the host genome is mediated by the viral enzyme *integrase* encoded by the *pol* gene. The integration mechanism is a multi-step procedure. Firstly, *integrase* produces a staggered cut at the *att* sites of the viral LTRs and the host genome, and then the viral DNA is inserted into host genome. This is followed by the repair of any gap between viral and host genome by host cell enzymes. However, during the proviral integration process, a significant amount of liner DNA is converted into two circular forms called episomes by nuclear proteins. These two episomal circular DNA are known as the by-products of the integration process. Episomal DNA circles are formed either by non-homologous end joining (NHEJ) or homologous recombination. NHEJ pathway ligates the 5′ and 3′ end of the liner DNA and form 2-LTR circles. While in the homologous recombination pathway viral 5′ and 3′-LTR can produce 1-LTR episomes (Cara *et al*., 1996). Due to the way episomes are generated, they contain one or two copies of the viral LTR and are metabolically stable. For a long time, viral episomes were thought of as non-active products (Li *et al*., 2001) but research showed these episomes have gene expression activity, although lower than the integration-proficient form (Wanisch & Yáñez-Muñoz, 2009). They do not carry any replication signals but they remain stable in non-dividing cells and are continuously diluted with successive rounds of cell divisions.

Mutations within the genetic structure of integrase in lentiviral vectors impair the proviral integration into the host genome. There are two classes of such mutation: class I and class II. In class I, specific amino acids of integrase are mutated to avoid integration. In this class, one of the three amino acids of the catalytic triad-aspartic acid placed at position 64, 116 and one glutamic acid residue at position 152 mutate to inhibit the proviral integration process. In class II, mutation is carried out by deletion into the

integrase region of *pol* gene: while both these mutations inhibit the integration process, class II also has a modifying effect on reverse transcription, replication, nuclear entry and gene transfer process in lentiviral vector. In class I, the mutation does not make any changes to normal DNA synthesis or reverse transcription process (Engelman, 1999).

Integration-deficient lentiviral vectors (IDLVs) produced by class I mutation (D64V) have been shown to mediate efficient transfer and expression of Enhanced Green Fluorescence Protein (*eGFP*) in ocular and brain tissue of rodents. In addition, no difference was noted between expressions obtained from either integrating or non-integrating viral vectors. Furthermore, long-term expression was mediated by IDLVs with high levels of transgene expression *in vivo* to rescue mouse models of retinal dystrophies (Yáñez-Muñoz *et al*., 2006). Therefore, IDLVs can be utilized to transfer genes into dividing and non-dividing cells without vector integration, which would reduce risk associated with insertional mutagenesis (Peluffo *et al*., 2013 and Saeed *et al*., 2014).

The main advantages of using IDLVs include (a) their relatively large transgene capacity (b) low immunogenicity (c) minimal risk of insertional mutagenesis (d) and RCL generation. As IDLVs remain episomal they do not integrate, but retain the transduction efficiency of standard integrating lentiviral vector both *in vitro* and *in vivo*. These important combinations make IDLVs efficient and safe delivery vectors and an attractive tool for clinical applications, particularly in quiescent tissues (Yáñez-Muñoz *et al*., 2006).

1.11 Applications of lentiviral vectors in gene therapy

Due to their unique advantages, lentiviral vectors are at the forefront of gene delivery systems for research and clinical applications. (Picanco-Castro *et al.*, 2012).

Genetically engineered animal models recapitulate aspects of the selected human disease. Animal models are employed in almost all fields of biomedical research to help researchers to discover disease mechanisms, understand gene function, model human diseases, biological process, validate drug target or to test therapeutic strategies.

Lentiviral vectors are applicable to engineer an animal model for *in vivo* research, which is an essential stage of investigation of a therapeutic strategy before moving on to a clinical trial (Jucker, 2010). Lentiviral vectors are utilised to generate transgenic animals successfully. Lentiviral vectors can generate animal models from a wide range of species including birds, cows, monkeys, pigs, and mice, because of their high gene transfer efficiencies into zygotes or early progenitor cells (Pfeifer & Hofmann, 2009 and Singer & Verma, 2008). Lentiviral vectors have been commonplace in experimental research and these viral vectors successfully treat or cure disease in animal models (Mátrai *et al.*, 2010).

Lentiviral vectors have been utilised to treat disease in several animal models, such as, SCID (Throm *et al.*, 2009), β-thalassemia (Zhao *et al.*, 2009), Wiskott-Aldrich syndrome (Mantovani *et al.*, 2009), metachromatic leukodystrophy (Biffi & Naldini, 2007), haemophilia (Brown *et al.*, 2007), Fanconi anaemia (Jacome *et al.*, 2009) and liver disease (Menzel *et al.*, 2009). Lentiviral vectors have now moved beyond the preclinical stage into clinical trials, and they have been successfully utilised in the clinical trial. The first lentiviral vector clinical trial was approved in December 2002 by the US Food and Drug Administration (D'Costa *et al.*, 2009) and then several other clinical trials have been approved to treat patients with both infectious and genetic diseases, using viral

vectors. Many human diseases have been targeted for treatment with lentiviral vectors, such as monogenic diseases (Metachromatic Leukodystrophy, Inherited Skin Disease Netherton Syndrome, X linked cerebral adrenoleukodystrophy, X-Linked Chronic Granulomatous Disease, Wiskott-Aldrich Syndrome, mucopolysaccharidosis type VII, Fanconi Anemia Complementation Group A, Hemophilia A, X-Linked Severe Combined Deficiency, Adenosine Deaminase Deficient Severe Combined Immunodeficiency, β-thalassemia, Sickle cell anemia, Parkinson's disease, various cancers and Stargardt Macular Degeneration (Naldini, 2015 and Tomás *et al*., 2013). Several clinical trials using lentiviral vectors are explained in detail in this section. The first ever Phase I clinical trial gene therapy using lentiviral vectors for AIDS was carried out in the US in 2003. The sponsor of this clinical trial was VIRxSYS Corporation, which is a private biotechnology company, which focused on the development of gene therapy treatments for diseases such as HIV/AIDS. The aim of this trial was to repopulate the immune system of the patient with genetically modified cells that can support a patient's immune system against HIV and other infections. In this pilot study, lentiviral vector expressed a 937-base long antisense gene (VRX496) against HIV *env*. The antisense used in this study blocks HIV replication in CD4 T-cells, and makes them resistant to the wild type HIV virus to prevent the destruction of CD4 T-cells cells by this virus Five HIV + patients participated in phase I of this trial and they received a single dose of approximately 1.0×10^{10} lentivirus-transduced CD4 T-cells. This study moved to multiple Phase II trials and 60 HIV + patients participated after successful completion of Phase I trial. The collected results demonstrated primary human T Cells transduced with VRX496 resistant to wild type HIV virus replication, thus the number of CD4 T-cells and immune response improved in treated HIV + patients. (Levine *et al*., 2006 and McGarrity *et al*., 2013). Lentiviral vector encoding *ABCD1* gene was used to

treat X- linked adrenoleukodystrophy (X-ALD). Cartier *et al*., 2009 treated two 7-year old boys who had X-ALD. Extracted bone-marrow cells from these two patients were transduced with lentiviral vectors with the *ABCD1* gene, then the subjects received an infusion of their own genetically modified stem cells carrying the normal gene. There was no ALD protein in blood cells of these patients before treatment, while nearly two years after transplantation 9-14% of peripheral blood cell of patients expressed ALD protein and neurological function had stabilised. Bank *et al*., 2010 carried out a phase I/II clinical trial utilising lentiviral vectors expressing β-globin gene to treat β – thalassaemia in Paris in 2007. The aim of this experiment was to harvest bone marrow from patients, then transduce isolated CD34+ cells with lentiviral encoding β-globin gene. Genetically modified cells were introduced into the patients as an autologous transplant. The treated patients have corrected β-globin gene and stable blood haemoglobin level approximately 3 years after treatment.

Lentiviral vectors were employed in 5.6% (n=135) of gene therapy clinical trials (http://www.wiley.com/legacy/wileychi/genmed/clinical/ on 17.09.2016).

Several gene therapy clinical trials based on the use of lentiviral vectors are on-going or approved. A list of some gene therapy trials using lentiviral vectors is provided in Table 1.7.

Table 1.7 : Summary of clinial trials using lentiviral vecotor by disease

Disease	Status	Transgene	Phase	Trial code
Lymphoma	Active, not recruiting	Encoding Multiple Anti-HIV RNAs	pilot clinical trial	NCT00569985
Fabry Disease	Recruiting	Alpha-Galactosidase A	I	NCT02800070
Leukemia, Myeloid, Acute	Unknown	CD80 and RFUSIN2-AML1	I	NCT00718250
Adenosine deaminase (ADA)-Deficient Severe Combined Immunodeficiency (SCID)	Active, not recruiting	human adenosine deaminase	I/II	NCT01852071
Acute Myeloid Leukemia	Recruiting	Interleukin 12	I	NCT02483312
Wiskott-Aldrich Syndrome	Recruiting	Wiskott–Aldrich syndrome	I/II	NCT02333760
X-linked Chronic Granulomatous Disease	Recruiting	G1XCGD	I/II	NCT02234934
Metachromatic Leukodystrophy	Active, not recruiting	Arylsulfatase A gene	I/II	NCT01560182
B-cell acute lymphoblastic leukemia (ALL)	Recruiting	Chimeric antigen receptors	I	NCT01044069
Cerebral Adrenoleukodystrophy (CCALD)	Active, not recruiting	ABCD1 (adenosine triphosphate (ATP)-binding cassette, subfamily D, member 1)	II/III	NCT01896102
Parkinson's Disease	Active, not recruiting	ProSavin	I/II	NCT01856439

1.12 *In utero* gene therapy

Many genetic diseases can cause a significant amount of irreversible damage to the fetus before birth, so treating these diseases in the very early stages of fetal development may be highly beneficial. At the current time, it is possible to diagnose genetic diseases at a very early stage of life, so it would be very reasonable to treat them immediately following diagnosis, in an attempt to avoid any further problems for patient. *In utero* gene therapy opens a very promising window to treat the disease before birth and it may allow the birth of a healthy infant who does not have special requirements or treatment, and can have a normal life. *In utero* gene therapy means delivering genes of interest to the fetus to correct genetic disease (Chauhan *et al*., 2004). There are many advantages for taking *in utero* gene therapy forward and improving the field, such as avoidance or slow of disease onset, less immune response against delivered vector or transgene products, high vector to cell ratio and possible chance of transducing stem and other progenitor cells (Chauhan *et al*., 2004 and Waddington *et al*., 2005).

1.12.1 Immune response

Producing antigen-specific immunity in a host body against delivered viral vectors and novel therapeutic gene products plays a substantial role in treatment and this is one of the difficulties of using gene therapy to treat adults. Each different vector has its own immunological hurdles for therapeutic usage. Type 2 adenovirus and adeno-associated virus (AAV) vectors are common vectors for gene therapy purposes, and deliver the gene of interest to the host body, but 50% of individuals worldwide have pre-existing humoral immunity to type 2 adenovirus and 35% to 80% have antibodies against AAV2 (Chirmule *et al*., 1999).

Innate immune responses are prompted almost immediately after vector delivery and, following that, an immune response mounted against the novel therapeutic gene product. The therapeutic transgenes are recognized as a foreign antigen by the immune system of the host and the reaction of the immune system against the vector can lead to short-term expression and thus the correction of deficiency can be short lived (Seppen *et al*., 2006). Nguyen & Ferry, 2004 and Wang *et al*., 2004 reported the immune response against therapeutic transgene when gene therapy is applied. Seppen *et al*., 2006 used lentiviral vector encoding UDP glucuronosyltransferase family 1 member A1 (*UGT1A1*) to treat hyperbilirubinemia in neonatal Gunn rats. The animals were injected at the day of birth and treated for up to 18 weeks. A rapid reduction in therapeutic affect was observed as well as high levels of antibody against human UGT1A1 when the animals were 18 weeks old. The immune response against GFP has been reported by other studies in adult mice, rats and baboons (Inoue *et al*., 2005; Morris *et al*., 2004 and Stripecke *et al*., 1999).

A possible solution to overcome this problem is to introduce the gene of interest in the fetal or neonatal period (Seppen *et al*., 2006). The *in utero* injection can be beneficial because it exploits the tolerization that occurs toward the antigens. This is because the immune system is not fully developed at these stages of life. For the first time ever in 1953, Billingham *et al*., showed that tolerization can be achieved when the antigens are introduced into the host before the development of the faculty of immunological response. Waddington *et al*., 2003 and 2004 are two important studies in the *in utero* gene therapy field, which employed adenoviral and lentiviral vectors to deliver the transgene to fetuses. The outcome of these two studies demonstrates that introducing the gene of interest through *in utero* injection can tolerise the host immune system to foreign therapeutic proteins. Waddington *et al*., 2003 uses adenovirus to deliver the human factor IX (hFIX) transgene into fetuses, which results in immune tolerance of the

transgenic product. In this study, adult mice received the viral injection again, after prenatal viral injection, and 5 out of 9 injected mice were found to have persistence of blood hFIX and absence of hFIX antibodies. In contrast, viral vectors carrying the hFIX were administered to the adult mice, which had not received any prenatal vector injection. The result of these injections showed rapid loss of expression of delivered gene and the level of hFIX antibody was found to be high in the injected animal. These results confirm that *in utero* injection can achieve immune tolerance of vectors and transgene products, which will be very beneficial for gene therapy. Seppen *et al*., 2006 found there is no antibody against GFP in sera from Gunn rats that received *in utero* injection of GFP lentiviral vectors.

However, it needs to be mentioned that a few studies reported the immune response in fetuses that had undergone *in utero* injection. An immune response was reported against CMV promoter in prenatal children infected with viral vector carrying CMV promoter (Marchant *et al*., 2003). Jerebtsova *et al*., 2002 demonstrated injection of adenovirus or adeno-associated viral vector mounts an immune response and low titer of antibody to beta galactosidase detected in injected animals. Seppen *et al*., 2006 suggested that *in utero* injection does not always lead to tolerization. In their studies, they injected fetal and neonatal rats with UGT1A1 and GFP lentiviral vectors. The immune systems of all animals injected with UGT1A1 lentiviral vectors responded against delivered transgene and animals developed antibodies to UGT1A1. In contrast, animal that had been injected with lentiviral vectors encoding GFP did not mount an immune response, and antibodies against GFP were not detected in injected animals. These results suggested UGT1A1 is a highly immunogenic protein.

1.12.2 Avoidance of disease onset

In many genetic diseases, the genetic default causes organ damage, which might be irreversible after birth, even if the genetic disease is treated in the affected individual. *In utero* gene therapy can be the only solution for those diseases such as urea-cycle disorder. Urea-cycle disorder is a genetic disorder caused by a single mutation, and this mutation will result in deficiency of one of the enzymes in the urea cycle. This disease occurs in 1 in 30,000. The urea cycle is not fully functional and the concentration of ammonia increases in the blood. The ammonia reaches the brain through the blood and this can cause irreversible brain damage, coma and/or death (Mian & Lee, 2002).

1.12.3 Infection of Stem Cells and Progenitors

In utero gene therapy can increase the possibility of transduction in stem or other progenitor cells which can result in expression of gene of interest in large number of daughter cells (Waddington *et al*., 2005). The permanent replacement of a faulty gene with a healthy gene into somatic stem cells would ensure daughter cells will carry the gene of interest, thus the genetic disorder is treated *in utero* and there is no need for further treatment for the affected individual (Larson & Cohen, 2000). Moreover, transduced progenitor cells *in utero* may give access to tissues that are difficult to access for treatment in adulthood (Waddington *et al*., 2005). MacKenzie *et al*., 2002 and Waddington *et al*., 2003 applied gene delivery *in utero*, injecting fetal mice with HIV-1 or EIAV lentiviral vectors intravascularly. Their result demonstrated the expression of marker genes in the liver in focal clusters, a finding that suggests that they may have arisen from individual progenitors.

1.12.4 Vector dose scaling

Another issue in the gene therapy field is the scaling of vector dose. It has been reported that it is difficult to scale the vector dose based only on the body mass of the host (Waddington *et al*., 2005). Two studies were done by Chuah *et al*., 2003 and Harding *et al*., 2004, in which a large animal model was used and demonstrated that scaling the vector dose based on animal weight would not have the same result as achieved in a small animal. In these two studies, dogs and mice were injected with high-capacity (HC) adenoviral vectors encoding FVIII (Chuah *et al*., 2003) and recombinant adeno-associated virus serotype 2 (rAAV2) vectors encoding human FIX (Harding *et al*., 2004). The dog model received an equivalent or higher dose of vectors per kilogram of body weight than mice, but the outcome showed reduction of circulating concentration of clotting factor in injected dog model than injected mice. In conclusion, the small body size of the fetus is useful for *in utero* gene therapy in terms of vector scaling and also the small body mass of the fetus increases the chance of vector biodistribution (Waddington *et al*., 2005).

1.12.5 Risks of *in utero* gene therapy

There are several critical safety concerns regarding the application of *in utero* gene therapy for clinical purposes, which must be addressed. The risks of this method can be divided into two groups: the risks in the first group relate to fetal intervention, which include infection, preterm labour, fetal loss and the possibility that manipulating the fetus can alter normal organ development. The second risk group is linked to the risk of gene transfer itself, and includes insertional mutagenesis, germline transmission and the

type of vector used in the treatment process to deliver the gene of interest (Almeida-Porada, Atala, & Porada, 2016; Recombinant DNA Advisory Committee, 2000; Pearson & Flake, 2013). Mattar *et al*., 2011 administered AAV delivered human Factor IX (hFIX) into nonhuman primate fetuses. The result demonstrated that the AAV vector could cross the blood/placental barrier, enter the maternal circulation, and transduce multiple tissues of the mother. These possible issues need to be investigated in more detail, and alternatives, such as the utility of different vectors, needs to be explored before heading into clinical trial (Almeida-Porada *et al*., 2016).

Taken together, the goal of using gene therapy is to treat a particular disease or abnormal function but as with any potential therapeutic modality, the field is facing difficulties. However, significant improvement has been made in this field over the last two decades, but there are many other hurdles to overcome before *in utero* gene therapy becomes a mainstream clinical modality (Almeida-Porada *et al*., 2016). The key factors needed to improve the prenatal gene therapy field are explained in following section.

1.12.5.1 Timing of *in utero* gene transfer

Time of *in utero* injection is a critical factor, which is key to the safety and transduction efficiency of fetal gene therapy. Tarantal *et al*., 2001 injected fetal rhesus monkeys with lentiviral vectors encoding enhanced green fluorescent protein (e*GFP*). The first trimester during the embryonic stage of development (55 days gestation) and the early second trimester (70 days gestation) had been chosen as two different time points to inject the fetuses. After analysing the transduction efficiency in animals that had received the injection at 55 days gestation, the outcome of the experiment demonstrated that many other organs had been transduced in addition to the targeted organ (lung).

Conversely, less transduction of non-pulmonary tissue was observed in animals injected during the early second trimester (70 days gestation) and injection was more restricted to the lung.

Thus, more information on organ-specific gene expression is needed to enhance the safety and usefulness of *in utero* injection. It is possible that increasing gestational age in addition to using tissue-specific promoters may be beneficial (Tarantal *et al*., 2001).

1.12.5.2 Choosing the appropriate vector for *in utero* gene transfer

An important key to achieving effective and safe fetal gene therapy is choosing the appropriate vector system (Coutelle *et al*., 2003).

1.12.5.2.1 Adenoviral Vectors for *in utero* gene transfer

Adenoviral vectors are a potential system to employ to transfer genes of interest into fetuses. These vectors have a very good infection capability and can infect a wide range of fetal tissue depending on the route of administration. These vectors do not integrate into the host genome; therefore they will be dilute rapidly in case of active cellular proliferation (Lipshutz *et al*., 1999).These vectors are also highly immunogenic but, in some cases, it has been reported by Lipshutz *et al*., 2000 and Waddington *et al*., 2003 that fetal administration demonstrated immune tolerance to the transgene and vector. Taken together, these vectors are not good candidates for permanent correction of a genetic disease. However, they are a good candidate to investigate an immune response against vectors and transgenes after *in utero* delivery, as they are highly immunogenic.

1.12.5.2.2 Adeno-associated Virus for *in utero* gene transfer

Another possible viral vector to employ for fetal gene therapy purposes is adeno-associated virus (AAV). AAV vectors have low toxicity or immunogenicity in comparison to early generation adenovirus vectors. Jerebtsova *et al*., 2002 observed immune response against viral vector or transgene product in mice after *in utero* injection of adeno-associated virus between 13 and 15 days post-conception. AAV vectors are a good potential candidate for *in utero* gene therapy as they demonstrated long term expression of the gene of interest in murine following fetal administration at day 14 gestation (Bouchard *et al*., 2003). However, as AAV vectors are non-integrating, in case of fetal gene therapy AAV vectors will lose the permanent or long term gene expression as a consequence of a rapidly expanding fetal cell population (Waddington *et al*., 2005).

1.12.5.2.3 Retroviral Vectors for *in utero* gene transfer

Retroviral vectors are another possible vector system to be used for fetal gene therapy purposes. Retroviral vectors were used by Stuhlmann's group for the first time in 1984 for *in utero* delivery. In their study, Moloney murine leukemia virus was used to deliver the bacterial *Eco gpt* gene as marker gene into mouse embryo, and they successfully transduced almost 50% of embryonic cells. Hatzoglou *et al*., 1990 employed retroviral vectors to transduce rat embryo with gene of interest. In this study, they observed the expression of delivered gene for up to 8 months after injection. Therefore, the fact that retroviral vectors are capable of integrating efficiently into the host genome makes them ideal candidates for long-term expression in the developing embryo. However, it must be mentioned that inserting the delivered gene into the host genome carries a risk of

insertional mutagenesis/oncogenesis. As mentioned earlier, lentiviruses have the ability to transduce a variety of dividing and non-dividing cells. Additionally, pseudotyped lentiviral vectors with other virus's envelope proteins can modify the tropism of lentiviral vectors and allow the vectors a wider tropism. An *in utero* study was carried out by MacKenzie *et al*., 2002, in which they investigated the efficacy of lentiviral vectors with different pseudotypes to transfer gene of interest into tissues. MacKenzie and her collaborates injected fetuses at 14-15 days gestation with lentiviral vectors carrying the transgene *lacZ* under the control of the CMV promoter. The lentiviral vectors were administered into fetal mice by intramuscular or intrahepatic injection. The lentiviral vectors were pseudotyped with vesicular stomatitis virus (VSV-G), with Mokola, or with Ebola envelope proteins. The result of their experiments demonstrated that Mokola and Ebola pseudotyped were more efficient in transducing myocytes whereas lentiviral vectors pseudotyped with VSV-G envelope proteins strongly transduced hepatocytes. Therefore, pseudotyped lentiviral vectors with different viral envelopes allow for various degrees of transduction efficiency and specificity.

1.12.6 Candidate Diseases for Prenatal Gene Therapy

The National Institutes of Health Recombinant DNA Advisory Committee published a report regarding what types of diseases can be candidates for prenatal gene therapy (Recombinant DNA Advisory Committee, 2000). This report concluded any candidate disease for prenatal gene therapy should have serious morbidity and mortality risks for the fetus either *in utero* or postnatally. The candidate disease should not have effective postnatal therapy. Also, a candidate disease should not be associated with serious abnormalities that are not corrected by the transferred gene. Moreover, the selected

disease for prenatal gene transfer is required to be definitively diagnosed in utero, and the genotype/phenotype relationship should be well defined. There must be demonstration of effective gene transfer *in utero* in an animal model that recapitulates the human disease or disorder (Recombinant DNA Advisory Committee, 2000). Some of the diseases that may be suitable for fetal treatment are listed in Table 1.8.

Table 1.8: Some candidate diseases for prenatal gene therapy.

Disease	Therapeutic gene product	Target cells/ organ	Age of onset	incident	Life expectancy	References
Cystic fibrosis	CF transmembrane conductance regulator	Airway and intestinal epithelial cells	In utero	1:2000	32 years	(Stallings, 2003)
Spinal muscular atrophy	Survival motor neuron protein	Motor neurons	6 months (type I)	1:10000	2 years	(Russman *et al.*, 2003)
Lysosomal storage disease	Glucocerebrosidase in Gaucher disease	Hepatocytes	9-11 years	1:9000 overall	<2 years	(Beutler, 2004)
Urea cycle defects	Ornithine transcarbamylase in ornithine transcarbamylase deficiency	Hepatocytes	2 days (severe neonatal onset)	1:30000	<1 month (severe neonatal onset)	(Mian & Lee, 2002)
Duchenne muscular dystrophy	Dystrophin	Myocytes	2 years	1:4500	25 years	(Eagle *et al.*, 2002)
Severe combined immunodeficiency (SCID)	γ *c* cytokine receptor (X-linked SCID); adenosine toxicity	Haematopoietic precursor cells	Birth	1:1000000	< 6 month if no bone marrow transplant	(Qasim *et al.*, 2004)

1.12.7 Animal models for *in utero* gene transfer

Different animal models have been used for *in utero* gene therapy. However, the mice model is the most common model used for prenatal vector delivery, due to their relative ease to use and handle. They need less complicated facilities for maintenance and breeding than any other animal. One of the advantages of using mice model is because of the number of fetuses, and how fetuses link together in each pregnancy. Mice usually carry between 6 and 12 fetuses in each pregnancy. Each individual fetus is surrounded by an interior amniotic membrane and provides nutrients to the exterior parietal yolk sac via the yolk sac vessels at 16 days gestation. Thus, each individual fetus can receive a different injection. In addition to mice model, rabbits and guinea pigs have been successfully used as small animal models for *in utero* gene therapy in some cases (Waddington *et al*., 2005). Different information like the length of gestation, mass of fetus at birth, size of litter and life span of animal are taken into consideration when choosing an animal model for in utero gene therapy, Table 1.9.

Using larger animal models can better address areas such as the safety of treatment, delivery techniques and expression time of delivered transgene (David & Waddington, 2012). Additionally, a large animal model has the advantage of serving as a more accurate model of a human pregnancy. Macaques (Tarantal *et al*., 2001) and sheep (Peebles *et al*., 2004) models have been successfully used for prenatal vector delivery. Longer-term gene transfers and a higher vector dose are required in large animals to translate the protein of interest compared to a small animal model. Therefore, it would give researchers a better understanding of how their hypothesis can be applied to humans (David & Waddington, 2012).

Table 1.9: Animal model for fetal studies. Taken from (Waddington et al., 2005).

Species	Gestation length in days [Median (range)]	Litter size	Approximate body weigh at birth (g)	Lifetime (years)
Mouse	19-21	6-12	1	1-3
Rabbit	29-35	4-10	30-80	5-8
Rat	20-22	7-11	5	2.5-3.5
Rhesus macaque	130-180	1	550	20-30
Sheep	144-151	1-3	5-5.5 kg	8-13
Dog	57-71	3-10	100-400	7-15
Guinea pig	60-65	2-4	70-90	4-6

1.13 Project aims

For more than two decades, efforts have made to understand the structure of lentivectors derived from HIV-1 and to modify them to produce a safe delivery vector. The biosafety of lentiviral vectors has improved remarkably, and they have been employed widely in clinical trials. Lentiviral vectors are regarded as attractive gene-delivery vehicles because of their unique properties, such as: (a) being able to transduce both dividing and non-dividing cells, (b) being capable of transducing a wide range of cells, including critical target cell types for gene therapies; (c) not expressing viral proteins after vector transduction; (d) providing long-term gene expression via stable vector integration into the host genome, although a possible downside is the raised risk of insertional mutagenesis, which can lead to undesirable outcomes. Introducing integration-deficient lentivectors has significantly improved the bio-safety of viral vectors, with lower risks associated with lentiviral vector integration while preserving transgene expression in quiescent cells.

The main goal of this study is to design and optimise lentiviral vectors to mediate therapeutic transgene replacement to ameliorate SMA disease.

To achieve this goal, the study aimed to:

(**A**) Design and produce different lentiviral vector configurations.

(**B**)Test the produced vectors in different *in vitro* models and perform a comparative study on the effect of different factors on achieving maximisation of full-length SMN protein production.

(**C**) Study the functional efficiency of SMN protein production from lentiviral vectors.

(**D**) Carry out a preliminary assessment of *in utero* technology and the optimum viral vectors using an *in vivo* SMA model.

Chapter 2   Materials and methods

2.1 Material

2.1.1 Biochemical reagents

All molecular biology grade laboratory chemicals, DNA and protein size markers and media, along with their suppliers, are listed in Appendix 1. The storage conditions of all reagents were carried out according to the manufacturer's recommendation.

2.1.2 Biological kits

The list of all biological kit which was used in this study is available in Appendix 1.

2.1.3 Buffer, solution and completed medium

A list of all buffers and solutions that were applied in western blot, bacterial work, immunofluorescences, lentivector production, agarose gel electrophoresis and buffer for isolation primary cells is provided in Appendix 2.

2.1.4 List of plasmids

Plasmid name	Description
pMDLg/pRREintD64V	A 8895 bp packaging plasmid containing cytomegalovirus (CMV) promoter and Integrase (IN) with class I mutation via replacing aspartic acid at position 64 with valine. The plasmid was made by Prof. Rafael J. Yáñez-Muñoz.
pMDLg/pRRE	A 8865 bp packaging plasmid containing CMV promoter and IN. Plasmid vector was a kind gift from Prof. Luigi Naldini's laboratory.
pRSV-Rev	A 4174 bp plasmid in a pUC19 vector backbone. The Rous Sarcoma virus (RSV) promoter drives the expression of REV which is necessary for the nucleic acid transfer from the nucleus to the cytosol. This plasmid was a kind gift from Prof. Luigi Naldini's laboratory.
pMD2.VSV-G	A 5824 bp envelope plasmid in a pUC18 vector backbone, which was encoded with the glycoprotein from the vesicular stomatitis virus (VSV-G). Virus pseudo-typed with this envelope can infect both mammalian and non-mammalian cells. Plasmid vectors were a kind gift from Prof. Luigi Naldini's laboratory.
pHR'sc_S_E_W	A 9662 bp plasmid contains mutated Woodchuck hepatitis virus post transcriptional regulatory element (mWPRE) downstream of enhanced green fluorescent protein (e*GFP*) and under control spleen focus-forming virus (SFFV) promoter. Plasmid vectors were a kind gift from Prof. Adrian Thrasher's laboratory.
pRRLsc_C_m*Smn*_eleGFP-W	A 9008 bp plasmid encoding mouse survival motor neuron (m*Smn*) tagged with FLAG sub-

	cloned downstream of CMV promoter, with e*GFP* under the action of an internal ribosome entry site (IRES). A WPRE was placed downstream of enhanced e*GFP*. The plasmid was made by Dr. Sherif Ahmed in Prof. Yáñez's laboratory.
pRRLsc_hSYN_*mSMN*_eleGFP_W	A 8894 bp plasmid encoding *mSmn* downstream of human synapsin (hSYN) promoter, with e*GFP* under the action of an IRES. A WPRE was placed downstream of e*GFP*. The plasmid was made by Ms. Anila Iqbal in Prof. Yáñez's laboratory.
pscAAV_Cagh_*SMN1*	A 4841 bp plasmid encoding human *Survival Motor Neuron 1* (*hSMN1*). The plasmid was made by Dr. Maria Gabriela Boza in Prof Yáñez's laboratory.
pscAAV_Cag_h*SMN1NtF*	A 4868 bp plasmid encoding h*SMN1* with a N terminal FLAG tag. The plasmid was made by Dr. Maria Gabriela Boza in Prof. Yáñez's laboratory.
pscAAV_Cag_h*SMN1CtF*	A 4865 bp plasmid encoding h*SMN1* with a C terminal FLAG tag. The plasmid was made by MS. Pavlina Petrova in Prof. Yáñez's laboratory.
pRRLsc_C_m*Smn*_mW	A 7690 bp plasmid encoding m*Smn* tagged with Flag downstream of the cytomegalovirus (CMV) promoter and upstream of mutated woodchuck hepatitis virus post transcriptional regulatory element (mWPRE).
pRRLsc_hSYN_*mSmn*_mW	A 7559 bp plasmid encoding *mSmn* downstream of the hSYN promoter and upstream of mWPRE.

pRRLsc_C_*hSMN1*-mW	A 7446 bp plasmid encoding *hSMN1* downstream of the hSYN promoter and upstream of mWPRE.
pRRLsc_C_*hSMN1_NtF*_mW	A 7611 bp plasmid encoding *hSMN1* with a N terminal flag-tag downstream of the CMV promoter and upstream of mWPRE.
pRRLsc_hSYN_*hSMN1_NtF*_mW	A 7473 bp plasmid encoding *hSMN1* with a N terminal flag-tag downstream of the hSYN promoter and upstream of mWPRE.
pRRLsc_C_*hSMN1_CtF*_mW	A 7470 bp plasmid encoding *hSMN1* with a C-terminal flag-tag downstream of the CMV promoter and upstream of mWPRE.
pRRLsc_hSYN_*hSMN1_CtF*_mW	A 7470 plasmid encoding *hSMN1* with a C-terminal flag-tag downstream of the hSYN promoter and upstream of mWPRE.
pRRLsc_C_*Co_hSMN1*_mW	A 7590 bp plasmid encoding codon-optimised *hSMN1* downstream of the CMV promoter and upstream of mWPRE.
pRRLsc_hSYN_*Co_hSMN1*_mW	A 7473 bp plasmid encoding codon-optimised *hSMN1* downstream of the hSYN promoter and upstream of mWPRE.

2.1.5 Accession number

The GenBank accession number for wild type *hSMN1* used in this study is NM_000344.3. Codon optimisation of wild-type *hSMN1* was carried out using GENEART's gene optimization system, however, the codon optimised *hSMN1* sequence is confidential and will not be disclosed in this thesis, as the results of the experiments are not yet published.

2.1.6 Cell lines

Cell line	Description
HEK293T	An adherent epithelial human embryonic kidney 293 cell line containing an integrated copy of the human adenovirus serotype 5, E1a gene and constitutive expression of SV40 Large T antigen (Graham *et al.*, 1977).
HeLa	Human cervical adenocarcinoma cell line (Scherer, 1954).
Chinese hamster ovary (CHO)	The Chinese hamster ovary cell line was originally established from an inbred female laboratory animal in 1957 (Tjio & Puck, 1958).

2.1.7 Primary cell culture:

Motor neurons	Motor neuron primary cells were isolated from 15 days old (SD) rat embryos by dissociation of spinal cord ventral horns. SD rats were purchased from Charles River, UK.
Cortical neurons	Cortical neuron primary cells were extracted from 18 days old CD1 mouse embryos. The CD1 mouse purchased from Charles River, UK.
Human control and SMA type I fibroblasts	These cells were obtained with informed consent from control or SMA patients in Santa Creu I Sant Pau Hospital by our collaborator Dr. Eduardo Tizzano (Barcelona, Spain).

2.1.8 Western blotting antibodies

Primary antibodies for western blot used in this project

Antibody	Commercial source	Catalogue number	Dilution
Mouse anti-survival motor neuron (SMN)	BD Biosciences, UK	610646	1 in 5000
Rabbit anti-Alpha tubulin	ABCAM, UK	AB4074	1 in 2000
Anti-GFP antibody	ABCAM, UK	ab290	1:1000

Secondary antibodies for western blot used in this project

Antibody	Commercial source	Catalogue number	Dilution
IRDye 800CW goat anti-mouse	LI-COR, UK	92632210	1 in 2000
Goat anti-Rabbit Alexa Fluor 680	Molecular Probes, UK	A21076	1 in 5000

2.1.9 Immunofluorescence antibodies

Primary antibodies for immunofluorescence staining used in this project

Antibody	Commercial source	Catalogue number	Dilution
Mouse anti-NeuN	Millipore, UK	MAB377	1:500
Goat anti gemin2 polyclonal antibody	Santa Cruz, UK	ab6084	1:200
goat IgG anti ChAT	Milipore, UK	AB144P	1:50
Mouse anti-survival motor neuron (SMN)	BD Biosciences, UK	610646	1:500

Secondary antibodies for immunofluorescence staining were used in this project

Antibody	Commercial source	Catalogue number	Dilution
Goat anti-Mouse AlexaFluor555	Invitrogen, UK	A-21424	1:1000
Goat anti-rabbit AlexaFluor680	Invitrogen, UK	A-21076	1:1000
Goat anti-mouse AlexaFluor488	Invitrogen, UK	A-11001	1:1000
Donkey anti-goat AlexaFluor555	Santa Cruz, UK	sc-362265	1:1000

2.1.10 List of restriction enzyme

Restriction enzyme	Commercial source	Catalogue number
*Xma*I	New England Biolabs (NEB), UK	R0180
*Eco*RI	NEB, UK	R0101
*Sac*II	NEB, UK	R0157
*Sma*I	NEB, UK	R0141
*Acc*65I	NEB, UK	R0599
*Nco*I	NEB, UK	R0193
*Hin*dIII	NEB, UK	R0104
*Age*I	NEB, UK	R0552
*Sbf*I	NEB, UK	R0642
*Pst*I	NEB, UK	R0140
*Nde*I	NEB, UK	R0111
*Apa*I	NEB, UK	R0114
*Bsr*GI	NEB, UK	R0575
*Ava*I	NEB, UK	R0152

2.1.11 Software

Software	Commercial source
Vector NTI	Invitrogen, UK
Rotor Gene Software 1.7	Corbett Life Science, UK
Odyssey Infrared Imaging System Application Software V1	Li-cor Bioscience, UK
Axio Vision 4.8.1	carl Zeiss Image solution, UK
BD FACSiva^{TM} Software	BD Bioscience, UK
GraphPad Prism	GraphPad, US

2.2 Molecular cloning method
2.2.1 Agarose gel electrophoresis

The amount of agarose and Tris-acetate-EDTA (TAE) to be used depends on the concentration of the gel required for analysis. Agarose gel of 0.8-1% concentration is normally adequate to analyse the polymerase chain reaction (PCR) products and DNA. To prepare 0.8 agarose gel, 0.4 g agarose powder (Invitrogen, UK) was dissolved in 49 mL of 1 × Tris-acetate-EDTA (TAE) buffer (see appendix 2). 0.5 µg/mL of Ethidium bromide (EtBr) (Invitrogen, UK) was added into the mixture. Then the mixture was poured into a gel caster set that had been prepared earlier. The gel was left to solidify at room temperature for at least for 30 minutes. Once solidified, the gel was placed into an electrophoresis tank filled with 1 × TAE buffer and then the samples of interest were loaded on gel and run at 50 V for 1 hour.

2.2.2 Restriction enzyme digestion

For a restriction enzyme digestion reaction with final volume of 20 µL, 1× of the corresponding buffer (NEB, UK), 100µg/mL of Bovin serum albumin (BSA) (NEB, UK) and 500 ng of DNA were mixed with 2.5-5 U of restriction enzyme (NEB, UK) and then the mixture was incubated at 37°C for 1 hour. After incubation time the reaction was inactivated at 65 °C for 20 minutes. The presence of DNA fragments was visualized by running the digested products on agarose gel.

2.2.3 Determination of nucleic acid concentration

The concentration and purity of DNA were measured using a Spectrophotometer. This technique is based on measuring the amount of light that was absorbed by the 1 μL of DNA solution placed in a NanoDropTM 1000 (Eppendorf, Germany). The purity of the sample was measured with wavelengths of 260 nm and 280 nm. A DNA sample is considered pure when its value assesses in a range of between $OD_{260/280}$ 1.8 to 2.0 $OD_{260/280}$. A reading of less than 1.8 or more than 2.0 indicates that the DNA sample might be contaminated.

2.2.4 Cloning in plasmid vector
2.2.4.1 Luria Bertani (LB) agar plates

For preparation of LB agar, 2% Luria broth (Sigma, UK) and 1.5% bacteriological agar was mixed with sterile dH_2O using a magnetic stirrer until the powder was completely dissolved (Sigma, UK).

The mixture was then autoclaved at 121°C, 100 kPa for 15 minutes. After the sterilization step was completed, the mixture was cooled down under running tap water. Next, 100 μg/mL of Ampicillin (Sigma, UK) was added to the mixture. It was mixed well before 20 mL of the liquid agar was poured into 100 mm bacterial Petri dishes in a bacterial safety cabinet. All the plates were left to solidify for roughly 2 hours and then stored at 4°C.

2.2.4.2 Luria Bertani (LB) broth

For preparation of LB broth medium, 2% Luria broth (Sigma, UK) was mixed with 1 L of sterile dH_2O using a magnetic stirrer until all the powder was completely dissolved. The LB broth medium was autoclaved 14 121°C, 100 kPa for 15 minutes.

2.2.4.3 Bacterial strains

One Shot ® TOP10 *E. coli* is a genetically modified *E. coli* strain to obtain high transformation efficiency (Invitrogen, UK).

Genotype: F– *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G.

2.2.4.4 Preparation of competent cells

2 μL of One Shot ® TOP10 *E. coli* bacterial stock was cultured in 10 mL LB broth at 37°C, with 220 rpm agitation for overnight. The next day, 1 mL of overnight culture was sub-cultured into a new 10 mL LB broth aliquot at 37°C with 220 rpm agitation for approximately 10 minutes until cell density reached OD600 value of 0.5. Once the desired cell density was reached, the sub-culture was transferred into a sterile 15 mL Falcon tube and left in ice for 30 minutes. After that, the samples were centrifuged at 1,000 X *g* for five minutes at 4°C. Next, the supernatant was decanted before 5 mL of Tfb I solution (see appendix 2) was added. The pellet formed was re-suspended by gentle inversion on ice. The suspension was then incubated in ice for 20 minutes and was centrifuged at 1,000 X *g* for 15 minutes at 4°C. After decanting the supernatant, the pellet was re-suspended with 2 mL of Tfb II (see appendix 2) by gentle inversion on ice.

100 µL of the competent cell suspension was liquated into sterile 1.5 mL microcentrifuge tubes and were snap-frozen in liquid nitrogen for a few seconds before being stored in -80°C for future use.

2.2.4.5 Polymerase chain reaction

Each PCR reaction consisted of 1× Phusion HF Buffer (Thermo Fisher Scientific, Germany), 200 µM dNTP (Thermo Fisher Scientific, Germany),

0.5 uM of reverse and forward primers (Sigma, UK), 0.02 Units Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Germany) and 10 ng DNA sample in the final volume of 25 µL.

PCR was carried out with initial denaturation set at 98°C for 5 minutes, annealing at 60°C for one minute and extension at 72°C for one minute, followed by six cycles of denaturation at 98°C for 30 seconds, annealing at 60°C for one minute, extension at 72°C for 15 seconds, then followed by 35 cycles of denaturation at 98°C for 20 seconds, annealing at 70°C for 1 minute, extension at 72°C for 15 second followed by final elongation step at 72°C for five minutes.

2.2.4.6 PCR purification

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, UK) following manufacture's recommendations.

2.2.4.7 Preparative restriction enzyme digestion

Restriction enzyme digestion was carried out in 50 µL of reaction volume in a sterile 1.5 mL microcentrifuge tube. For a single reaction, 1× of corresponding Buffer (NEB, UK), 100 µg/mL of BSA (NEB, UK) and appropriate concentration of DNA (5 µg of backbone plasmid or 10 µg donor plasmid) were mixed with the appropriate volume of restriction enzyme (NEB, UK) and then the mixture was incubated at 37°C for 3 hours. After incubation time, the reaction was inactivated by heating at 65°C for 20 minutes.

2.2.4.8 Alkaline phosphatase treatment of DNA fragments

For phosphatase reaction, 1µL of Antarctic phosphatase enzyme (NEB, UK) and 5 µL of Phosp buffer were added to the sample. Sample was then incubated at 37°C for 30 minutes followed by inactivation at 65°C for 5 minutes.

2.2.4.9 Purification of DNA by gel extraction

DNA was purified using QIAquick Gel Extraction Kit (Qiagen, UK) following manufacture's recommendations.

2.2.4.10 DNA ligation

The ligation reaction was set up in a final volume of 10 µL using 3 units of DNA ligase (Promega, UK), 1×T4 ligation buffer, and the appropriate volume of backbone and insert. The reaction was kept at 18°C overnight. The maximum number of corrected

clones was obtained by using 3:1 insert/vector molar ratios for cohesive termini while for blunt-end ligation reaction 5:1 insert/vector molar ratios were used.

2.2.4.11 Heat–shock transformation method

Half of the ligation product was mixed with 100 μL of competent cell before the tube was placed in ice for 30 minutes. The tube was then placed in a 42°C water-bath for 45 seconds and immediately returned to ice for ten minutes. Subsequently, 900 μL of sterile LURIA broth (LB) broth medium was added into the tube and followed by incubation at 37°C for one hour with agitation at 180 rpm. Finally, cells were seeded onto agar plates containing an appropriate selective antibiotic for overnight.

2.2.4.12 Screening of transformants

Colonies were selected from the plates and each one was used to set up a 4 mL mini-culture in LB with 100 μg/mL of Ampicillin (Sigma, UK). The samples were incubated at 37°C agitation at 220 rpm overnight. After incubation, DNA plasmid was extracted from 1.5 mL overnight culture using the QIAprep Spin Miniprep Kit (Qigen, UK). DNA plasmid was then subjected to restriction enzyme digestion for checking the plasmid as described in section 2.2.

2.2.4.13 Purification of plasmid DNA

Plasmid DNA was extracted in Mini, Maxi and Mega scales using QiAprep Spin Miniprep Kit, EndoFree Plasmid Maxi Kit and Endofree Plasmid Mega Kit (Qiagen,

UK) respectively. The experiments were carried out according to manufacturer's instructions.

2.2.4.14 Storage of bacteria

Once successful transformed plasmid was confirmed using sequencing technology and analysing the sequencing results, then it was subjected to storage in -80°C for future work. 500 µL of the miniculture was mixed with 500 µL of 30% sterile glycerol stock to make glycerol stock of selected clone and stored it in -80°C.

2.3 Tissue culture methods

All cell culture and related works were carried out in sterile conditions in a class II biosafety cabinet, unless otherwise stated. Cells were maintained in a tissue culture incubator at 37°C, 5% CO_2 or frozen in aliquots stored in liquid nitrogen.

2.3.1 Thawing frozen cells

In order to retrieve frozen cells, the cell cryovial was placed into a 37°C water bath for approximately 3 minutes. The content of the vial was transferred into 9 mL of Dulbecco's modified Eagle medium (DMEM) with stable glutamin and 4.5 g/l glucose (PAA, Austria) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies, UK), 100 U/mL penicillin and 100 µg/mL streptomycin (Pen& strep) (PAA, Austria) (this basic culture medium will be titled to as full DMEM in next section), followed by centrifuging at 350 X g for 5 minutes. The supernatant was

removed after centrifuging, and the cells were re-suspended in full DMEM medium and transferred into a flask and incubated at 5% CO_2 and 37°C.

2.3.2 Cell lines
2.3.2.1 Culture of HeLa and HEK293T cells

HeLa and HEK293T cell were cultured in full DMEM. The cell line was incubated at 5% CO_2 and 37°C on 75 cm^2 flask. Cells were passaged every 2-4 days once cells were 80-90% confluent to avoid over-confluence of cells and to maintain a normal growth condition for cells. In order to split the cells, they were washed with Dulbecco's PBS and then cells were detached from the culture surface using 0.1% trypsin (diluted in PBS/ EDTA) at 37°C for roughly 3 minutes. After incubation time, cells were subsequently collected in full DMEM and spun at 350 X *g* for 5 minutes. After centrifugation, the medium was removed and the cells were re-suspended in 10 mL of full DMEM. The required number of cells was transferred to the new flask with appropriated final volume of full DMEM. In order to count the number of cells, 10 μL of the re-suspended cells were mixed with 10 μL of 0.4% trypan blue then 10 μL of this mixture was placed in a haemocytometer and then the number of cells was counted under the microscope.

2.3.2.2 Culture of CHO cells

The standard medium used to grow CHO cells was DMEM, supplemented with 10% FBS, 0.02 g/l L-proline, 100 units/mL penicillin, and 100 μg/mL streptomycin. The maintenance conditions and the rest of general cell culture procedure, such as splitting

the cells and counting the number of cells for CHO cells, followed the same description as outlined in the tissue culture methods section.

2.3.2.3   CHO cell-cycle arrest

DMEM without methionine supplements with 2% FBS, 0.02 g/L- proline, 100 units/ mL penicillin, and 100 µg/mL streptomycin were used on purpose to arrest CHO cell line (Kymäläinen *et al*., 2014). The maintenance condition followed the same description in the tissue culture methods section.

2.3.2.4   Culture of human control and SMA type fibroblasts

The growth medium to culture fibroblast cells line contained 64.8% DMEM high glucose with stable glutamin 21% M-199 (Lonza, UK), 10% FBS, 10 ng/mL Fibroblast growth factor 2 (FGF2) (Miltenyi, Germany), 25 ng/mL Epidermal growth factor (EGF) (Miltenyi, Germany), 1µg/mL gentamicine. The maintenance condition and the rest of the general cell culture procedure, such as splitting the cells and counting number of cells for CHO cell followed the same description as outlined in the tissue culture methods section.

2.3.2.5   Storage of cells

Freezing medium was supplemented with 10% Dimethylsulfoxide (DMSO) to prevent ice crystals forming in cells, 50% FBS and 40% DEME (without any supplements) was used to freeze cells. Following the tryptinisation of a 80% confluent cultured cells, they

were stored by re-suspending in freezing medium. 10^6 of cell in 1 mL freezing medium was liquated into a cryovial and then the tubes were transferred into an isopropanol freezing container and stored at -80°C for overnight. The following day, the cryovials were transferred to a liquid nitrogen storage tank for long-term storage.

2.3.3 Primary cells

2.3.3.1 Isolation and culture of E18 mouse cortical neurons

A CD1 (Charles River, UK) pregnant mouse on day 18 was killed using CO_2 chamber, followed by cervical dislocation to confirm death. The embryos were transferred immediately into an ice-cold dissection buffer and kept on ice. The cortices were isolated using dissection of the brain. Mouse cortices were dissociated by 0.05% trypsin/EDTA in 37°C water bath for 20 minutes. The supernatant was aspirated and tissue pieces were washed with neuronal differentiation medium (see appendix 2) and then tissues were titred through pre-coating with inactivated FBS p100, p 200, p 10. In the next step, the cell suspension was then passed through a 100 μm cell strainer and cells were collected into a Falcon tube. The collected cells were centrifuged at 80 ×g for 5 minutes. 0.2% trypan blue was used to count the number of live cell suspension. Then 3×10^6 was seeded into a pre-coated 24-well plate with Poly D-Lysine (Sigma, UK). The primary cells were not passaged at all during experiments and they remained on pre-coated plates, which they seeded after isolation procedure.

2.3.3.2 Preparation of pure embryonic motor neuron primary cultures

Embryos were extracted from a E 15 Sprague-Dawley (SD) (Charles River, UK) pregnant rat under sterile condition. The head and the tail were separated from embryo body to extract the spinal cord by introducing fine forceps into the spinal cord canal. Ventral spinal cords were stored in L15 medium (Invitrogen, UK). In the next stage, the isolated ventral spinal cords were cut into 10-15 pieces and transferred into 15 mL tubes and washed with PBS (PAA, UK). The PBS was discarded and 1 mL of PBS + Trypsin 0.05% was added into samples and incubated for 12 minutes at 37C ° with frequent mixing. The trypsin + PBS was discarded and then 0.8 mL L15c medium (Invitrogen, UK) supplemented with 7.5% Sodium Bicarbonate (Sigma, UK), 7.2 % glucose (BDH Chemical, UK), 1% penicillin/ streptomycin (CSL, UK), 0.1% putrescine (ICN Biochemicals, UK), 1% conalbumin (Sigma, UK), 2% horse serum (CSL, UK), 0.1 % progesterone (Sigma, UK), 1% Insulin (Invitrogen, UK), 0.1% sodium selenite + 0.1mL BSA 4% (Sigma, UK) + 0.1 mL DNAse I (1000U/mL) (Promega, UK) was added into the samples and then the sample was mixed for 3 minutes until DNAse separated tissue fragment. In the next stage, tissue fragments were allowed to settle into the bottom of the tube and then the supernatant was transferred into 15 mL tubes. 0.9 mL L15c + 0.1 mL BSA 4% + 20 µL DNAse I (1000U/mL) was added into samples and using 1 mL micropipette was pipetted gently six times. Supernatant was transferred into 15 mL tubes and then 11 mL of L15c was added into it. Before the samples were centrifuged at 300 g for 10 minutes, 1 mL of BSA 4% was very slowly added into the bottom of the tubes using a glass Pasteur pipette. After centrifuging, the supernatant was discarded and the pellets were re-suspended 10 times into 1 mL L15c + 20 µL DNAse (1000U/mL). 2 mL L15c was added and then spilt into two 15 mL tubes with 1.5 mL each (1 tube per 4-5 spinal cords). 2 mL of Optiprep (1:10 in L15) was very slowly added at the bottom of

each tube. In the following step, the samples were centrifuged at 800 xg for 15 minutes. 0.9 mL of medium over the band of cells was discarded using 1 mL micropipette. The band of motor neurons was collected in 1 mL and added to the immunopanning plate containing secondary antibody against rat IgG (ICN, UK) and IgG192 (in-house made monoclonal antibody supernatant, against the rat p75 neurotrophin receptor, (see appendix 2)) and incubated at room temperature for 40 minutes. The plate was washed three times with L15, and then 4 mL of IgG 192 was added to the plate to detach the motor neurons. The detached neurons were transferred into a 15 mL tubes which were then filled with 11 mL of L15c. 1 mL of BSA 4% was added at the bottom of the tubes with a glass Pasteur pipette and the samples were centrifuged at 300 g for 10 minutes. The pellet was resuspended into 0.5 mL of L15c and then the number of cells was counted. The cells were seeded at a 300 cells/cm^2 on a 12 well plate coated with 10 g/mL poly-d-lysine (Sigma, UK) and 10 g/mL laminin (Sigma, UK).

2.4   Lentivector production
2.4.1 Preparation of HIV vectors by calcium phosphate transfection

HEK293T cells were seeded at 3×10^6 per 15 cm plate in 20 mL of full DMEM. The cells were transfected when they were 50-60% confluent. The media was replaced two hours prior to transfection. The DNA ratio of third generation vectors was followed 1:1:1:2 (PACKAGING, REV, ENV, TRANSFER), so 12.5 µg of the packaging plasmid (pMELg/ pRRE (integration proficient) or pMDLg/ pRRE-intD64V (integration-deficient through point mutation which inactivates the catalytic site of integrase), 7µg of ENV plasmid (pMD2.VSV- G) and 25 µg of TRANSFER plasmid was mixed together and made up the DNA mixture 1125 µL with TE buffer. After that, 125 µL of 2,5 M Calcium Chloride (CaCl$_2$) was added to sample, the mixture vortexed and incubated for

5 minutes at room temperature. 1250 μL of 2× HBS (see appendix 2) was added to sample drop-wise while vortexing DNA-CaCl2, then the mixture was added to the cell immediately and mixed with medium. Afterward, the sample was incubated at 37°C and 5% CO2 for 16 hours. After incubation time, the medium was removed and replaced with 18 mL/ plate of fresh medium and subsequently the medium was harvested 48 and 72 hours post-transfection. After the first harvest, 18 mL fresh medium was added to the cell. Harvested medium was centrifuged at 1000 X g for 10 minutes, room temperature and then supernatant was filtered through a 0.22μm Nalgene filter. After that, filtered medium was transferred to high-speed polyallomer centrifuge tubes and was centrifuged at 50,000 X g, 4 °C for 2 hours. After discarding the supernatant, 50 μL/tube of DMEM without supplement was added to the viral pellets and mixed by pipetting up and down ~ 10 times to re-suspend the virus and transferred to an Eppendorf tube. The sample was centrifuged for 10 minutes at 1000 X g at room temperature to remove any debris. After that, supernatant was transferred into a new Eppendorf tube. The vector stock was adjusted to 10 mM (Magnesium Chloride) MgCl2 with 1M MgCl2 by adding 0.5 μL/50 μL vector stock. 5 Unit/mL of DNAse was added to each sample, and then the samples were incubated at 37°C for 30 minutes. The viral vectors were liquated and frozen at -80°C.

2.4.2 Lentivector titration by flow cytometry (eGFP)

1×10^5 HeLa cells were seeded per well on a 6-well plate and incubated at 37°C and 5% CO2 overnight to be titrated. The next day, 10-fold vector dilution was made in full DMEM. The dilution range was from 10^{-3} to 10^{-6}. The medium was removed from wells and 1 mL of DMEM 16 μg/mL polybrene was added to each well. Then 1 mL of

adequate virus dilution was added to each well too, and 1 mL DMEM for mock. After 72 hours transduction, cells were harvested by trypsin digestion. Cells were washed with 1 mL (phosphate buffer saline) PBS and then 150 μL of trypsin-Ethylenediaminetetraacetic acid (EDTA) were added to each well and incubated at 37°C for a few minutes to detach cells. After detaching the cells, 850 μL DMEM was added to each well to transfer all cells into fluorescent activated cell sorting (FACS) tubes. The samples were spun at 350 X g for 5 minutes. The supernatant was aspirated very gently and then the pellet was re-suspended in 200 μL of PBS 1% formaldehyde. The samples were run through flow cytometer, using the mock to set negative population and highly positive sample to set compensation. The viable cell population was selected in the forward scatter (FCS) versus side scatter (SSC) plot, and used FL1 (eGFP) versus FL2 (red autofluorescence) to identify positive population.

Titer was calculated using dilution with 1-10% green cells. The formula for calculation is eGFP transducing units

(TU)/mL=%green cells $\times 10^5$ (cells/well on day 0) × 1/vector dilution.

2.4.3 Lentivector titration by quantitative real time PCR

1×10^5 HeLa cells were seeded per well in a 6-well plate and incubated at 37°C and 5% CO_2 overnight to be titrated by quantitative real time PCR (qPCR). The following day, 2 vector dilutions (5×10^4 and 5×10^5) were made to transduce HeLa cells. Next, 1 mL of virus dilution was added to each well and moreover, 1 mL of DMEM 16 μg/mL polybrene was added to wells and incubated at 37°C and 5% CO_2 overnight. The next day, cells were harvested by trypsin digestion then 850 μL of full DMEM was added to samples, and cells were transferred into Eppendorf tubes. The sample was spun at 350

X g for 5 minutes then the supernatant was removed. In the next part, the pellet was re-suspended with 500 μL of PBS. One more time, the samples were spun at 350 X g for 5 minutes and the pellet was re-suspended into 200 μL of PBS. The samples were then subjected to DNA extraction with DNeasy tissue Qiagen kit (Qiagen, UK). Two qPCR reactions were set up using SYBR Green (Bioline, UK) but with different primers. DNA standard samples, which were used in these two qPCR reactions, were made in QIAGEN DNA elution buffer with dilution 10^2-10^7 and were prepared in triplicates. The first reaction was human β-actin qPCR, the reaction was carried out in final reaction volume of 20 μL. Every reaction contains 10 μL of SYBR green (Bioline, UK), 0.02 μL of 100 μM forward primer (actin-f: 5′-TCACCCACACTGTCCCATCTACGA-3′), 0.02 μL of 100 μM of reverse primer (actin-r: 5′-CAGCGGAACCGCTCATTGCCAATGG-3′), 5 μL of DNA sample and a sufficient amount of dH_2O to make up final volume of 20 μL. The second reaction was a late reverse transcript reaction, which followed the above concentration and volume but used different primer. The forward primer was LRT-f:5′-TGTGTGCCCGTCTGTTGTGT-3′ and reverse primer was LRT-r: 5′GAGTCCTGCGTCGAGAGAGC-3′. PCR was carried out with initial denaturation step at 95°C for 10 minutes, followed by 40-50 cycles of denaturation at 95°C for 15 second and 60°C for 45 seconds.

Viral titre was calculated based on the formula below:

Viral vector titre = viral DNA copies / number of total cells × 10e5 seeded cells × 1/ vector dilution.

2.5 *In vitro* experiment

2.5.1 Study of the transduction efficiency of generated lentivectors

2.5.1.1 Transduction of growth-arrested CHO cells

1×10^5 CHO cells in full DMEM were seeded in 6-well plates. The following day, the cells were arrested (according the description in section 2.3.2.3) and transduced with produced lentivector at with q-PCR MOI 100 and 500. 72 hours after transduction, efficiency of transduction was examined using western blot method as described in section 2.5.3.

2.5.1.2 Transduction of motor and cortical neurons in primary culture

Transduction on motor neuron was carried out two hours post seeding while transduction procedure for cortical neuron was carried out three weeks after seeding the cells. The transduction procedure was followed by the collection of all the medium of primary cell culture into 50 mL Falcon tubes. The collected medium was mixed very well and 1 mL or 200 μL of collected medium was added into cortical neurons or motor neurons respectively. In the following step, the primary cortical neuron cells were transduced with 30 and 100 qPCR MOI of integrating and non-integrating lentivectors, which encoded the gene of interest. However, embryonic motor neuron primary cells were transduced with 30, 60 and 100 qPCR MOI of generated vectors. Analysis of vector's transduction efficiency in motor neuron was performed three days post transduction using immunostaining. Transduction efficiency in the cortical neuron was analysed three days post transduction using western blot method.

2.5.2 Functional effect of produced SMN protein in fibroblast cell line

2.5.2.1 Fibroblast transduction

1×10^5 of fibroblast cells in full DMEM were seeded in 12-well plates. Three days post seeding, the cells were transduced with lentivector at q-PCR MOI 30, 60 and 100. The samples were subject to immunostaining to study the functionality of produced SMN protein using generated lentivectors three days post transduction.

2.5.3 Western blotting

2.5.3.1 Isolation of protein

Cells were gently washed twice with ice-cold PBS and 200 µL of ice-cold RIPA buffer containing a cocktail to inhibit proteases (Roche, Germany) was added to cells. Samples were incubated at 4°C for 10 minutes. In the next step, cells were harvested using a cell scraper and collected into an Eppendorf tube. The samples were then centrifuged at 14000 X g, 4°C for 15 minutes to remove large debris. After 15 minutes of centrifuge, the supernatant was transferred to a new Eppendorf tube and kept at -80°C.

2.5.3.2 Protein assay

Bio-Rad protein Assay Kit (Bio Rad, USA) was used to measure the concentration of protein in each sample. The experiment followed the instruction of the DC protein assay.

2.5.3.3 SDS-PAGE and western blotting

Up to 5 µg of each protein sample were mixed with 5 µL of 5 × sample buffer (see appendix 2) and incubated at 95°C for 10 minutes. Then the samples were loaded onto a 12% SDS-polyacrylamid gel (see appendix 2). The gel was then run with 1× running buffer (see appendix 2) for 150 min at 150 V. After that, the proteins were transferred to membrane using a mini trans-Blot Cells at 150 mA for 2 hours using a fresh, cold 1× WB transfer buffer (see appendix 2). The efficiency of transference was assessed at 5 minutes incubation with 10 mL Poneau red solution (see appendix 2). After a successful transfer, the membrane was incubated with WB blocking buffer for two hours on gentle agitation at room temperature and then overnight at 4°C with the primary antibody diluted in 10 mL of WB blocking buffer. The following day, the membrane was washed 4 × with TBS_T (see appendix 2) and gentle agitation. In the next step, the membrane was incubated with secondary antibody diluted in 5 mL WB blocking buffer for 2 hours at room temperature. The membrane was washed four times with TBT-T and gentle agitation. Protein of interest was detected on membrane using infrared imaging (Odyssey system). The exposure time when scanning the membrane was the same for each individual experiment.

2.5.3.4 Quantification of western blots

Western blotting is a method used to confirm the presence or absence of a protein of interest in a mixture of proteins. It is based on electrophoretic separation: separated proteins are transferred to a membrane and then stained with specific antibodies. The primary antibody is directed against protein and recognizes a specific amino-acid sequence of a particular protein, while the secondary antibody recognizes the primary

antibody, and is conjugated with an enzyme or fluorescent dye. Detected proteins can be quantified by generated light or by fluorescent signal through the secondary antibody.

In regard to protein quantification, the fluorescent detection method has advantages over other methods. Detection is more consistent and straightforward, and quantification is more accurate. Fluorescent conjugates avoid the enzyme kinetics and substrate availability which are the limitation of chemiluminescence.

The intensity of generated fluorescence is indicative of the amounts of protein on the membrane, which has been detected using antibodies (LI-COR, 2012).

The membrane was scanned and visualized in the 800 and 600 channel using Odyssey Infrared Imaging System (LI-COR Biosciences, UK). The saved image was then used to quantify the proteins by drawing a rectangular box around the band of interest to get the reading for each individual band. The software can automatically deduct the background from the signal for each band. For each individual sample in this experiment, a rectangular box was drawn around a loading control (α tubulin protein) and targeted protein (SMN). The signal values for the sample are divided per signal values of relevant loading control. The result is a normalised value, and stands for fluorescence intensity which is directly proportional to the amount of target protein on each shape drawn in arbitrary fluorescent units. The background will automatically be deducted from the signal.

2.5.4 Immunofluorescence

2.5.4.1 Immunofluorescence staining

Cultured cells in 12-well plate or 8-well chamber slide were washed in ice-cold $1 \times$ PBS and then in the following step, the cells were fixed using 4% Paraformaldehyde for 15 minutes and then washed twice with ice-cold 1×PBS. The cells were permeabilized 1× TBS-T (see appendix 2) for 10 minutes and incubated for 30 minutes in 1% BSA blocking buffer (see appendix 2). The cells were incubated with primary antibody diluted in 1% BSA blocking buffer for overnight at 4°C. The following day, the samples were washed in ice-cold $1 \times$ PBS for 3 times with gentle agitation. After washing, the samples were incubated for 2 hours at room temperature with the secondary antibody diluted in 1% BSA blocking buffer. Then the samples were washed again in ice-cold $1 \times$ PBS 3 times with gentle agitation. In the following stage, the samples were incubated 1 μg/mL 4,6-diamidion-2-phenylindole (DAPI) for 15 minutes in the dark at the room temperature. For the last time, the cells were washed in ice-cold $1 \times$ PBS three times with gentle agitation, and then the images of the samples were captured and quantified. The same exposure time was used to capture the images for all replicates.

It is important to note that the microscopy images were taken from cells seeded on cell culture plasticware in buffer. As most plasticware used for cell culture has high autofluorescence, and the cells were covered with buffer, this may help explain the blurry images obtained in some cases.

2.5.4.2 Measurement of SMN intensity by immunofluorescence

In fluorescence microscopy, the intensity value of a pixel is used to determine the local concentration of fluorophores present at the targeted area. Fluorescence from a

fluorophore tagged to an antigen of interest is often used to measure the quantity of the tagged protein.

Samples were selected for analysis based on identification of single motor neuron cells per field. Analysis of the samples was performed blind to vector type and gene of interest, and using MOI. Regions of interest are created around the motor neuron cell body and fluorescence pixel intensities were measured within this region of interest. The area outside the regions of interest is considered a background, and is subtracted from the fluorescence intensity. The subtracted fluorescence pixel intensities were divided by the selected region of interest area and the value stand as intensity mean value and expressed as a.u./µm2. The analysis was performed using AxioVision software (Carl Zeiss, UK).

2.6 *In vivo* studies

All the animal procedures were performed according to United Kingdom Home Office regulations.

2.6.1 Animal strain

The wild type mice were CD1. The SMA mice used in this study were 'Taiwanese' mouse model of severe SMA (genotype $Smn^{-/-}$; $SMN2^{tg/0}$) and their breeding was according to the breeding strategy developed by Riessland *et al*., 2010.

2.6.2 Animal feeding

A normal chow diet was used to feed animals.

2.6.3 *In utero* surgery

In this study, inhalational anaesthetic was chosen between many types of available anaesthesia for mouse surgery, as a vaporizer releasing isoflurane results in rapid sedation and recovery. The mouse was in an induction chamber with 400-500 mL of 4% isoflurane per minute vaporised into it, and the pregnant mouse was kept in the induction camper until she lost her righting reflex. Following the induction of anaesthesia, mice received an analgesic (Flunixin Meglumine, 2.5 mg/kg) *via* subcutaneous injection. Operation procedure was started when the unconscious E16 pregnant female mouse was maintained via face mask ~ 270 mL/ minute of 2.7% isoflurane and then an incision was made in the abdominal cavity using fine scissors. The uterus was exposed carefully by pinching gaps between foetuses.

It is very tricky not to damage either placenta or the uterus blood vessels, and it is very important to keep the uterus wet during procedure by dropping pre-warm PBS.

Hamilton needle was filled with viral vectors and all foetuses received either 2 µL of viral vector into their spinal cord or 10- 20 µL of viral vector into their intraperitoneal cavity.

After completing the injection, the uterus was placed back into the abdominal cavity and the surgical incision was sutured using interrupted stitches. The injected pregnant females recovered in a warm cage.

2.6.4 Physical check and behaviour test
2.6.4.1 Righting reflex

The behaviour test carried out in this study was righting reflex. Righting reflex is a simple and rapid test to assess locomotor ability. The strategy of this assay is to score and measure a mouse's ability to turn themselves on to their paws when they are placed in a supine position. The result of this physical activity is a performance of the general body strength, the straightness and weakness of muscles and improvement by therapeutic compounds.

To assess the righting reflex, the mouse was normally placed on its back on a flat surface, then the time taken for them to right themselves was measured. This test was carried out every day after the pups were born. The SMA mice are not very good at doing this, so this assay is use as a readout of their motor performance.

2.6.4.2 Checking weight

The weight of the animal was recorded every day for any therapeutic improvement, and to find out the ending point of the running experiment. According to United Kingdom Home Office regulations the ending point of an experiment is when the animal loses 20% of their weight. Therefore, in this study, the weight of pups was recorded when they were born and they were weighed daily.

2.6.5 Collection of tissues

The experiment was terminated by intraperitoneal injection of sodium pentobarbital into injected mouse at 2 weeks post injection. Tissue collection was started after confirming the dam's death using neck dislocation.

Whole spinal column was removed, and a 23-gauge needle connected to a 5 mL filled syringe with PBS was inserted into the sacral end of the column. The PBS was gently flushed through the spinal column to remove the spinal cord from the cervical end of the column.

Liver, brain and muscles tissues were harvested as well.

The aim was to analyse collected tissue with western blot and immunofluorescence methods. Thus, the tissues were divided into two parts. One part was transferred into Eppendorf tubes and stored into -80 to be analysed with western blot (full description is available in section 2.6.8). The other part of the tissue was fixed with 4% paraformaldehyde at 4°C for 24 hours for more investigation using immunofluorescence method (full description is available in section 2.6.7).

2.6.6 Immunofluorescence
2.6.6.1 Sectioning

The tissues of interest were fixed in 4% paraformaldehyde (PFA) in PBS overnight, and the following day the PFA was removed and the tissue was washed twice with PBS. In the next part, fixed tissue was incubated into 30% sucrose (Sigma, UK) in PBS overnight at 4 C° to cryoprotect the tissues. The tissues were dissected and embedded separately in optimal cutting temperature (OCT) compound (CellPath, UK) then frozen on dry ice. In the next stage, the tissues were sliced at 25 µm thickness using a cryostat (Leica

CM3050S, UK) and every 4th section collected onto poly-L-lysine coated slides (Thermo Scientific, UK). Excess OCT compound around section was removed using PBS and a narrow paintbrush. Slides were left to dry before processing for immunohistochemistry.

2.6.6.2 Immunofluorescence staining

Sectioned tissue was washed three times with 1×PBS for 5 minutes. In the next stage, the tissue was permeabilised with 1×PBS-T (see appendix 2) for 10 minutes. After the incubation time, the sample was blocked using 1% BSA blocking buffer (see appendix 2) for half an hour to avoid non-specific staining between the primary antibodies and the tissue. The samples were incubated with diluted primary antibody in 1% BSA buffer overnight. After the incubation time, the slides were washed with 1×PBS for 5 minutes three times. Then the tissues were incubated with diluted secondary antibody in 1% BSA buffer for 2 hours. Following the incubation time, the sample was washed three times with 1×PBS for 5 min. Then the tissue was incubated in the dark for 15 minutes with 1 μg/mL DAPI. After staining the tissue with DAPI, the samples were washed 3 times with 1×PBS. For the last time, the cells were washed in ice-cold 1× PBS for 3 times. The section was mounted with mounting solution (see Appendix 2) then covered with coverslips and stored at 4 °C in the dark.

2.6.7 Western blotting
2.6.7.1 Tissue lysis

Stored tissues at -80°C were defrosted on ice. Then the tissue was cut into smaller pieces while it was kept on ice and a proper size of tissue was transferred into an Eppendorf

tube to undergo lysis. Then 100 µl of RIPA buffer (Thermo Fisher Scientific, UK) containing proteinase inhibitor solution (Thermo Fisher Scientific, UK) was added to samples. After that, the tissue was thoroughly homogenized using a plastic pestle. Then the sample was incubated on ice for 10 minutes. Following the incubation time, the sample was centrifuged at 14,000 X g at 4 °C for 10 minutes. After centrifuge time, the supernatant was transferred into new Eppendorf tube while the sample was kept on ice. The extracted protein was stored at -80°C for future usage.

2.6.7.2 Protein assay

The procedure was carried out according to the description in section 2.5.3.2.

2.6.7.3 SDS-PAGE and western blotting

The western blot method to analyse *in vivo* an experiment was followed as per the description in section 2.5.3.3.

2.6.8 Statistical Analysis

Prism 5 software (GraphPad, California, US) was used for all statistical analyses. All data is presented as mean ± standard error of the mean (SEM) and normalisation was not performed when presenting data. In *in vitro* studies "n" refers to the number of independent experiments performed to collect data and to analyse them statistically. However, in the case of *in vivo* experiments "n" refers to the number of animals per group. When analysing data collected from transduced primary motor neuron and SMA fibroblasts, a single number was generated (by averaging) for each independent

experiment and the means and SEMs are based on the numbers from individual experiments. Comparisons of statistical significance were assessed by one-way ANOVA followed by Bonferoni or Dunnett *post-hoc* tests. Dunnett *post-hoc* was employed to compare mock groups with each individual transduced group while Bonferoni *post-hoc* was employed to run a comparison between transduced groups. Differences were considered statistically significant if P<0.05 (*), P<0.01 (**) and P<0.001 (***).

Chapter 3   Cloning of lentiviral transfer plasmids and vector production

3.1 Introduction

Lentiviral vectors have been extensively analysed and used as promising tools for the genetic modification of targeted cells in biomedical research and gene therapy applications. Lentiviral vectors have been used successfully in clinical trials and they are increasingly being used as gene therapy vectors for the treatment of acquired and inherited diseases. Their key attractive properties above other viral vectors are: a comparatively larger packaging capacity, the ability to transduce both dividing and non-dividing cells, broad tissue tropisms, no expression of viral proteins after vector transduction, sustained gene expression through stable vector integration into host genome, similar transduction efficiency to adeno-associated virus vectors, reduced immunogenicity during *in vivo* administration and lack of prior immunity (Blömer *et al*., 1997; Merten *et al.* 2016 and Sakuma *et al.*2012). Despite the integration features of lentiviral vectors, which provide stable gene expression through integration of their genome into the host DNA, the integration property can have unintended consequences, such as a risk of insertional mutagenesis (Shaw & Cornetta, 2014). Preventing the integration of viral transgenes into the host genome is the most straightforward way to overcome this problem. The integrating mechanisms of lentiviruses have been studied extensively and significant efforts have been made to develop non-integrating lentiviral vectors by mutating the integrase gene (Sarkis *et al.*2008). Several studies have reported efficient gene expression using both integrating and non-integrating lentiviral vectors *in vitro* and *in vivo* (Cornu & Cathomen, 2007; Peluffo *et al*., 2013; Wong, Goodhead, *et al*., 2006; Wong, Yip, *et al*., 2006 and Yáñez-Muñoz *et al*., 2006). Therefore, in this study both integration-proficient lentiviral vectors (IPLVs) and integration-deficient lentiviral vectors (IDLVs) were

employed as potent gene delivery vehicles to transfer the gene of interest into the targeted host.

3.2 Aims of the chapter

This chapter describes the work undertaken to produce IPLVs and IDLVs expressing the gene of interest. My specific objectives are:

(A) Cloning the different type of *hSMN1* genes into different lentiviral plasmid backbones.

(B) Producing paired IPLVs and IDLVs with identical structure but differing in integration proficiency.

(C) Titrating the lentiviral vectors in HeLa cells by qPCR and, for *eGFP* vectors, by flow cytometry.

3.3 Summary of experiment and method

Cloning of DNA molecules into plasmid vectors is one of the most commonly employed techniques in molecular biology. Most methods for cloning DNA fragments in the laboratory share certain general features. To clone the gene of interest into lentiviral vector's backbone, both the insert and backbone plasmid are digested using restriction enzyme to cleave the DNA fragments at specific location.

The restriction enzymes used in this chapter recognised specific six-base-pair-sequence of DNA and then cleaved the sugar-phosphate backbones in the two DNA strands in a staggered manner. The backbone and donor plasmid were cut with the same enzyme(s) and had complementary cohesive ends, which let the purified plasmid fragments ligate together using DNA ligase and created a new recombinant DNA. More details of the procedure are provided in sections 2.2. Cohesive-end ligation is over 100X more efficient than blunt-end ligation.

Lentiviral vectors were generated by transfecting the transgene and packaging plasmids into HEK293T cells. Virus particles were collected twice, 48 and 72 hours post transduction. At this time, maximal vector production occurred. More details can be found in section 2.4.1. Lentiviral vectors were concentrated by ultracentrifugation. Produced vectors were subjected to titration by qPCR and flow cytometry to determine the amount of particles in viral vector stock, more details in sections 2.4.2 and 2.4.3.

3.4 Cloning of potentially therapeutic transgenes into lentiviral backbone

In order to produce lentiviral vectors carrying the relevant transgenes, the first step was to clone the gene of interest into lentiviral plasmid backbones. The genes of interest were wild –type h*SMN1*, h*SMN1* with a C-terminal flag-tag (h*SMN*1-*CtF*), h*SMN1* with an N-terminal flag-tag (*hSMN1-NtF*) and codon-optimised h*SMN1* (*Co*-h*SMN1*). In this study, cytomegalovirus (CMV) or human synapsin (hSYN) promoters drove the transgene. The design and construction strategies of each lentiviral transgene plasmid are detailed in the relevant sections, and indicated elements on plasmid maps are listed in Table 3.1.

Table 3.1: List of abbreviation on plasmid maps

Abbreviation	Full name
AmpR	Ampicillin resistance
CAG	Short version of ubiquitous cytomegalovirus enhancer/ chicken beta actin promoter
CMV	Cytomegalovirus promoter
Co-h*SMN1*	Codon-optimized human *Survival Motor Neuron 1*
cPPT/cTS	Central polypurine tract/central termination sequence
dLTR	LTR with partial deletion of U3 sequences removing lentiviral promoter and enhancer
eGFP	Enhanced green fluorescent protein
eIeGFP	Enhanced green fluorescent protein gene under the action of an internal ribosome entry site
gag	Group specific antigen
hSMN1	Human *survival motor neuron* 1
hSMN1_CtF	Human *Survival Motor Neuron 1* with C-terminal FLAG tag
h*SMN1 NtF*	Human *Survival Motor Neuron 1* with N-terminal FLAG tag
hSYN	Human synapsin promoter
ITR	Inverted terminal repeat
IRES	Internal ribosome entry site
LTR	Long terminal repeat
m*Smn*	Mouse *survival motor neuron*
mW	Mutated woodchuck hepatitis virus post transcriptional regulatory element
PBS	Primer binding site
Poly (A)	Poly (A) tail
PPT	Polypurine track
RRE	Rev response element
RSV	Rous Sarcoma Virus promoter
RU5	Repeat and U5 regions of lentiviral LTR
SV40prom/enh	Simian virus-40 promoter/enhancer;
SFFV	Spleen focus-forming virus
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element

3.4.1 Construction of pRRLsc_C_m*Smn*_mW

The maps of the recipient transfer plasmid expressing m*Smn* under the control of CMV promoter (pRRLsc_C_m*Smn*_eIeGFP_W), donor plasmid containing mutated woodchuck hepatitis virus post transcriptional regulatory element (mWPRE) (pHR'SIN cPPT SE mW) and the new construct containing m*Smn* and mWPRE (pRRLsc_C _m*Smn*_mW) are provided in Figure 3.1.

The construction of the lentiviral transfer plasmid expressing m*Smn* under the control of CMV promoter and containing mWPRE (pRRLsc_C_m*Smn*_mW) was performed as follows: mWPRE (~600bp) was amplified from donor plasmid (pHR'SIN cPPT SE mW) using PCR and then digested with *Xma*I and *Eco*RI, (restriction sites were introduced using PCR oligos). IRES, eIeGFP, and wild-type WPRE elements were digested using *Xma*I and *Eco*RI from starting plasmid (pRRLsc_C_m*Smn*_eGFP_W) and the rest of plasmid (pRRLsc_C _m*Smn*) was used as backbone.

Digested and gel-purified mWPRE was ligated to pRRLsc_C_m*Smn* to generate the new plasmid (pRRLsc_C_m*Smn*_mW). The new construct pRRLsc_C_m*Smn*_mW was verified by digestion with *Sac*II, *Eco*RI and *Sma*I.

Backbone Plasmid

pRRLsc C mSmn eIeGFP W
9008 bp

Donor plasmid

pHR'SIN cPPT SE mW
9662 bp

Recombinant plasmid

pRRLsc C mSmn mW
7690 bp

A

B

C

140
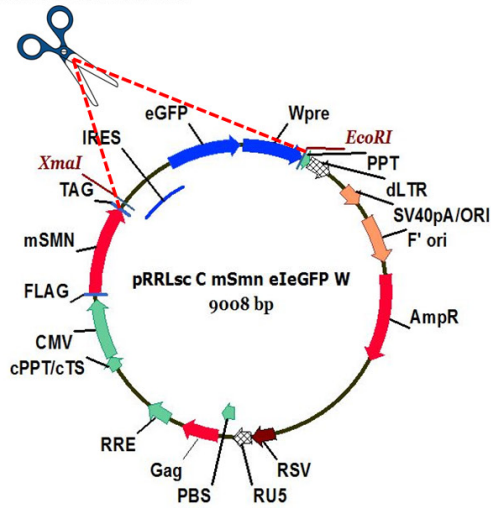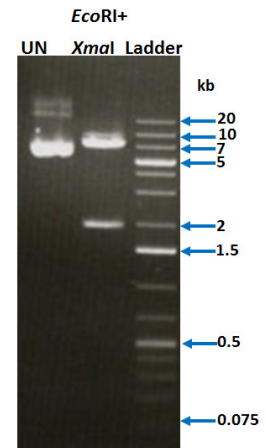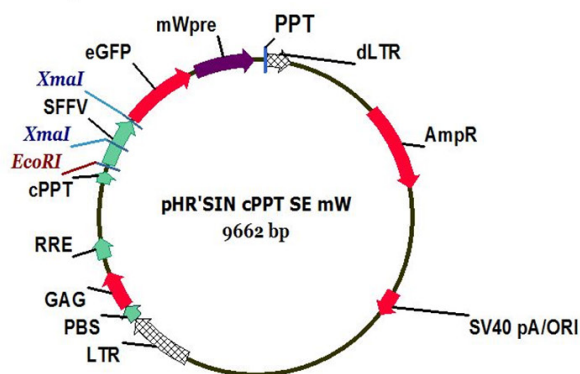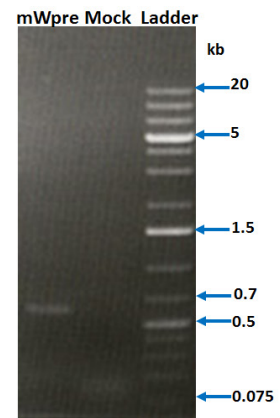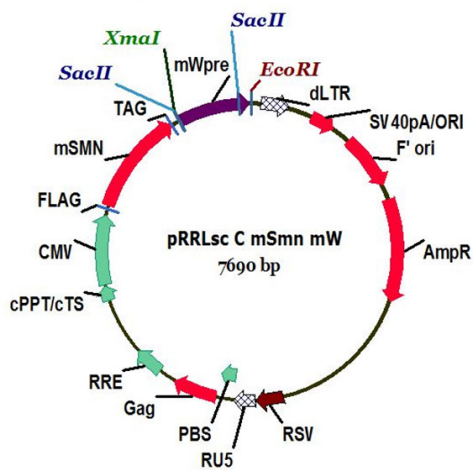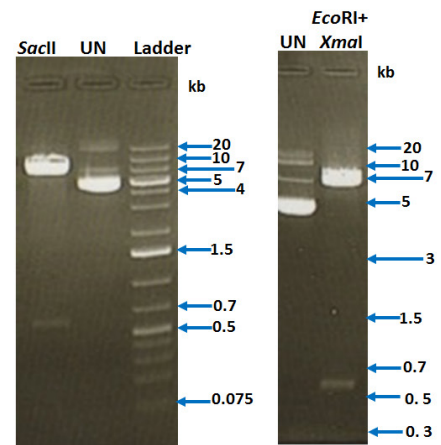
Figure 3.1: Cloning strategy for pRRLsc_C_m*Smn*_mW.

All plasmid maps are presented with relevant restriction sites. The main elements of the plasmids are listed in Table 3.1.

(**A**) *Xma*I and *Eco*RI double digestion was used to remove the IRES, eGFP and wild-type WPRE (1927 bp) from the recipient plasmid (pRRLsc_C_m*Smn*_eIeGFP_W).

(**B**) mWPRE (~600bp) was amplified from original plasmid (pHR'SIN cPPT SE mW) using forward primer and reverse primers. The forward primer was designed to introduce an *Xma*I restriction site at the 5' end of mWPRE (GCAATTAACCCGGGCCTGCAGGTAATCAACCTCTGGATTACA). The reverse primer was designed to introduce an *Eco*RI restriction site at the 3' end of mWPRE (CTTAATTAGAATTCATCGATACCGTCGACCTCGA) and then the amplified fragment was digested using *Xma*I and *Eco*RI. Then the digested mWPRE was inserted into the digested recipient transfer plasmid (pRRLsc_C_m*Smn*).

(**C**) Restriction endonuclease analysis of generated pRRLsc_C_m*Smn*_mW (7690 bp) construct confirmed the structure of the new plasmid. The enzymatic digestion with *Sac*II produced 7168 bp and 522 bp fragments, and *Eco*RI and *Sma*I double digestion produced 7083 bp and 602 bp fragments from the generated plasmid.

Sequencing was also performed to confirm mWPRE sequence (data not shown).

UN: Undigested plasmid.

Ladder was 1Kb plus ladder (Thermo Scientific, Germany).

3.4.2 Cloning of pRRLsc_hSYN_m*Smn*_mW

The maps of recipient transfer plasmid expressing m*Smn* under the control of hSYN promoter (pRRLsc_hSYN_m*Smn*_eIeGFP_W), donor plasmid containing mWPRE (pHR'SIN cPPT SE mW) and the new construct containing m*Smn* and mWPRE (pRRLsc_hSYN_m*Smn*_mW) are provided in Figure 3.2.

The construction of the lentiviral transfer plasmid expressing m*Smn* under the control of hSYN promoter and containing mWPRE (pRRLsc_hSYN_m*Smn*_mW) was performed as follows: mWPRE (~600bp) was amplified from the donor plasmid (pHR'SIN cPPT SE mW) using PCR and then digested with *Acc*65I (restriction sites were introduced in the PCR oligos). IRES, eIeGFP and Wild-type WPRE elements were digested using *Acc*65I from starting plasmid (pRRLsc_hSYN_m*Smn*_eIeGFP_W) and the rest of the plasmid (pRRLsc_hSYN_m*Smn*) was used as backbone.

Digested and gel-purified mWPRE was ligated to pRRLsc_hSYN_m*Smn* to generate the new plasmid (pRRLsc_hSYN_m*Smn*_mW). The new construct pRRLsc_hSYN_m*Smn*_mW was verified by digestion with *Sac*II, *Acc*65I, *Nco*I, *Hin*dIII and *Eco*RI.

Backbone Plasmid

Donor plasmid

Recombinant plasmid

143
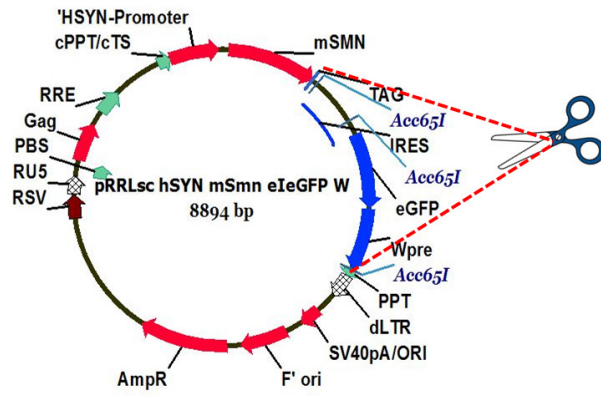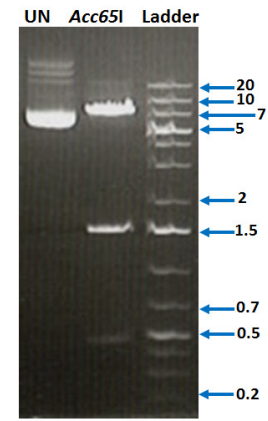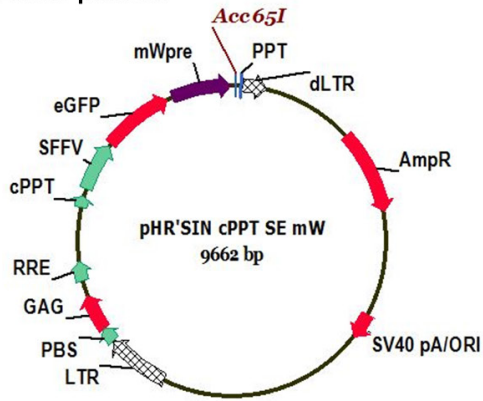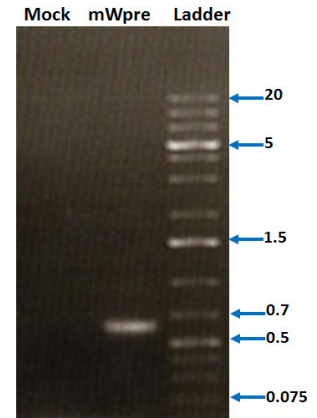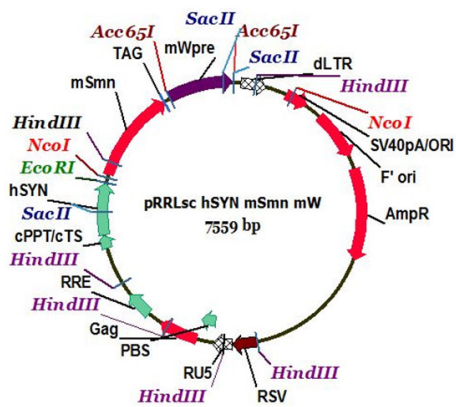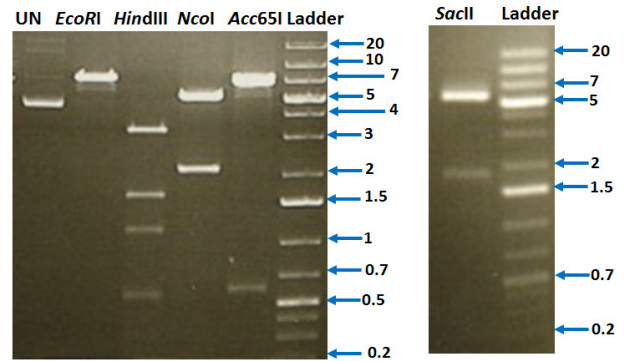
Figure 3.2: Construction and characterisation of pRRLsc-hSYN-m*Smn*-mW

All genetic plasmid maps are presented with relevant restriction sites. The abbreviations of indicated elements in the plasmid maps are explained in Table 3.1.

(**A**) *Acc*65I digestion was used to remove the IRES, eGFP and wild-type WPRE (1487 and 463 bp) from recipient plasmid (pRRLsc_hSYN_m*Smn*_eIeGFP_W).

(**B**) mWPRE (~600bp) was amplified from the original plasmid (pHR'SIN cPPT SE mW) using forward primer and reverse primer. The forward primer was designed to introduce an *Acc*65I restriction site at the 5' end of mWPRE (GCAATTAAGGTACCCCTGCAGGTAATCAACCTCTGGATTACA). The reverse primer was designed to introduce an *Acc*65I restriction site at 3' end of mWPRE (CTTAATTAGGTACCGCGGGGAGGCGGCCCAAAGGGAGAT) and then the amplified fragment was digested using *Acc*65I. Then the digested mWPRE was inserted into digested recipient transfer plasmid (pRRLsc_hSYN_m*Smn*).

(**C**) Restriction endonuclease analysis of generated pRRLsc_hSYN_m*Smn*_mW construct confirmed the structure of new plasmid. The enzymatic digestion with *Sac*II produced 5680 bp, 1794 bp and 85 bp fragments, *Acc*65I produced 6955 bp and 604 bp fragments, *Nco*I produced 5449 bp and 2110 bp fragments. *Hin*dIII produced 3341 bp, 1613 bp, 1155 bp, 584 bp, 553 bp and 313 bp fragments and *Eco*RI produced 7548 bp fragment from the generated plasmid.

Sequencing was also carried out to ensure the correct sequence of mWPRE (data not shown).

UN: undigested plasmid.

Ladder was 1Kb plus ladder (Thermo Scientific, Germany).

3.4.3 Cloning of pRRLsc_C_*hSMN1*_mW

In order to express h*SMN1* under the control of CMV promoter in lentiviral plasmid backbone, the m*Smn* gene in pRRLsc_C_m*Smn*_mW was replaced by the *hSMN1* transgene. Thus, the resulting plasmid was pRRLsc_C_*hSMN1*_mW. This particular cloning was performed by retrieving the gene of interest (*hSMN1* (896 bp)) from donor plasmid (pscAAV_CAG_*hSMN1*) using *Age*I and *Sbf*I. Double digestion (*Age*I and *Sbf*I) of the backbone plasmid yielded two fragments of different sizes. One fragment was m*Smn* gene (1002 bp) and the other was a recipient fragment (pRRLsc_C_mW (6688 bp)). The recipient backbone was de-phosphorylated with Antarctic phosphatase to avoid the re-circularisation of backbone plasmid during ligation and then was retrieved from a 1% agarose gel. The ligation reaction was set up to ligate 1002 bp fragment (*hSMN1*) into 6688 bp recipient transfer plasmid (pRRLsc_C_mW). Following transformation, random colonies were screened for the presence of the correct plasmid. The new construct pRRLsc_C_*hSMN1*_mW was verified by digestion with different restriction enzymes.

A diagram showing the designing of cloning strategy for this particular construct is provide in Figure 3.3.

Backbone Plasmid

pRRLsc C mSmn mW
7690 bp

Donor plasmid

pscAAV CAG hSMN1
4841 bp

Recombinant plasmid

pRRLsc C hSMN1 mW
7584 bp

A

*Age*I+

UN   *Sbf*I   Ladder

10
8
6
5
4

2

1

0.6

0.2

B

*Age*I +
*Sbf*I

10
8
6
5
4

3

1

0.8

0.2

C

*Pst*I   *Nco*I   UN   Ladder

10
8
6
5
4

1.5

1
0.8
0.6

0.2

146
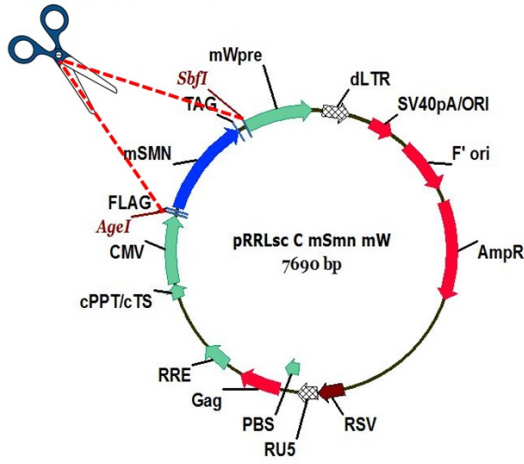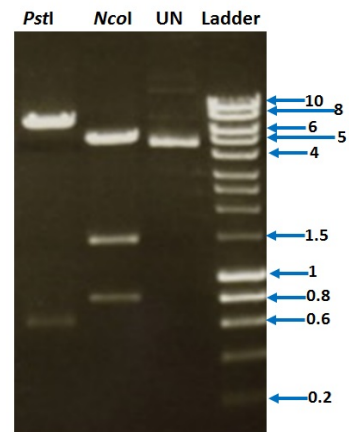
Figure 3.3: Construction and characterisation of pRRLsc_C_*hSMN1*_mW.

Maps of backbone plasmid pRRLsc_C_m*Smn*_mW (7690 bp), donor pscAAV_CAG_*hSMN1* (4841 bp) plasmid and the recombinant plasmid pRRLsc_C_*hSMN1*_mW (7584 bp) are presented with common elements and the restriction sites that are either used for cloning the new genes or to verify the resulting plasmid. The abbreviation of indicated elements in the plasmid maps are explained in to Table 3.1.

(**A**) Digestion of pRRLsc_C_m*Smn*_mW with *Age*I and *Sbf*I produced two different fragments sizes: the 1002 bp fragment was m*Smn* gene and the 6688 bp fragment was pRRLsc_C_mW.

(**B**) The donor plasmid was digested with the same restriction enzyme (*Age*I and *Sbf*I) to release the gene of interest, which was *hSMN1* (896 bp). (**C**) The recombinant plasmid was digested with *Nco*I generating fragment sizes of 5230 bp, 1488 bp and 866 bp and *Pst*I generating fragment sizes of 6951 bp, and 633 bp fragments.

Sequencing was also carried out to ensure the correct sequence of *hSMN1* (data not shown).

UN: Undigested plasmid.

Ladder was 1Kb ladder (Bioline, UK).

3.4.4 Construction of pRRLsc_hSYN_ *hSMN1*_mW

Here the aim was to construct a plasmid encoding *hSMN1* under the control of hSYN promoter. The maps of backbone transfer plasmid (pRRLsc_hSYN_m*Smn*_mW), donor plasmid expressing *hSMN1* (pscAAV_CAG_*hSMN1*), and the resulting plasmid (pRRLsc_hSYN_*hSMN1*_mW) are shown in Figure 3.4. The construction of lentiviral transfer plasmid expressing *hSMN1* under the control of hSYN promoter (pRRLsc_hSYN_*hSMN1*_mW) was performed as follows: m*Smn* gene (1015 bp) was removed from recipient transfer plasmid using *Eco*RI and *Sbf*I and the rest of plasmid, pRRLsc_ hSYN _ mW, (6544 bp) was de-phosphorylated with Antarctic phosphatase before separation by electrophoresis and extraction from 1% gel and used as backbone to generate new construction. pscAAV_CAG_*hSMN1* (4841 bp) was digested with *Eco*RI and *Sbf*I and 902 bp fragment (h*SMN*1) was retrieved from a 1% agarose gel, and ligated to backbone plasmid. The new construct pRRLsc_hSYN_*hSMN1*_mW (7446 bp) was verified using different restriction enzymes: *Eco*RI expected pattern (7446 bp), *Pst*I expected pattern 6077 bp, 736 bp and 633 bp fragments, *Nco*I expected pattern 5970 bp, 1476 bp fragments and *Nde*I expected pattern 6827 bp and 619 bp fragments.

Backbone Plasmid

pRRLsc hSYN mSmn MW
7559 bp

mWPRE
SbfI
TAG
dLTR
SV40pA/ORI
F' ori
mSMN
EcoRI
AmpR
'HSYN-Promoter
cPPT/cTS
RRE
Gag
PBS
RU5
RSV

A

EcoRI +
UN   SbfI   Ladder
10
8
6
5
1.5
1
0.8
0.4
0.2

Donor plasmid

pscAAV CAG hSMN1
4841 bp

AAV 5' ITR
CMV
CAG
Eco RI
pbr 322 origin
Insert
hSMN1
AmpR
SbfI
lacZ a
AAV 3' MUT ITR
Poly A

B

EcoRI +
SbfI   UN
10
8
6
5
4
3
2.5
2
1.5
1
0.8
0.4
0.2

Recombinant plasmid

pRRLsc hSYN hSMN1 mW
7446 bp

Eco RI
hSYN
Nde I
Pst I
hSMN1
Pst I
Nco I
cPPT/cTS
Nde I
RRE
Pst I
Gag
mWpre
PBS
dLTR
RU5
Nco I
RSV
SV40pA/ORI
F' ori
AmpR

C

*Eco*RI *Pst*I *Nco*I *Nde*I UN Ladder
10
8
6
5
4
2
1.5
1
0.8
0.6
0.4
0.2

149

Figure 3.4: Cloning strategy of pRRLsc_hSYN_*hSMN1*_mW

Maps of the starting plasmid pRRLsc_hSYN_m*Smn*_mW, donor plasmid pscAAV_CAG_*hSMN1* and recombinant plasmid pRRLsc_hSYN_*hSMN1*_mW are presented with common elements and the restriction sites that were used either for cloning the new genes or for verifying the resulting plasmid. The main elements of the plasmids are listed in Table 3.1.

(**A**) Double digestion (*Eco*RI and *Sbf*I) was employed to remove m*Smn* (1015 bp) from pRRLsc_hSYN_m*Smn*_mW and then replaced with h*SMN*1 (902 bp) which isolated using the same cloning enzymes (*Eco*RI and *Sbf*I) from pscAAV_CAG_*hSMN1*. (**B**). (**B**)The band at 5kb is due to partial digestion (only cut with a single restriction enzyme), 4kb is the donor plasmid without insert and 0.8kb is the insert. (**D**) In order to verify the resulting plasmid, pRRLsc_hSYN_*hSMN1*_mW (7446 bp) was digested with *Eco*RI, *Nde*I, *Pst*I, *Nco*I.

The sequence of *hSMN1* gene, which was under the control of hSYN, was confirmed by DNA sequencing (data not presented).

Ladder was 1Kb ladder (Bioline, UK).

UN: undigested plasmid.
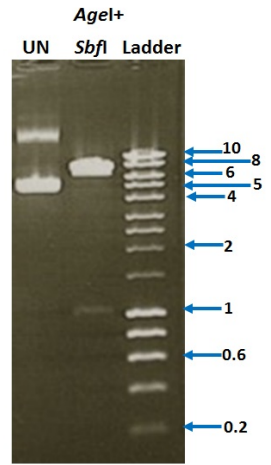
3.4.5 Cloning of pRRLsc_C_*hSMN1_NtF*_mW

In order to express *hSMN1_NtF* under the control of CMV promoter in lentiviral plasmid backbone, the m*Smn* gene in pRRLsc_C_m*Smn*_mW was replaced by *hSMN1_NtF*. The resulting plasmid was pRRLsc_C_*hSMN1_NtF*_mW. This particular cloning was performed by retrieving the gene of interest, *hSMN1_NtF* (923 bp), from donor plasmid (pscAAV_CAG_*hSMN1_NtF*) using *Age*I and *Sbf*I. Double digestion (*Age*I and *Sbf*I) of backbone plasmid yielded two fragments in different sizes. One fragment was m*Smn* gene (1002 bp) and the other fragment was pRRLsc_C_mW (6688 bp). Backbone fragment was de-phosphorylated with Antarctic phosphatase to avoid the re-circularisation of backbone plasmid during ligation and then was retrieved from a 1% agarose gel. The ligation reaction was set up to ligate 923 bp fragment (*hSMN1*) into a 6688 bp recipient transfer plasmid (pRRLsc_C_mW). Following transformation, random colonies were screened for the presence of the correct plasmid. The new construct pRRLsc_C_*hSMN1_NtF*_mW (7611 bp) was verified using different restriction enzymes. A diagram showing the designing of cloning strategy for this particular construct is provide in Figure 3.5.
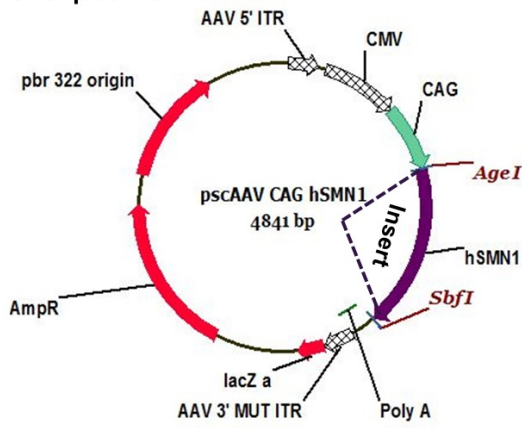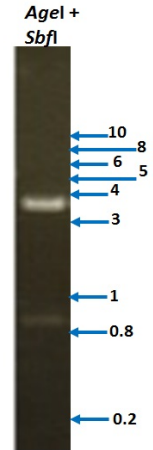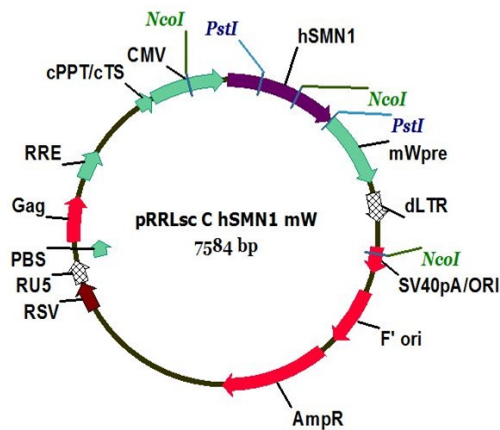
Backbone Plasmid

pRRLsc C mSmn mW
7690 bp

Labels: *Sbf*I, TAG, mWpre, dLTR, SV40pA/ORI, F' ori, AmpR, RSV, RU5, PBS, Gag, RRE, cPPT/cTS, CMV, *Age*I, FLAG, mSMN

Donor plasmid

pscAAV CAG hSMN1 NtF
4868 bp

Labels: AAV 5' ITR, CMV, CAG, *Age*I, Falg tag, hSMN1, *Sbf*I, Poly A, AAV 3' MUT ITR, lacZ a, AmpR, pbr 322 origin, Insert

Recombinant plasmid

pRRLsc C hSMN1 NtF mW
7611 bp

Labels: *Nco*I, CMV, Flag tag, *Pst*I, hSMN1, *Nco*I, *Pst*I, mWpre, dLTR, SV40pA/ORI, F' ori, AmpR, RSV, RU5, PBS, Gag, RRE, cPPT/cTS

A

*Age*I+

UN | *Sbf*I | Ladder

10, 8, 6, 5, 4, 2, 1, 0.6, 0.2

B

*Age*I + *Sbf*I

10, 8, 6, 5, 4, 3, 1, 0.8, 0.2

C

*Sbf*I | *Nco*I | UN

10, 8, 6, 5, 4, 3, 2, 1.5, 1, 0.8, 0.6, 0.2

152

Figure 3.5: Cloning of *hSMN1_NtF* in to Lentiviral backbone

Maps of backbone plasmid pRRLsc_C_m*Smn*_mW (7690 bp), donor pscAAV_CAG_*hSMN1_NtF* (4868 bp) plasmid and the recombinant plasmid pRRLsc_C_*hSMN1_NtF*_mW (7611 bp) are presented with common elements and the restriction sites that were used either for cloning the new genes or for verifying the resulting plasmid. The abbreviation of indicated elements in the plasmid maps are explained in to Table 3.1.

(**A**) Digestion of pRRLsc_C_ m*Smn*_mW with *Age*I and *Sbf*I resulted in two different fragment sizes. The 1002 bp fragment was m*Smn* gene and the 6688 bp fragment was backbone plasmid (pRRLsc_C_ mW).

(**B**) The donor plasmid was digested with same restrictions enzyme (*Age*I and *Sbf*I) to release the gene of interest which is *hSMN1_NtF* (923 bp).

(**C**) The recombinant plasmid (pRRLsc_C_*hSMN1_NtF*_mW) was digested with *Nco*I, generating fragment sizes of 5230 bp, 1488 bp and 893 bp and *Pst*I, generating fragment sizes of 6978 bp, and 633 bp fragments.

The sequence of *hSMN1_NtF* under the control of CMV was confirmed by DNA sequencing (data not presented).

Ladder was 1Kb ladder (Bioline, UK).

UN: Undigested plasmid.

3.4.6 Construction of pRRLsc_hSYN_*hSMN1_NtF*_mW

Lentiviral transfer plasmid containing *hSMN1_NtF* under the control of hSYN promoter (pRRLsc_hSYN_*hSMN1_NtF*_mW) was constructed as follows: 1015 bp m*Smn* was digested using *Eco*RI and *Sbf*I from starting plasmid (pRRLsc_hSYN _m*Smn*_mW) and the rest of plasmid (pRRLsc_hSYN_mW (6544 bp)) was used as backbone to generate the new recombinant plasmid.

hSMN1_NtF (929 bp) was released from donor plasmid (pscAAV_CAG_*hSMN1_NtF*) using *Eco*RI and *Sbf*I. Isolated *hSMN1_NtF* was ligated into de-phosphorylated recipient transfer plasmid (pRRLsc_hSYN_mW) to generate the new plasmid. The new construct pRRLsc_hSYN_*hSMN1_NtF*_mW (7473 bp) was verified by digestion with *Pst*I, *Nco*I and *Nde*I.

The maps of recipient transfer plasmid expressing m*Smn* under the control of hSYN promoter (pRRLsc__hSY_m*Smn*_mW), donor plasmid expressing *hSMN1_NtF* (pscAAV_CAG_*hSMN1_NtF*) and the new construct expressing *hSMN1_NtF* under the control of hSYN promoter (pRRLsc_hSYN_*hSMN1_NtF*_mW) are provided in Figure 3.6.

Backbone Plasmid

pRRLsc hSYN mSmn MW
7559 bp

SbfI
TAG
mWPRE
dLTR
SV40pA/ORI
F' ori
mSMN
EcoRI
AmpR
'HSYN-Promoter
cPPT/cTS
RRE
Gag
PBS
RU5
RSV

Donor plasmid

pscAAV CAG hSMN1 NtF
4868 bp

AAV 5' ITR
CMV
CAG
EcoRI
Flag tag
pbr 322 origin
Insert
hSMN1
SbfI
AmpR
lacZ a
Poly A
AAV 3' MUT ITR

Recombinant plasmid

pRRLsc hSYN hSMN1 NtF mW
7473 bp

NcoI
NdeI
hSMN1
PstI
PstI
NdeI
mWPRE
Flag tag
dLTR
hSYN
NcoI
PstI
SV40pA/ORI
cPPT/cTS
F' ori
RRE
AmpR
Gag
PBS
RU5
RSV

A
EcoRI +
UN SbfI Ladder
10
8
6
5
1.5
1
0.8
0.4
0.2

B
EcoRI +
SbfI UN Ladder
10
8 6
5 4
3
2.5
2
1.5
1
0.8
0.4
0.2

C
NdeI NcoI PstI UN Ladder
10
8
6
5
4
2
1.5
1
0.8
0.6
0.4

155

Figure 3.6: Construction and characterization of pRRLsc-hSYN-*hSMN1_NtF*-mW.

The above figure presents the maps of pRRLsc_hSYN_m*Smn*_mW (backbone plasmid), pscAAV_CAG_*hSMN1_NtF* (donor plasmid) and pRRLsc_hSYN_*hSMN1_NtF*_mW (resulting plasmid). Relevant restriction sites are indicated on the plasmids map and a list of the plasmids' main elements are provided in Table 3.1.

(**A**) *Eco*RI and *Sbf*I double digestion was used to remove the m*Smn* (1015 bp) from recipient plasmid (pRRLsc_hSYN_m*Smn*_mW).

(**B**) *Eco*RI and *Sbf*I double digestion was used to isolate the *hSMN1_NtF* (929 bp) from donor plasmid (pscAAV_CAG_*hSMN1_NtF*).

(**C**) Restriction digestion analysis was used to confirm the structure of generated plasmid. Digestion with *Pst*I produced 6077bp, 763 bp and 633 bp fragments, *Nco*I produced 5997 bp and 1476 bp fragments and *Nde*I produced 6854 bp and 619 bp fragments.

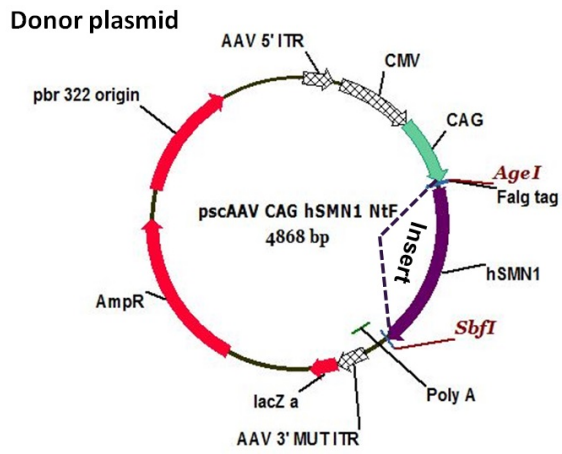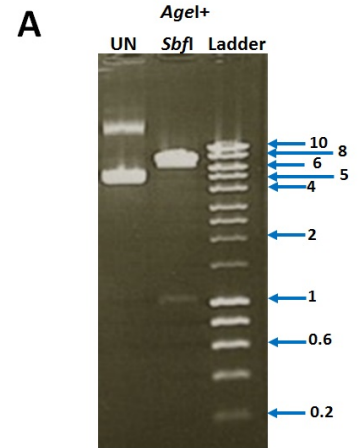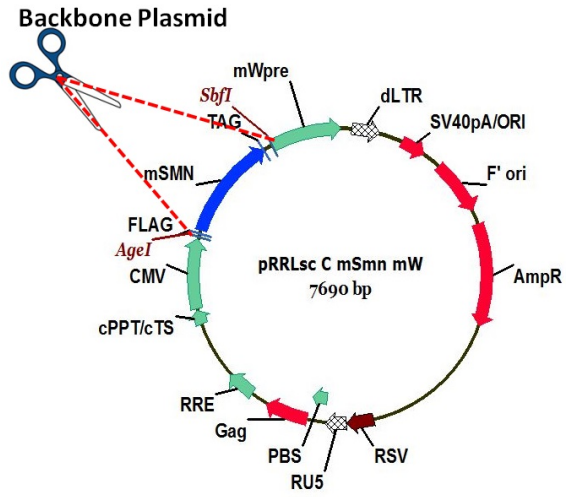The sequence of *hSMN1_NtF* under the control of hSYN promoter was confirmed by DNA sequencing (data not presented).

Ladder was 1Kb ladder (Bioline, UK).

UN: undigested plasmid.

3.4.7 Cloning of pRRLsc_C_*hSMN1_CtF*_mW

Here the aim was to construct a plasmid encoding *hSMN1_CtF* under the control of CMV promoter. The maps of backbone transfer plasmid (pRRLsc_CMV_m*Smn*_mW), donor plasmid (pscAAV_CAG_*hSMN1_CtF*), and the resulting plasmid expressing (pRRLsc_C_*hSMN1_CtF*_mW) are shown in Figure 3.7. The construction of lentiviral transfer plasmid expressing *hSMN1_CtF* under the control of CMV promoter (pRRLsc_C_*hSMN1_CtF*_mW) was performed as follows:

m*Smn* gene (1002 bp) was removed from recipient transfer plasmid using *Age*I and *Sbf*I and the rest of plasmid, pRRLsc_CMV_mW, (6688 bp) was de-phosphorylated with Antarctic phosphatase and then used as backbone to generate new construction. pscAAV_CAG_*hSMN1_CtF* (4865 bp) was digested with *Age*I and *Sbf*I, and 920 bp fragments (*hSMN1_CtF*) were retrieved from a 1% agarose gel, and ligated to backbone plasmid. The new construct pRRLsc_CMV_*hSMN1_CtF*_mW (7608 bp) was verified by digestion using *Eco*RI expected pattern (7608 bp), *Pst*I expected pattern 6951 bp, 657 bp fragments, *Nco*I expected pattern 5230 bp, 1512 bp and 866 bp fragment and *Nde*I expected pattern 6436 bp, 619 bp and 553 bp fragments.
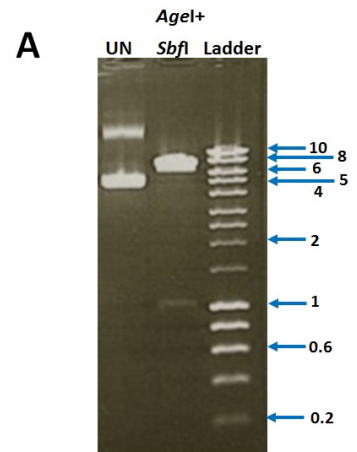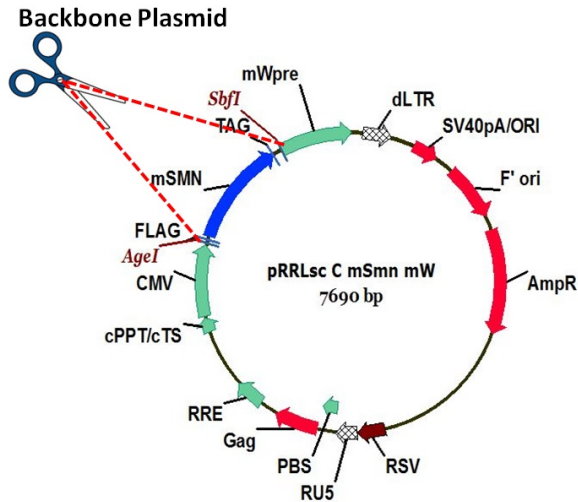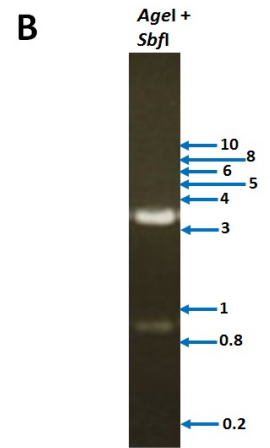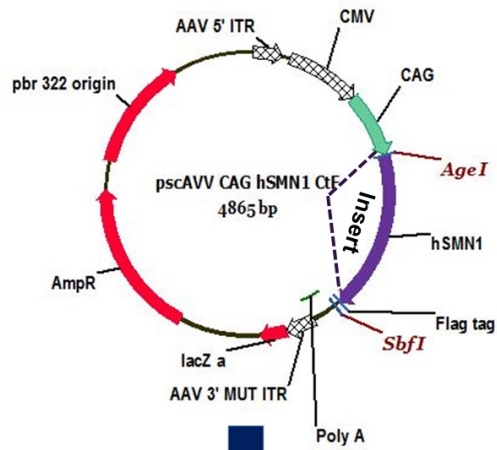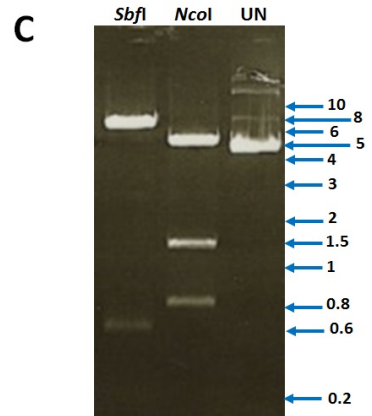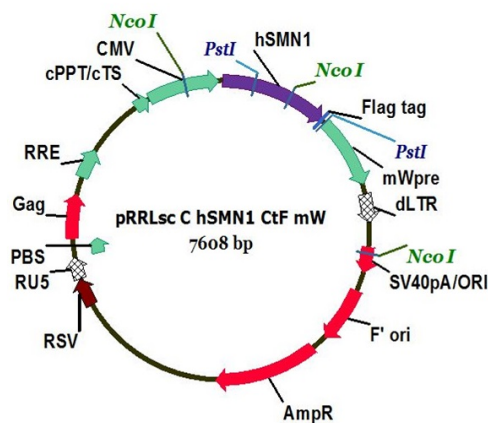
Figure 3.7: Cloning strategy for pRRLsc_CMV_*hSMN1_CtF*_mW.

Maps of the starting plasmid (pRRLsc_C_m*Smn*_mW), donor plasmid (pscAAV_CAG_*hSMN1_CtF*) and recombinant plasmid (pRRLsc_C_*hSMN1_CtF*_mW) are presented with common elements and the restriction sites that were used either for cloning the new genes or for verifying the resulting plasmid. The main elements of the plasmids are listed in Table 3.1.

(**A**) Double digestion (*Age*I and *Sbf*I) was employed to remove m*Smn* (1002 bp) from pRRLsc_m*Smn*_mW and then replaced with *hSMN1_CtF* (920 bp), which isolated from 4865 bp donor plasmid (pscAAV_CAG_*hSMN1_CtF*) using the same cloning enzymes (*Age*I and *Sbf*I).

(**B**) *Age*I and *Sbf*I double digestion was used to isolate the *hSMN1_CtF* (920 bp) from donor plasmid (pscAAV_CAG_*hSMN1_CtF*).

(**C**) In order to verify the resulting plasmid, pRRLsc_C_*hSMN1_CtF*_mW (7608 bp) was digested with *EcoRI, PstI, NcoI* and *Nde*I.

Sequencing was also performed to confirm *hSMN1_CtF* sequence (data not shown).

Ladder was 1Kb ladder (Bioline, UK).

UN: Undigested plasmid.

3.4.8 Construction of pRRLsc_ hSYN_ *hSMN1_CtF*_mW

In order to express *hSMN1_CtF* under the control of hSYN promoter in lentiviral plasmid backbone, the m*Smn* gene in pRRLsc_hSYN_m*Smn*_mW was replaced by the *hSMN1_CtF*. Thus, the resulting plasmid was pRRLsc_hSYN_*hSMN1_CtF*_mW. This particular cloning was performed by retrieving the gene of interest *hSMN1_CtF* (926 bp) from the digested donor plasmid (pscAAV_CAG_*hSMN1_CtF*), using *Eco*RI and *Sbf*I. Double digestion (*Eco*RI and *Sbf*I) of backbone plasmid yielded two fragments of different sizes. One fragment was m*Smn* gene (1015 bp) and the other fragment was backbone plasmid (pRRLsc_C_mW (6544 bp)). The backbone fragment was de-phosphorylated with Antarctic phosphatase to avoid the re-circularisation of backbone plasmid during ligation and then was retrieved from a 1% agarose gel. The ligation reaction was set up to ligate a 926 bp fragment (*hSMN1_CtF*) into 6544 bp recipient transfer plasmid (pRRLsc_hSYN_ mW). Following transformation, random colonies were screened for the presence of the correct plasmid. The new construct pRRLsc_hSYN_*hSMN1_CtF*_mW was verified by digestion with different restriction enzymes. A diagram showing the designing of cloning strategy for this particular construct is provide in Figure 3.8.
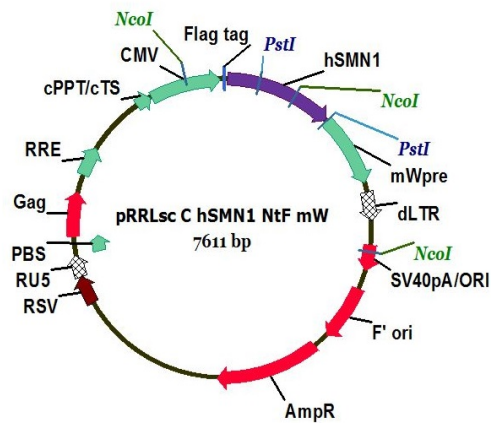
Backbone Plasmid

pRRLsc hSYN mSmn MW
7559 bp

Donor plasmid

pscAAV CAG hSMN1 CtF
4865 bp

Insert

Recombinant plasmid

pRRLsc hSYN hSMN1 CtF mW
7470 bp

A

*Eco*RI + *Sbf*I

UN   *Sbf*I   Ladder

10
8   6
5

1.5

1
0.8

0.4

0.2

B

*Eco*RI + *Sbf*I   UN

10
8

3
2.5
2
1.5

1
0.8

0.4

0.2

C

*Nco*I   *Pst*I   UN   Ladder   *Nde*I

10   8
6
5
4
2.5
2
1.5
1
0.8
0.6
0.2

161
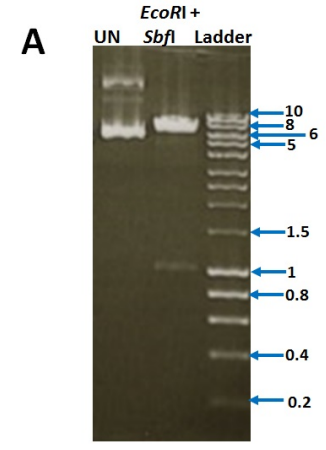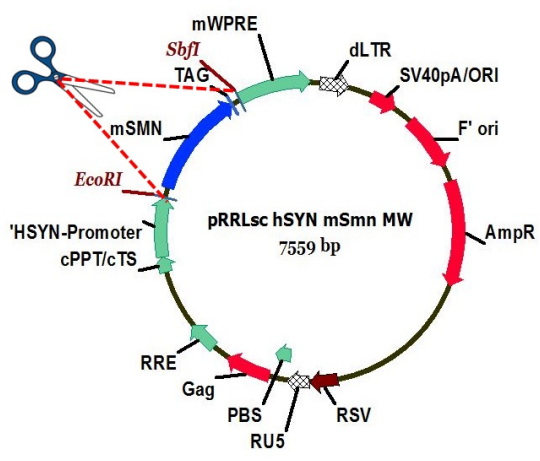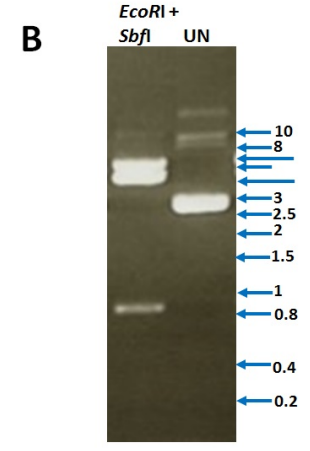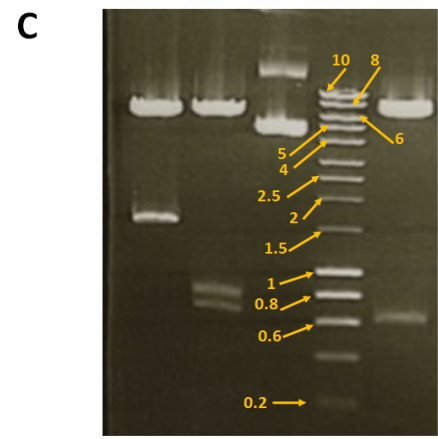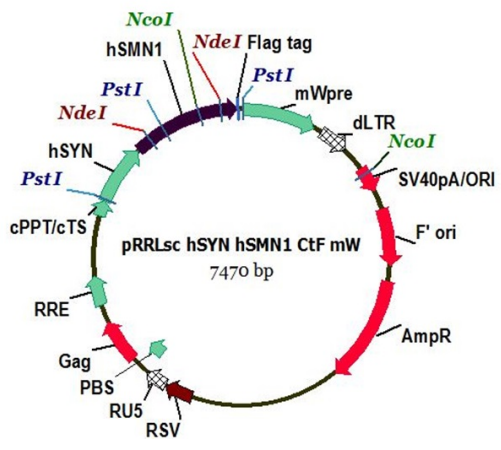
Figure 3.8: Construction and characterization of pRRLsc-hSYN-*hSMN1_CtF*-mW.

Maps of backbone plasmid pRRLsc_hSYN_m*Smn*_mW (7559 bp), donor pscAAV_CAG_*hSMN1_CtF* (4865 bp) plasmid and the recombinant plasmid pRRLsc_hSYN_*hSMN1_CtF*_mW (7470 bp) are presented with common elements and the restriction sites that were used either for cloning the new genes or for verifying the resulting plasmid. A list of plasmid elements is provided in Table 3.1.

(**A**) Digestion of pRRLsc_hSYN_m*Smn*_mW with *Eco*RI and *Sbf*I resulted in two different fragment sizes. The 1015 bp fragment was m*Smn* gene and the 6544 bp fragment was recipient plasmid (pRRLsc_hSYN_mW).

(**B**) 4865 bp donor plasmid was digested with same restrictions enzyme (*Eco*RI and *Sbf*I) to release the gene of interest, which was *hSMN1_CtF* (926 bp).
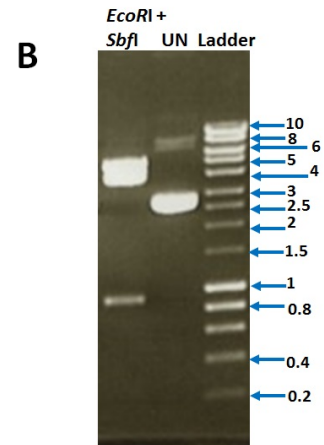
(**C**) Restriction endonuclease analysis of generated pRRLsc_hSYN_h*SMN*1_CtF_mW construct confirmed structure of new plasmid. The enzymatic digestion with *Pst*I produced 6077 bp, 736 bp and 657 bp fragments, *Nco*I produced 5970 bp and 1500 bp fragments and *Nde*I produced 6851 bp and 619 bp fragments.

The sequence of *hSMN1_CtF* under the control of hSYN was confirmed by DNA sequencing (data not presented).

Ladder was 1Kb ladder (Bioline, UK).

UN: Undigested plasmid.

3.4.9 Construction of pRRLsc_C_*Co-hSMN1*_mW

Here the aim was to construct a plasmid encoding codon optimised version of *hSMN1* under the control of CMV promoter. The maps of backbone transfer plasmid (pRRLsc_CMV_m*Smn*_mW), donor plasmid expressing *Co-hSMN1* (pMA-RQ Co-*hSMN1*), and the resulting plasmid expressing (pRRLsc_C_*Co-hSMN1*_mW) are shown in Figure 3.9. The construction of lentiviral transfer plasmid expressing *Co-hSMN1* under the control of CMV promoter (pRRLsc_C_*Co-hSMN1*_mW) was performed as follows: m*Smn* gene (1002) was removed from recipient transfer plasmid using *Age*I and *Sbf*I and the rest of plasmid, pRRLsc_hSYN_mW, (6688 bp) was de-phosphorylated with Antarctic phosphatase before extraction from 1% gel electrophoresis and used as backbone to generate new construction. 3252 bp donor plasmid (pMA-RQ Co-*hSMN1*) was digested with *Age*I and *Sbf*I and 908 bp fragment (*Co-hSMN1*) was retrieved from a 1% agarose gel, and ligated to de-phosphorylated lentiviral backbone plasmid. The new construct pRRLsc_C_*Co-hSMN1*_mW (7596 bp) was verified by digestion with different restriction enzymes.

Backbone Plasmid

pRRLsc C mSmn mW
7690 bp

Labels: mWpre, SbfI, TAG, dLTR, SV40pA/ORI, F' ori, mSMN, FLAG, AgeI, CMV, cPPT/cTS, RRE, Gag, PBS, RU5, RSV, AmpR

Donor plasmid

pMA-RQ Co-hSMN1
3252 bp

Labels: AmpR, AgeI, Insert, Co-hSMN1, SbfI, Col E1 origin

Recombinant plasmid

pRRLsc C Co-hSMN1 mW
7596 bp

Labels: ApaI, Co-hSMN1, BsrGI, AvaI, CMV, cPPT/cTS, AvaI, RRE, Gag, PBS, RU5, RSV, mWpre, dLTR, SV40pA/ORI, F' ori, AmpR

A

AgeI+

UN   SbfI   Ladder

Ladder bands: 10, 8, 6, 5, 4, 2, 1, 0.6, 0.2

B

AgeI+
SbfI   UN   Ladder

Ladder bands: 10, 6, 5, 4, 3.5, 3, 2.5, 2, 1, 0.75, 0.5

C

ApaI   BsrGI   AvaI   UN

Ladder bands: 10, 8, 6, 5, 3, 1.5, 1, 0.5, 0.25

164

Figure 3.9: Cloning strategy for pRRLsc_C_*Co-hSMN1*_mW

Maps of backbone plasmid pRRLsc_C_m*Smn*_mW (7690 bp), donor pMA-RQ *Co-hSMN1* (3252 bp) plasmid and the recombinant plasmid pRRLsc_C_*Co-hSMN1*_mW (7596 bp) are presented along with their common elements and the restriction sites that were used either for cloning the new genes or for verifying the resulting plasmid. The abbreviation of indicated elements in the plasmid maps are explained in Table 3.1.

(**A**) Digestion of pRRLsc_C_m*Smn*_mW with *Age*I and *Sbf*I resulted in two different fragment sizes. The 1002 bp fragment was m*Smn* gene and the 6688 bp fragment was backbone plasmid (pRRLsc_C_mW).

(**B**) The donor plasmid was digested with the same restrictions enzyme (*Age*I and *Sbf*I) to release gene of interest which is *Co-hSMN1* (908 bp).

(**C**) The recombinant plasmid (pRRLsc_C_*Co-hSMN1*_mW) was digested using *Bsr*GI, which generated fragment sizes of 7596 bp, using *Apa*I, which generated fragment sizes of 7596 bp and using *Ava*I, which generated fragment sizes of 6703 and 893 bp.

The sequence of *Co-hSMN1* under control CMV confirmed by DNA sequencing (data not presented).

Ladder was 1Kb ladder (Bioline, UK).

UN: Undigested plasmid.

3.4.10 Cloning of pRRLsc_hSYN_*Co_hSMN1*_mW

In order to express a codon-optimised version of *hSMN1* under the control of hSYN promoter in lentiviral plasmid backbone, the m*Smn* gene in pRRLsc_hSYN_m*Smn*_mW was replaced by the *Co-hSMN1*. The resulting plasmid was pRRLsc_hSYN_*Co-hSMN1*_mW. This particular cloning was performed by retrieving the gene of interest, *Co-hSMN1* (902 bp), from 3252 bp donor plasmid (pMA-RQ *Co-hSMN1*) using *Eco*RI and *Sbf*I. Double digestion (*Eco*RI and *Sbf*I) of backbone plasmid yielded two fragments of different sizes. One fragment was m*Smn* gene (1015 bp) and the other fragment was pRRLsc_hSYN_mW (6544 bp). Backbone fragment was de-phosphorylated with Antarctic phosphatase to avoid the re-circularisation of backbone plasmid during ligation and then was retrieved from a 1% agarose gel. The ligation reaction was set up to ligate 902 bp fragment (*Co*-h*SMN1*) into 6544 bp recipient transfer plasmid (pRRLsc_C_mW). Following transformation, random colonies were screened for the presence of the correct plasmid. The new construct pRRLsc_hSYN_*Co*-h*SMN1*_mW was verified using different restriction enzymes. A diagram showing the designing of cloning strategy for this particular construct is provide in Figure 3.10.

Backbone Plasmid

pRRLsc hSYN mSmn MW
7559 bp

Donor plasmid

pMA-RQ Co-hSMN1
3252 bp

Recombinant plasmid

pRRLsc hSYN CohSMN1 mW
7446 bp

A

*Eco*RI +
UN   *Sbf*I   Ladder

10
8
6
5

1.5

1
0.8

0.4

0.2

B

*Eco*RI +
*Sbf*I   UN

10
8
6
5
4
3.5
2.5
2
1.5
1
0.75
0.5

C

*Apa*I   *Bsr*GI   UN

10
8
6
5
4

1.5
1
0.75

0.25

167
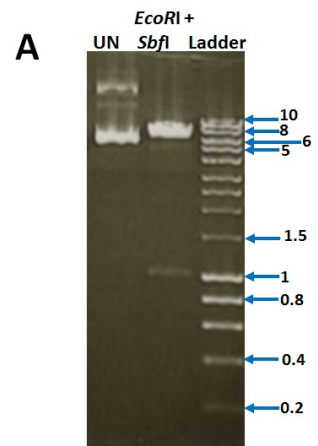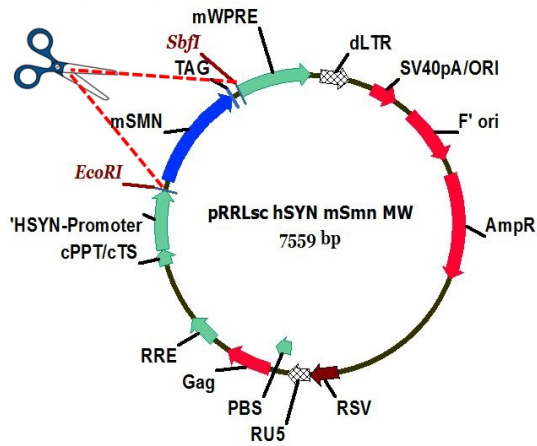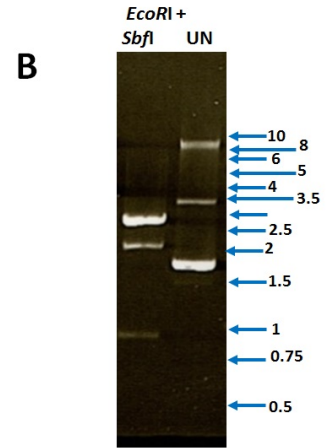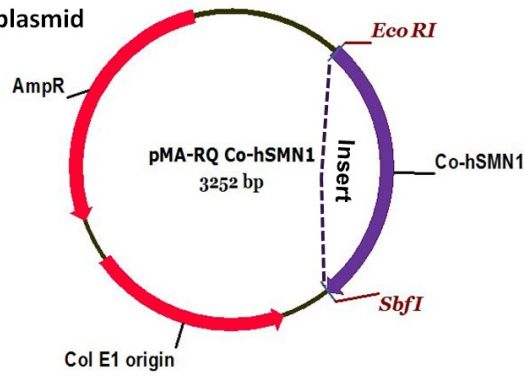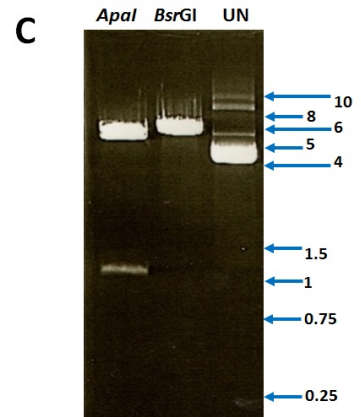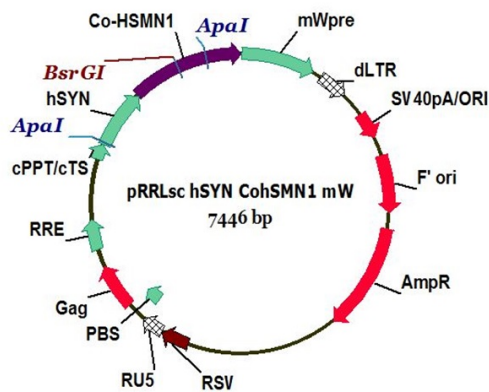
Figure 3.10: Construction and characterization of pRRLsc-hSYN_*Co*-h*SMN1*-mW.

Maps of backbone plasmid pRRLsc_hSYN_m*Smn*_mW (7559 bp), donor pMA-RQ *Co*-h*SMN1* (3252 bp) plasmid and the recombinant plasmid pRRLsc_hSYN_*Co*-h*SMN1*_mW (7446 bp) are presented with common elements and the restriction sites that were used either for cloning the new genes or for verifying the resulting plasmid. The abbreviation of indicated elements in the plasmid maps are explained in the Table 3.1.

(**A**) Digestion of pRRLsc_hSYN_m*Smn*_mW with *Eco*RI and *Sbf*I resulted in two different fragments sizes. The1015 bp fragment was m*Smn* gene and the 6544 bp fragment was recipient plasmid (pRRLsc_hSYN_mW).

(**B**) The donor plasmid was digested with same restrictions enzyme (*Eco*RI and *Sbf*I) to release gene of interest, which was *Co*-h*SMN1* (902 bp).

(**C**) The 7446 bp recombinant plasmid (pRRLsc_hSYN_*Co*-h*SMN1*_mW) was digested with *Bsr*GI, generating fragment sizes of 7446 bp, with *Apa*I, generating fragment sizes of 6356 bp and 1090 bp, and with *Ava*I, generating fragment sizes of 6691 bp, 525 bp and 230 bp.

The sequence of *Co*-h*SMN1* under the control of hSYN was confirmed by DNA sequencing (data not presented).

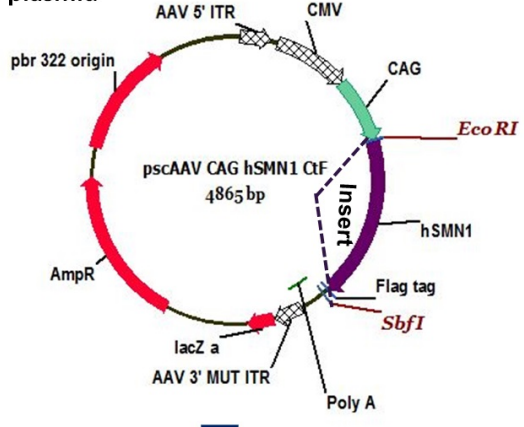**Ladder** was 1Kb ladder (Bioline, UK).

UN: Undigested plasmid.

3.5 Lentiviral vector production

After successfully cloning potentially therapeutic transgenes into lentiviral vector backbone plasmids, vectors were produced via the calcium phosphate plasmid transfection method. In addition, integrating and integration-deficient lentiviral vectors expressing *eGFP* were developed for use as positive control for vector production procedure. This reporter gene made it possible to check the transfection efficiency using fluorescence microscopy. Finally, all produced lentiviral vectors were titrated through transduction into HeLa cells followed by qPCR. In addition, stock titers of lentiviral vectors that carry a marker transgene (*eGFP*) were quantified by flow cytometry as well.

A summary of all lentiviral vectors with their gene of interest, relevant promoter, integration configuration and titre value is provided in Table 3.2 and 3.3.

Table 3.2: Main features of the lentiviral vectors prepared in the current study.

Vector	Transgene	Promoter	Integration configuration	qPCR titer	
				Day 1 harvest	Day 2 harvest
CMV_m*Smn*_mW	m*Smn*	CMV	IPLV	2.35E+10	7.54E+09
CMV_m*Smn*_mW			IDLV	1.58E+09	5.01E+08
hSYN_m*Smn*_mW		hSYN	IPLV	3.65E+10	1.20E+09
hSYN_mSmn_mW			IDLV	1.06E+09	1.15E+08
CMV_*hSMN1*_mW	*hSMN1*	CMV	IPLV	1.13E+10	9.9E+09
CMV_*hSMN1*_mW			IDLV	1.96E+09	1.01E+08
hSYN_*hSMN1*_mW		hSYN	IPLV	3.22E+10	2.71E+09
hSYN_*hSMN1*_mW			IDLV	1.98E+09	1.01E+08
CMV_*hSMN1_NtF*_mW	*hSMN1_NtF*	CMV	IPLV	6.17E+10	4.14E+09
CMV_*hSMN1_NtF*_mW			IDLV	1.88E+09	2.12E+08
hSYN_*hSMN1_NtF*_mW		hSYN	IPLV	5.14E+09	4.40E+08
hSYN_*hSMN1_NtF*_mW			IDLV	2.25E+09	1.19E+08
CMV_*hSMN1_CtF*_mW	*hSMN1_CtF*	CMV	IPLV	1.65E+11	7.12E+10
CMV_*hSMN1_CtF*_mW			IDLV	9.87E+10	4.18E+08
hSYN_*hSMN1_CtF*__mW		hSYN	IPLV	5.76E+09	2.84E+08
hSYN_*hSMN1_CtF*_mW			IDLV	3.74E+10	2.04E+09
CMV_*Co-hSMN1*_mW	*Co-hSMN1*	CMV	IPLV	3.20E+09	5.82E+08
CMV_*Co-hSMN1*_mW			IDLV	1.0E+09	2.19E+08
hSYN_*Co-hSMN1*_mW		hSYN	IPLV	2.89E+11	7.15E+10
hSYN_*Co-hSMN1*_mW			IDLV	1.09E+10	2.23E+09

Table 3.3: Summary of eGFP lentiviral vectors

Vector	Transgene	Promoter	Integration Configuration	qPCR titre		Flow cytometry titre	
				Day 1 harvest	Day 2 harvest	Day 1 harvest	Day 2 harvest
CMV_e*GFP*-W	*eGFP*cassette	CMV	IPLV	2.11E+09	1.10E+09	7.00E+08	6.00E+08
CMV_e*GFP*-W			IDLV	1.80E+09	1.05E+09	5.00E+08	4.00E+08

3.6 Discussion

The standard lentiviral vector production method relies on the transient transfection of HEK293T cells with a packaging plasmid, an envelope glycoprotein-encoding plasmid, a Rev plasmid and a lentiviral transfer vector plasmid using the calcium phosphate co-precipitation method (Pham *et al*., 2001 and Tang *et al*., 2015). This method is fast, reliable and inexpensive for lentiviral vector production, and was used to produce all lentiviral vectors during this project.

Successfully generated lentiviral transfer plasmids containing wild-type *hSMN1*, *hSMN1_CtF*, *hSMN1_NtF*, and *Co-hSMN1* driven by CMV and hSYN promoters were used as transfer plasmids in vector production. Two types of lentiviral vectors were employed in this study. The first type was integration-proficient lentiviral vector, which encode an active integrase and integrate into the host genome. The second type were integration-deficient and encoded a catalytically inactive viral integrase. Lack of integration in the latter is due to a single point mutation in the catalytic active site of IN introduced in the parental packaging plasmid (Wanisch & Yáñez-Muñoz, 2009).

Determining the amount of vector in viral vector preparation is an essential step for any further experiments. There are different titration methods to determine the quantity and quality of lentiviral vector production. These methods can be categorised into two group: physical or functional titration methods (Geraerts *et al*., 2006). Measuring the amount of concentration of p24 protein (pg p24/mL) in vector stock by ELISA assay is a physical titration method. p24 ELISA is a very straightforward and rapid method, and can be completed in few hours. A major drawback of this method is that the p24 ELISA measures all p24-containing vector particles, regardless of whether these particles carry a viral genome or not, or whether they are biologically viable. Therefore, the viral titre achieved via this method can be overestimated, as

quantification can include defective viral particles as well (Geraerts *et al*., 2006) . Another method belonging to the physical titration category is the reverse transcription-polymerase chain reaction (RT-PCR) method. In this assay, primers are designed to target for amplification a viral element of interest following in vitro reverse transcription of genomic RNA from vector stocks. The measurements achieved by RT-PCR may include defective particles unable to enter cells or complete transduction and express the transgene, which may result in overestimating the number of active vector particles in a sample. This is a key disadvantage of using this method.

Functional methods are more accurate than the physical method as they measure infectious viral particles. The principle of these methods is to transduce live cells with the generated vectors. Flow cytometry and qPCR are two functional titration methods, which were predominantly used to determine the titration of lentiviral vector production in my study (Geraerts *et al*., 2006).

Flow cytometry is a simple method to titrate vectors including a fluorescent reporter gene or gene products that can be detected with a fluorescent probe, in which cells are transduced with different serial dilutions of a vector stock, as described in the Materials and Methods section (2.4.2). The percentage of fluorescent target cells that express the transgene can be used to estimate the viral vector titre. To avoid underestimation of viral titres, the dilution chosen to calculate vector titre should have 1-10% of positive cells, to minimise the risk of multiple transduction events in a single cell. A disadvantage of flow cytometry is that it is restricted to titration of vectors encoding fluorescent reporter proteins, or others that can be detected through fluorescent probes. However, in principle it is an excellent method because it uses the final product of transduction, the transgenic protein, to estimate the titre, hence only

detecting the viral vector particles able to complete the full transduction process (Nightingale *et al*., 2006).

An alternative method of measuring viral vector titres is qPCR for detection of a vector sequence after reverse transcription is completed in transduced cells. As explained in 2.4.3 section, the qPCR method combines two different reactions. In one reaction, qPCR primers targeting an element specific to vector backbones, such as LTRs, *gag*, WPRE, antibiotic resistance-genes, or the gene of interest are used to estimate the number of vector molecules that have completed reverse transcription. As transduced cells contain a mixture of reverse transcription products, it is critical to use a vector sequence target guaranteeing that reverse transcription has been completed, and we use a region called the late-reverse transcript (LRT) for this purpose (Butler *et al*., 2001). In another reaction, the number of cells in the reaction is quantified using qPCR primers targeting a housekeeping gene, such as β-actin. This method does not rely on transgene expression and hence is ideal to compare (i) vectors encoding different transgenes; and (ii) IPLV and IDLV vectors, as the latter generally mediate lower transgene expression levels in proliferating cells like those used for titration. It is critical that cells are harvested 24 h after transduction, before the non-replicating vector episomes start diluting in proliferating cells, for an accurate estimation of IDLV titre. It should be noted that the qPCR method is quite labor intensive (Geraerts *et al*.2006 and Yoder & Fishel, 2008).

As stated previously, titre values of lentiviral vectors generated during this study were determined using qPCR. In addition to the qPCR method, flow cytometry was utilised to titre lentiviral eGFP expression vectors. As a rule of thumb, for eGFP vectors qPCR titres were around 3-fold higher than eGFP titres, indicating that not all particles that complete reverse transcription are able to express the transgenic reporter protein.

Charrier *et al*. (2011) quantified the copy numbers of lentiviral vectors in individual cell clones. The number of vector insertion sites were confirmed by Southern-blot and the results of this study clearly demonstrated that cells containing a single copy of the integrated lentiviral vector encoding *eGFP* can produce detectable levels of fluorescence. Moreover, their results demonstrated that there is a direct correlation between vector copy number and mean fluorescence intensity.

Chapter 4 SMN expression in growth-arrested CHO cells, cortical neurons and motor neurons

4.1 Introduction

The lentiviral vectors generated in this project have been tested in three different *in vitro* models –growth-arrested Chinese Hamster Ovary (CHO) cells, primary cortical neurons and primary motor neurons, from mouse and rat respectively– to investigate *SMN* expression and the effect of different parameters on efficient protein production. The following section provides an introduction on why these models were selected to investigate *SMN* expression in this project.

CHO cells were originally derived from Chinese hamster ovary. For the first time in 1957, Dr. Theodore T. Puck, of the Department of Medicine at the University of Colorado, isolated an ovary from a female Chinese hamster and established the cells in culture plates (Jayapal *et al*., 2007). CHO cell lines are very well characterized and have become a standard expression system in different areas of research, such as biology, and medical and pharmaceutical sciences. Their small size, rapid growth rate, high productivity, and low chromosome number (2n = 22) of CHO cells, in addition to their flexibility and adaptability to various culture conditions, make them a great system for tissue culture and use as an *in vitro* model. Moreover, CHO cells are efficient at post-translational modification, which is often essential for full biological activity and to produce human-compatible recombinant proteins. CHO cells are frequently used in biomedical fields to produce recombinant products for therapeutic applications. Jayapal *et al*., 2007 reported almost 70% of all recombinant therapeutic protein is produced in CHO. Altogether, the CHO cell line is ideal for use as an *in vitro* model for examining the transduction efficiency of viral vectors and investigating the protein production from them. However, an important point that needs to be considered regarding the use of CHO cells here is that this project uses non-integrating lentiviral vectors, which will be progressively diluted out due to cell division of CHO cells, which have a doubling time

of about 16 hours. Growth-arrested CHO cell models, which have been halted in their cycling, have been well-established in Prof. Yáñez's laboratory by Dr. Hanna Kymäläinen, a previous PhD student in our group (Kymäläinen *et al*., 2014), and would be an ideal model to employ for testing the non-integrating lentiviral vectors produced. As previously stated, it was initially believed that SMA is only a motor neuron disease, but recent and increasing evidence suggests that other cells and tissues are affected in a case of SMA disease. Depleted levels of SMN protein alter the development of multiple neuronal subpopulations. Cortical neuronal, hippocampus and neuronal loss is not strictly selective for spinal motor neurons (d'Errico *et al*., 2013). Cortical neurons are another neuronal cellular population involved in SMA, which contribute to the complex SMA pathology and are affected by the deficiency of SMN protein. The death of cortical neurons was reported in an SMA mouse model by Liu *et al*., 2010 . In addition, d'Errico *et al*., 2013 observed a decrease in the number of cortical neurons in an SMA model, which is indicative of cortical neurons degenerating as a consequence of low SMN protein. Continued investigation of the effect that insufficient levels of SMN protein has on neurons in various brain areas is required (Taylor *et al*.2013). In order to develop an effective therapeutic strategy for SMA, other cells such as cortical neurons need to be targeted in addition to spinal motor neurons. Thus, primary mouse cortical neurons have been chosen to be used as *in vitro* model to investigate the transduction efficiency of generated vectors and subsequent SMN expression.

Motor neurons are the most profoundly affected cells in SMA disease and loss of motor neurons is the main pathological feature of SMA. Degeneration of motor neuron cells due to low levels of SMN protein has been observed in human SMA patients, but why motor neurons are particularly vulnerable to low SMN levels is not clear yet (Burghes & Beattie, 2009; Hamilton & Gillingwater, 2013 and Szunyogova *et al*., 2016). In

addition to the loss of motor neurons due to low levels of SMN protein, insufficient levels of SMN protein arrests the post-natal development of the neuromuscular junction (NMJ). NMJ defects were confirmed in human patients affected by SMA, so NMJ defects are another key component of SMA pathology (Kariya *et al*., 2008). As the specific loss of motor neurons is the main pathological feature of SMA, a large body of research has been focussed on investigating the reason behind selective death of lower motor neurons due to low levels of SMN protein and targeting them to develop an effective therapy to treat SMA patients (Gogliotti *et al*., 2012).

To achieve the main goal of this study, which is optimising and producing potential therapeutic lentiviral vectors for SMA, it is essential to transduce primary motor neuron cultures *in vitro* before attempting any *in vivo* experiment.

4.2 Aims of the chapter

The main goal of this chapter was to test the vectors produced in chapter 3 in different *in vitro* models and investigate the effect of different parameters on the level of full-length SMN protein production. To achieve this goal, the experiments in this chapter were performed to investigate the following specific aims:

(**A**) To determine the efficiency of transgenic SMN production from the lentiviral vectors produced.

(**B**) To compare IPLVs and IDLVs in terms of transgene expression.

(**C**) To investigate the effect of codon-optimisation on *hSMN1* over-expression.

(**D**) To compare the ubiquitous CMV, with the relatively neuron-specific hSYN promoter for expression of *hSMN1* variants.

(**E**) To study the vector dose-dependence of transgenic *hSMN1* expression.

(**F**) To study the robustness of transgenic *hSMN1* expression across three growth-arrested or quiescent cell culture systems.

4.3 Summary of experiments and methods

To address the above aims, a range of integrating and non-integrating lentiviral vectors have been produced (please refer to section 3.5). The vectors were designed to carry different types of transgenes to investigate the advantages of using codon-optimised transgenes rather than wild-type versions for efficient protein expression. These transgenes were placed downstream of either CMV or hSYN promoter.

Following vector production and titration in chapter 3, the first experiment undertaken was to test generated vectors in growth-arrested CHO cells, to investigate the aims of this chapter. The CHO cell lines were maintained and cultured in a standard growth medium (as described in section 2.3.2.2). When the cell reached 70% confluency the cell cycle was arrested using methionine-free DMEM (as described in section 2.3.2.3). The transduction using the generated vectors was followed by a short period of induced cell cycle arrest in the presence of polybrene (8 μg/mL). In a bid to maximise transgene expression from generated lentiviral vectors, the multiplicity of infection (MOI) was increased to transduce the growth-arrested CHO cells.

To better achieve the aims of this chapter, a further investigation of lentiviral vector transduction efficiency, transgene expression, usage of the CMV versus the hSYN promoters, vector dose-dependent increases and the effect of different types of *hSMN1* transgenes for an effective SMN production was carried out in E18 primary mouse cortical neuron culture. Cortical neurons were isolated from brains of E18 mouse embryos. The procedure protocol was obtained from Boulos *et al*., 2006 and explained in section 2.3.3.1. In brief, the procedure can be divided into two parts: dissection and plating, carried out under sterile conditions. During dissection, the brains of the embryos were removed and cortical tissue was separated from other parts of the brain to reduce the number of unwanted non-neuronal cells. Neurons were dissociated by incubation

with trypsin, and following that, the neurons were triturated to form a single cell suspension. Cells were cultured in neurobasal medium that was supplemented with 2% B27, 0.25% GlutaMax, 1% FBS, 100 μM ascorbic acid (added prior to use) and maintained at 37°C in a 5% CO_2. Cultures were transduced with produced vectors at qPCR MOI 30 or 100 three weeks after cell seeding.

Protein extractions were performed three days after transduction in growth-arrested CHO cells and primary cortical neurons. Harvested protein was evaluated using western blotting methods through separating the protein based on their molecular weight and then probing the protein of interest with the relevant antibody. The results of the western blot method were analysed based on the quantification of fluorescence intensity of detected protein (section 2.3.1).

E15 rat motor neuron culture was another primary *in vitro* model used to investigate the aims of this chapter. Spinal motor neuron cultures were extracted from E14–15 rat embryos, according to the description in section 2.3.3.2. The procedure includes extracting the spinal cords from E15 rat embryos, dissociating spinal cord tissue, separating cells by density gradient centrifugation, collecting motor neurons by immunopanning using monoclonal antibody IgG192 (in-house made monoclonal antibody supernatant, see appendix 2) and plating and culturing lower motor neurons. The IgG 192 antibody can be used to detect all cells expressing p75NTR on their surface, such as motor neurons in the spinal cord. Camu & Henderson, 1992 demonstrated that the IgG 192-immunopanning purification procedure can enrich embryonic motor neurons cultures.

Cells were cultured in neurobasal medium supplemented with 25 mM 2-mercaptoethanol, 0.5 mM L-glutamine, B27, 25 mM L-glutamate, 2% horse serum and 1 ng/mL BDNF. The primary motor neuron cultures were incubated at 37°C in 5% CO_2

for 3 days. Cultured embryonic rat motor neurons were transduced 2 hours after seeding using generated vectors following the descriptions in 2.5.1.2 section with a range of multiplicity of infection (qPCR MOI 30, 60 and 100). The immunostaining method described in 2.5.4.1 was applied to stain SMN protein in motor neuron cultures using anti-SMN antibody. Finally, the Axio Observer D1 microscope connected to an AxioCam captured random images. AxioVision software (Carl Zeiss, UK) was used to analyse the captured images, more details provided in section 2.5.4.2.

It should be noted that all the cells in a single well for each condition were counted in each individual experiment and each experiment was repeated three times.

4.4 Results

4.4.1 *SMN* expression in growth-arrested CHO cells

Growth-arrested CHO cells were transduced with either IPLV or IDLV vectors which were designed to carry different types of *hSMN1* transgene– wild-type *hSMN1*, *Co-hSMN1*, and *hSMN1-NtF* – under the control of either the CMV or hSYN promoter. Cells were transduced at a range of multiplicity of infections (qPCR MOIs 100 and 500). The cells were harvested and analysed by western blot three days post-transduction (Figure 4.1A, 4.2A and 4.3A). The transduction efficiency and expression of SMN protein was assessed through analysing the results of western blot experiment. The data revealed that all generated vectors were expressing the transgene of interest and the fluorescence intensity of SMN increased in a MOI-dependent manner and was highest at MOI 500. When comparing transduced samples using IPLVs and IDLVs, higher transgene expression was observed in transduction using IPLVs. Between promoters, the CMV promoter demonstrated stronger expression of the gene of interest than the hSYN promoter. Conversely, *Co-hSMN1* revealed a higher level of SMN fluorescence than the wild-type *hSMN1* transgene (Figure 4.1B, 4.2B, 4.3B and Table 4.1).
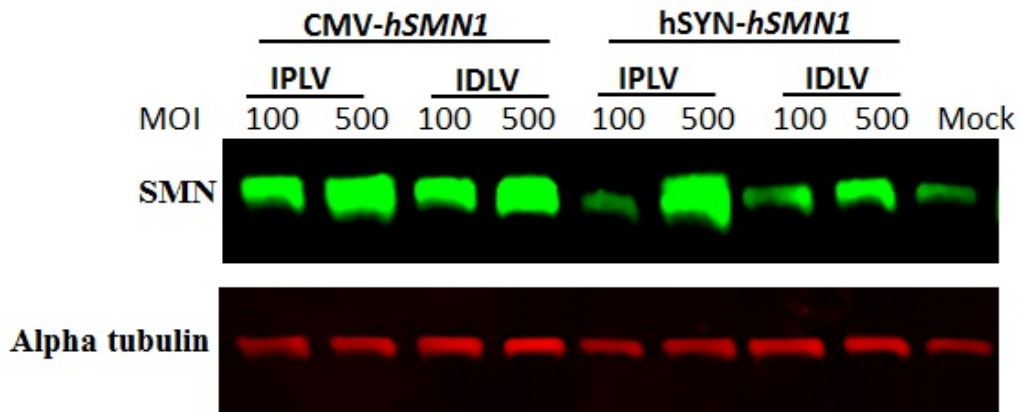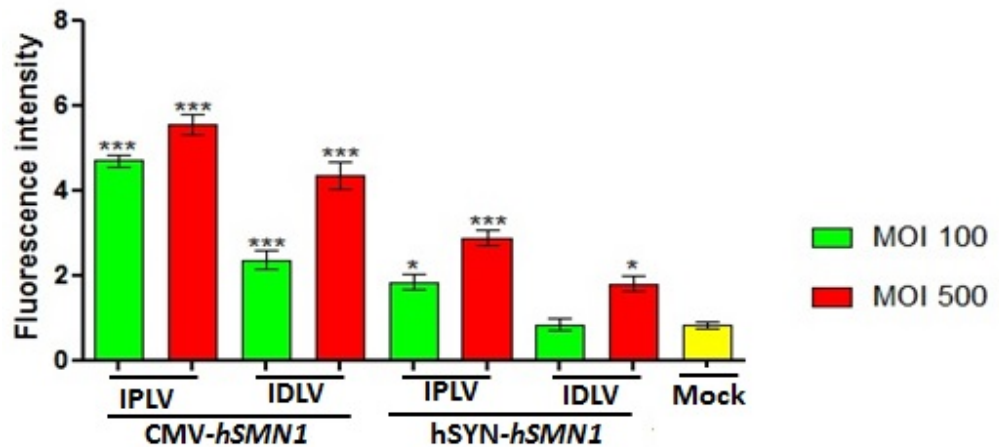
Figure 4.1: Lentivector-mediated wild-type *hSMN1* expression in growth-arrested CHO cell model.

(**A**) Arrested CHO cells were transduced at the indicated qPCR MOIs with either IPLVs /IDLVs containing CMV/hSYN and wild-type *hSMN1*. 72 hours post-transduction, transduced and non-transduced cells were lysed, and extracted proteins were subjected to protein assay for measuring total protein concentration of collected sample. 5 μg of total extracted protein was subject to western blot method to detect and analyse proteins of interest. SMN protein (~ 35 kDa) and α tubulin protein (~ 55 kDa) are our target protein here to detect using purified mouse anti-SMN and mouse α-tubulin antibody respectively. (**B**) Quantification of SMN fluorescence from analysed protein of transduced or un-transduced growth-arrested CHO cells was scored and plotted. Each transduced set of data was compared to un-transduced using one-way ANOVA and Dunnett's post-hoc test. Significant differences were observed in all samples, except those that were transduced using MOI 100 of IDLV-CMV-*hSMN1*. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.
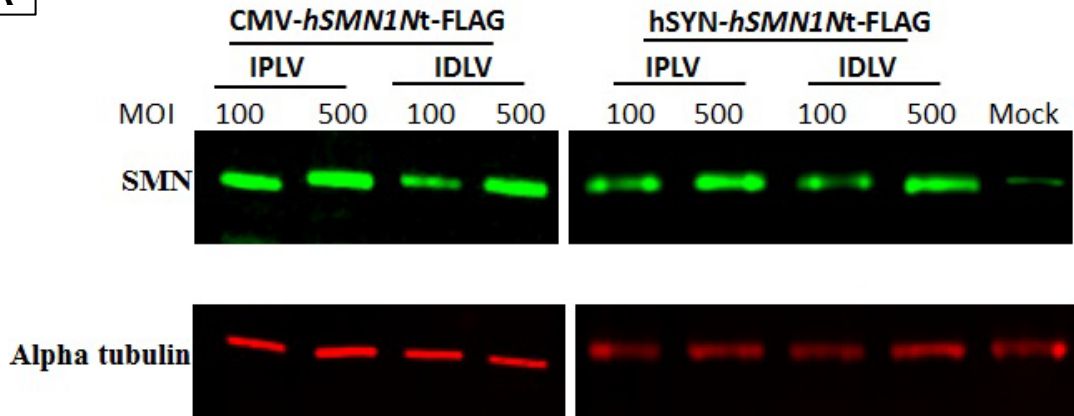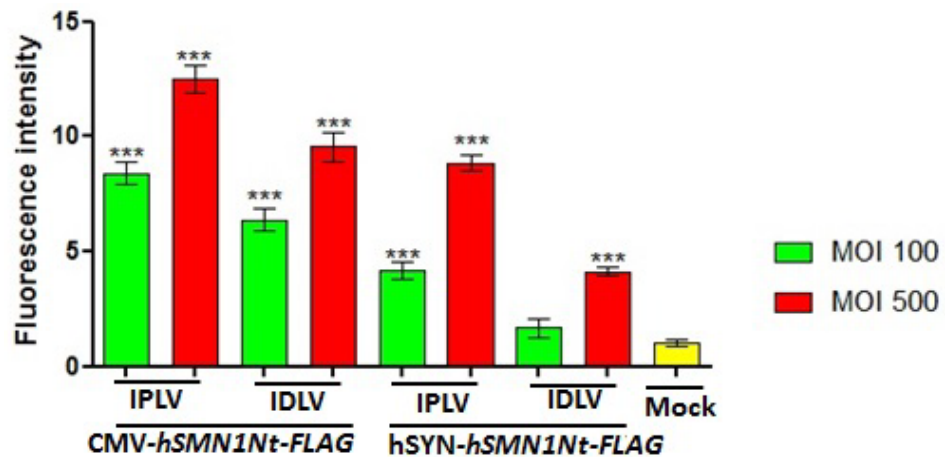
Figure 4.2: Lentivector-mediated FLAG-tagged *hSMN1* expression in growth-arrested CHO cell model .

(**A**) Representative western blot showed an overexpression of SMN protein in transduced cells. Arrested CHO cell cultures were transduced with IPLV/IDLV _hSMN1-NtF under control of CMV/hSYN promoter. qPCR MOIs 100 and 500 were used to transduce the cells. The mock culture was treated following the same manner of transduced culture, but it did not receive any vector. Protein extraction was performed at 72 hours after transduction. 5 µg of extracted protein was subjected to western blot and loaded onto a 12% SDS-PAGE gel. Loaded protein was separated based on molecular weight and then the protein of interest (SMN (~ 35 kDa) and alpha tubulin (~55 kDa)) were detected using relevant antibodies. Alpha-tubulin was used as a loading control. (**B**) Quantification of SMN fluorescence from analysed protein of transduced or un-transduced growth-arrested CHO cells was scored and plotted. Statistical analysis was performed between individual transduced and un-transduced growth-arrested CHO cells to determine significant differences between them. Significant differences were observed in all transduction samples except the sample that was transduced using MOI 100 of IDLV-hSYN-*hSMN1N-tFLAG*. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.
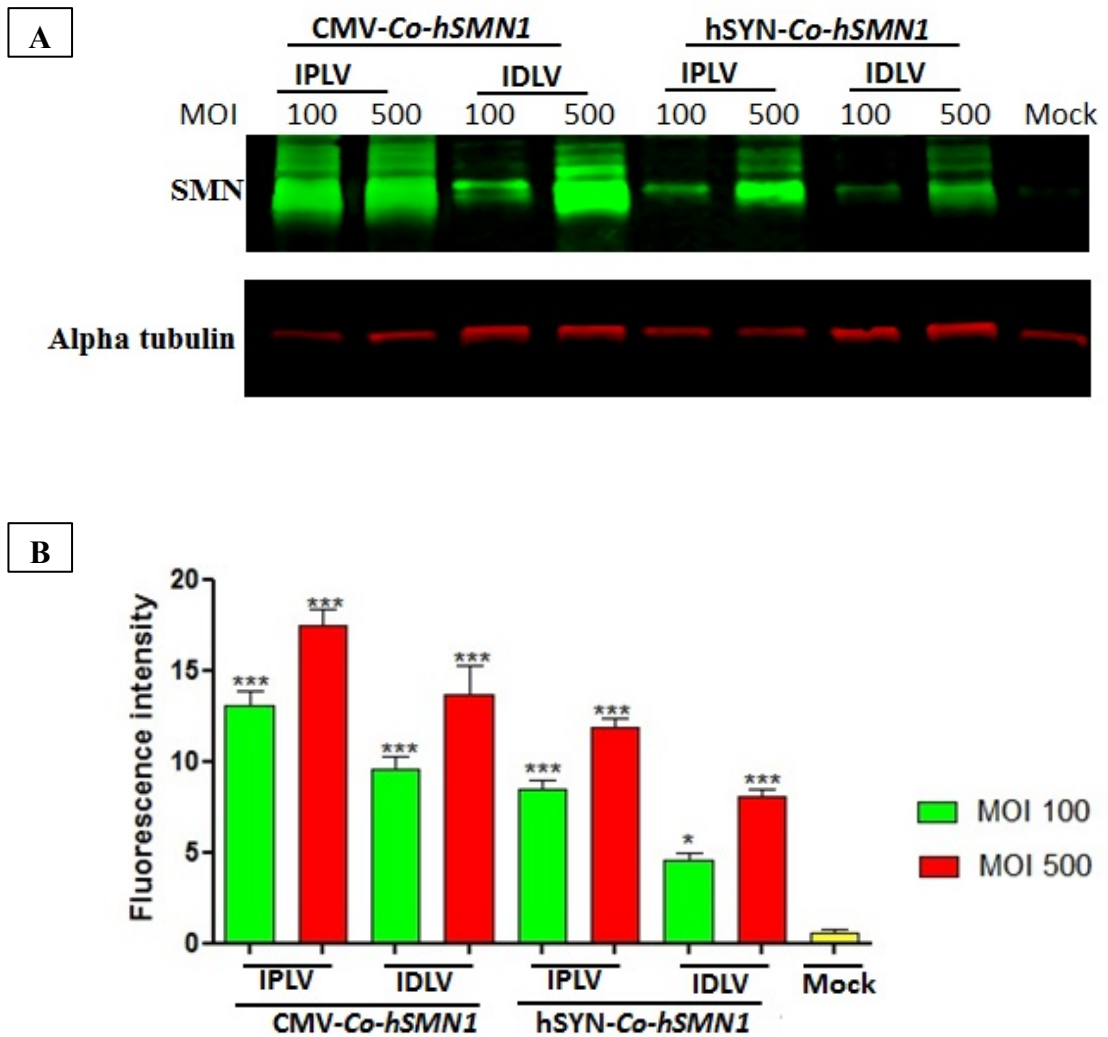
Figure 4.3: Lentivector-mediated expression of Co-*hSMN1* in growth-arrested CHO cell model .

Arrested CHO cells were transduced with qPCR MOI 100/500 of IPLVs or IDLVs encoding wild-type *hSMN1/Co-hSMN1* under the control of CMV / hSYN. MOI, type of vector and transgene of interest is indicated on each relevant lane on the above image. The transduced and non- transduced cells were lysed at 72 hours post-transduction. 5 μg of total protein extraction was loaded onto a 12% SDS-PAGE gel for western blot analysis with an antibody against the SMN protein and another antibody to detect alpha tubulin protein, which was used as a loading control. (**B**) Intensity of SMN fluorescence was evaluated 3 days post transduction. All transduced growth-arrested CHO cells showed significant differences to un-transduced cells. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3. The multiple bands for SMN protein in panel A are likely related to the very high level of over expression in growth arrested CHO cells transduced with lentiviral vector encoding *Co-hSMN1*.

Table 4.1: Comparison of SMN protein production from all vectors in transduced growth-arrested CHO cells.

Collected data from transduced growth-arrested CHO cells was subject to analysis using one-way ANOVA and Bonferroni's post-hoc test to determine whether there was a dose-dependent increase among each group. Moreover, the below table presents significant differences between various types of promoters, vectors, and transgenes and their effects on SMN protein production. Values represent mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, stars represent level of significance, n=3.

Transgene	Promoter	Vector	Tansgene	hSMN1								hSMN1_NtF								Co-hSMN1							
			Promoter	CMV				hSYN				CMV				hSYN				CMV				hSYN			
			Vector	IPLV		IDLV		IPLV		IDLV		IPLV		IDLV		IPLV		IDLV		IPLV		IDLV		IPLV		IDLV	
			MOI	100	500	100	500	100	500	100	500	100	500	100	500	100	500	100	500	100	500	100	500	100	500	100	500
hSMN1	CVM	IPLV	100		*	***		***				***	*			***				**	*			**			
			500				***		***						**		***				*				***		
		IDLV	100			***				***				***				***				**				**	
			500								***								***								***
	hSYN	IPLV	100					*	*							***	**							*	*		
			500								**								***								*
		IDLV	100							*								***								*	
			500																								
Co-hSMN1	CVM	IPLV	100	***								***															
			500		***								***														
		IDLV	100			***								**													
			500				***								***												
	hSYN	IPLV	100					***								***											
			500						***								**										
		IDLV	100							***								*									
			500								***								***								

■ Dose-dependent increase	■ CMV VS hSYN	■ IPLV VS IDLV	■ *hSMN1* VS *Co-hSMN1*

4.5 *SMN* expression in primary cortical neuronal cultures
4.5.1 Characterisation of cortical neuron cell cultures

Mouse primary cortical neuron cultures were employed as another *in vitro* model to explore the aims of this chapter. Cortical neuron cultures were produced from brains of E18 mouse embryos as described in 2.3.3.1. The procedure of extraction cortical cells has been well established in Prof. Yáñez's laboratory by Dr. Ngoc Lu-Nguyen, a previous PhD student. Dr. Ngoc Lu-Nguyen carried out extensive study to characterise the cortical neuron cultures. Neuronal cultures were originally characterised by immunocytochemistry at day 4 *in vitro* using mouse anti-NeuN antibody (for total neurons), or anti-GFAP (for total astrocytes). The result revealed that on average 60% of cells were neurons (NeuN+) and 13% astrocytes (GFAP+). Further characterisation of neuronal subpopulations demonstrated that 39% were GABAergic (GAD67+), 15% glutamatergic (VGLUT1+), and 4% dopaminergic neurons (TH+) (Lu-Nguyen *et al*., 2015). Therefore, in this study, prior to transduction cortical neuron, a pilot experiment was performed to demonstrate the presence of cortical neuron cells in *in vitro* culture (Figure 4.4). Characterisation of cell cultures was performed on day 4 post-seeding using a Neuronal nuclear (NeuN) antibody to identify the presence of cortical neuron cells in the culture. NeuN is a soluble nuclear protein, which is localized in the nuclei and perinuclear cytoplasm of most of the neurons in the central nervous system (Gusel'nikova & Korzhevskiy, 2015). This protein is not produced in any tissue other than nervous tissue, and has not been detected in glial cells (Gusel'nikova & Korzhevskiy, 2015 and Mullen *et al*.,1992). Therefore, NeuN is highly specific as a marker of neuronal culture and can detect neuron cell in primary cerebellar cultures.
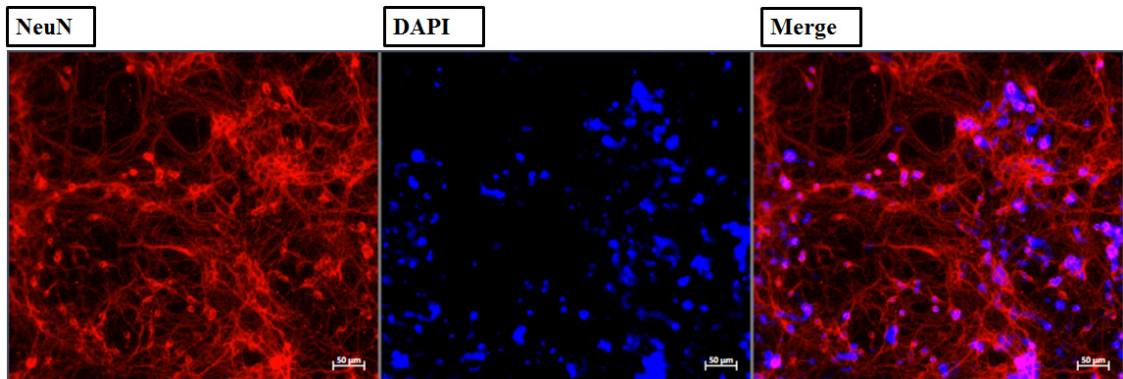
Figure 4.4: Cell cultures were characterised by immunocytochemistry method.

6 day old cortical neuron culture *in vitro* was fixed and stained with neuron marker (NeuN). Nuclei were stained blue with DAPI. Scale bars = 100 μm.

4.5.2 Lentiviral vectors transduce cultured purified primary cortical neuronal cultures

Integrating and non-integrating viral vectors used to transduce cortical neuronal cultures containing either a novel *Co- hSMN1*, a wild-type *hSMN1* or a *hSMN1_NtF* transgene. These transgenes were encoded under the control of the either the CMV or hSYN promoter. A direct correlation between increasing vector MOIs and expression of the gene of interest was investigated by transducing the three week old cortical neuron culture at different doses of vectors (qPCR MOI 30 and 100). Protein was extracted in a RIPA buffer with protease inhibitor cocktail three days after adding the vectors. Western blotting was performed following the standard procedures and as previously described in section 2.5.3.3. The primary antibodies used were anti-SMN and anti-α-tubulin. Odyssey secondary antibodies were added according to the manufacturers' instructions and then the blots were imaged using an Odyssey Infrared Imaging System (Li-COR; Biosciences) (Figure 4.5A, 4.6A and 4.7A). The western blot images were quantified according to the description stated previously (section 2.3.1). The transduction result demonstrated that the produced lentiviral vectors were expressing transgene of interest (Figure 4.5B, Figure 4.6B and Figure 4.7B). A direct correlation was observed between increasing vector MOIs and enhancing the fluorescence intensity of SMN. When considering IPLVs compared with IDLVs, IDLVs showed less transgenic expression. Between CMV and hSYN, CMV led to higher expression of the transgene of interest. Between *hSMN1* and *Co-hSMN1*, *Co-hSMN1* increased the transgene expression compared to the wild type protein (Table 4.2).

A
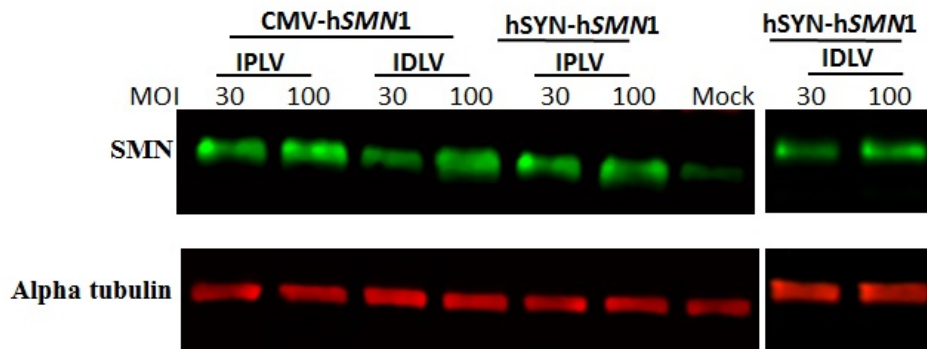
B
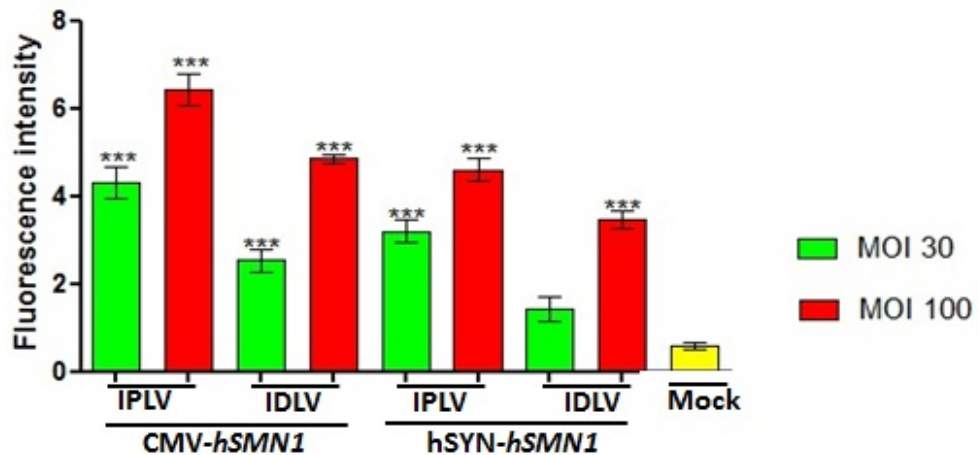
Figure 4.5: Transduction with *hSMN1*-expressing LVs in mouse primary cortical neuronal cultures.

(**A**) 3 week old mouse primary cortical culture was transduced with IPLV/ IDLV CMV-*hSMN1* or IPLV/ IDLV hSYN-*hSMN1* with qPCR MOI 30 and 100. Cells were lysed at 72 hours post transduction and 25 µg of extracted protein was run onto 12% SDS-Page gel. SMN protein with almost 35kDa molecular weight was detected using purified mouse anti-SMN antibody. Alpha tubulin protein (55 kDa) was used as loading control and detected by mouse α-tubulin antibody. Western blotting was performed by the Odyssey infrared imaging system. (**B**) Quantification of SMN fluorescent intensity from western blot was scored and plotted. Significant differences between transduced set of data versus un-transduced were determined using one-way ANOVA and Dunnett's post-hoc test. Significant differences were observed in all samples except those that were transduced using MOI 30 of IDLV-CMV-*hSMN1*. Values represent mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, stars represent level of significance, n=3.
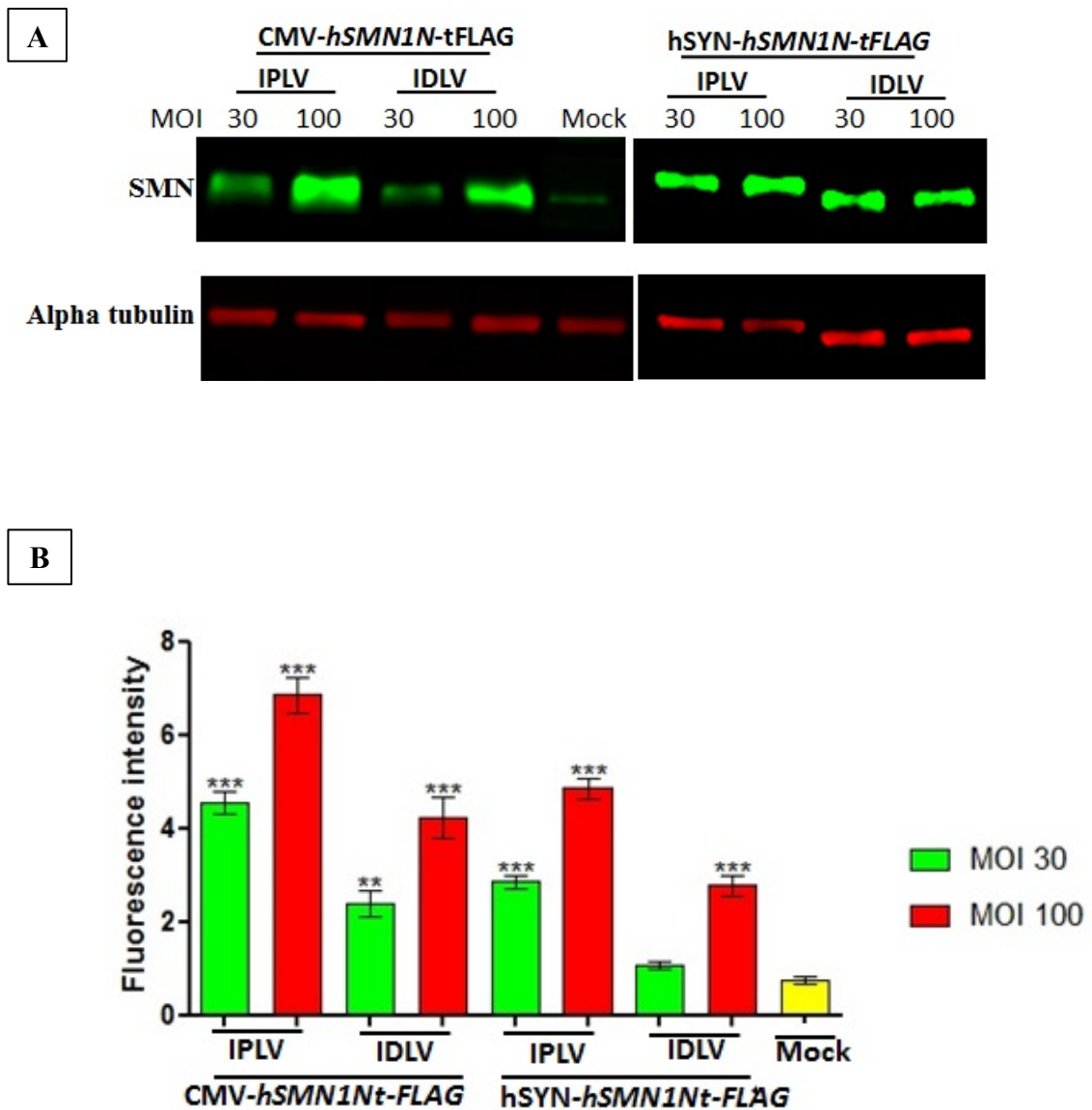
**Figure 4.6: Transduction of cortical neuron cultures using lentiviral vectors expressing FLAG-tagged *hSMN1*.**

(**A**) Primary cortical neurons cells were transduced with IPLV or IDLV encoding the *hSMN1_NtF* under the control of CMV and hSYN promoter at three weeks post seeding. Cells were harvested and lysed three days after transduction and then 25 µg of harvested protein was run on SDS-Page gel to separate proteins based on their molecular weight. Two proteins of interest in this western blot are full length SMN protein (~ 35 kDa) and Alpha tubulin protein (55 kDa) (used as loading control). These two proteins were detected using purified mouse anti-SMN and mouse α-tubulin antibody respectively. (**B**) Quantification of SMN fluorescent intensity from western blot was scored and plotted. Statistical analysis was performed between individual transduced and un-transduced primary cortical neurons to determine significant differences between them. Significant differences were observed in all transduction samples except the sample that was transduced using MOI 30 of IDLV-hSYN-*hSMN1_NtF*. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, stars represent level of significance, n=3.
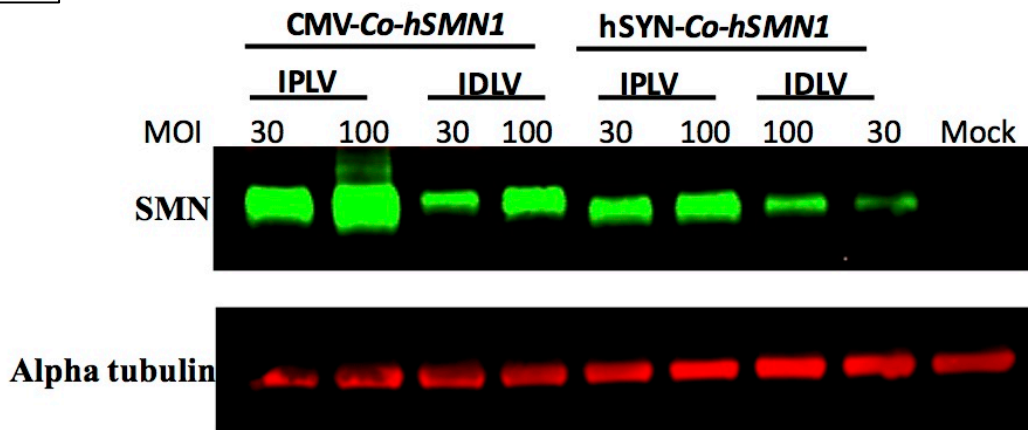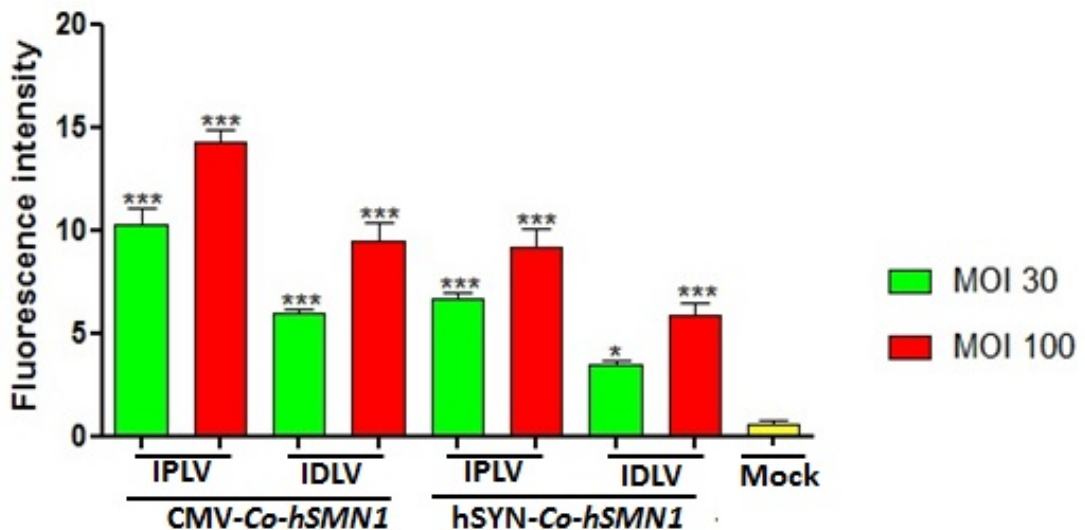
Figure 4.7: LV-mediated *Co-hSMN1* expression in primary cortical neuronal cultures.

(**A**) Mouse primary cortical cultures were transduced with IPLV/ IDLV CMV-*Co-hSMN1* or IPLV/IDLV hSYN-*Co-hSMN1* vectors at two different MOIs (infection dose and type of vector used is indicated in each relevant lane on above figure). SMN expression was evaluated by western blotting 3 days post-transduction. Extracted proteins from transduced and non-transduced cells were stained with purified mouse anti-SMN antibody to detect SMN protein (~35 kDa) and mouse α-tubulin antibody to detect loading control (Alpha tubulin, 55 kDa). Western blotting was completed by the Odyssey infrared imaging system. (**B**) Intensity of SMN fluorescence was evaluated 3 days post-transduction. All transduced rat primary cortical neurons showed significant differences to un-transduced. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.

Table 4.2: Comparison of SMN protein production from all vectors in cultured mouse cortical neurons.

One-way ANOVA and Bonferroni's post-hoc test were used to determine significant differences between obtained statistics from transducing mouse cortical neurons. The analysed data presents the effect of different parameters such as types of vectors, transgenes and promoters on protein production. Additionally, the data was analysed to determine whether there was a dose-dependent increases among each group. Values represent mean ± SEM, $*p<0.05$, $**p<0.01$, $***p<0.001$, stars represent level of significance, NS stand for significant different, n=3.

Transgene	Promoter	Vector	Tansgene	hSMN1								hSMN1_NtF								Co-hSMN1							
			Promoter	CMV				hSYN				CMV				hSYN				CMV				hSYN			
			Vector	IPLV		IDLV		IPLV		IDLV		IPLV		IDLV		IPLV		IDLV		IPLV		IDLV		IPLV		IDLV	
			MOI	30	100	30	100	30	100	30	100	30	100	30	100	30	100	30	100	30	100	30	100	30	100	30	100
hSMN1	CVM	IPLV	30	***	**			*				***	***			**				**	***			**			
			100				**		**					***	***								***		***		
		IDLV	30			***				*				***				*				**				*	
			100								*								**								**
	hSYN	IPLV	30					*	**							***	**							*	**		
			100								*								***								**
		IDLV	30							***								**								*	
			100																								
Co-hSMN1	CVM	IPLV	30	***								***															
			100		***								***														
		IDLV	30			***								***													
			100				***								***												
	hSYN	IPLV	30					***								***											
			100						***								***										
		IDLV	30							*								**									
			100								**								***								

■ Dose-dependent increase	■ CMV VS hSYN	■ IPLV VS IDLV	■ *hSMN1* VS *Co-hSMN1*

4.6 *SMN* expression in primary motor neuron
4.6.1 Characterisation of rat primary motor neurons cell cultures

Cultured embryonic rat motor neurons are one of the quiescent cell culture models that have been employed in this project to test produced vectors. Culturing of motor neuron cells was well-established and characterised in Prof. Yáñez's laboratory by Dr. Sherif Ahmed (Peluffo *et al*., 2013). Following the established protocol, motor neurons were produced from E14–15 rat embryos as described in 2.3.3.2. A pilot experiment was performed to confirm the presence of embryonic motor neuron cells in *in vitro* culture (Figure 4.8). The seeded cells were stained with Anti-Choline Acetyltransferase antibody to identify motor neurons after 3 days in culture. Choline acetyltransferase (ChAT) is one of the enzymes involved in the synthesis of the neurotransmitter acetylcholine. This enzyme is expressed by mature motor neurons, so ChAT antibody was used to positively identify the motor neurons. The average purity of the motor neuron population was approximately 70%, as expected (Cassina *et al*., 2002).
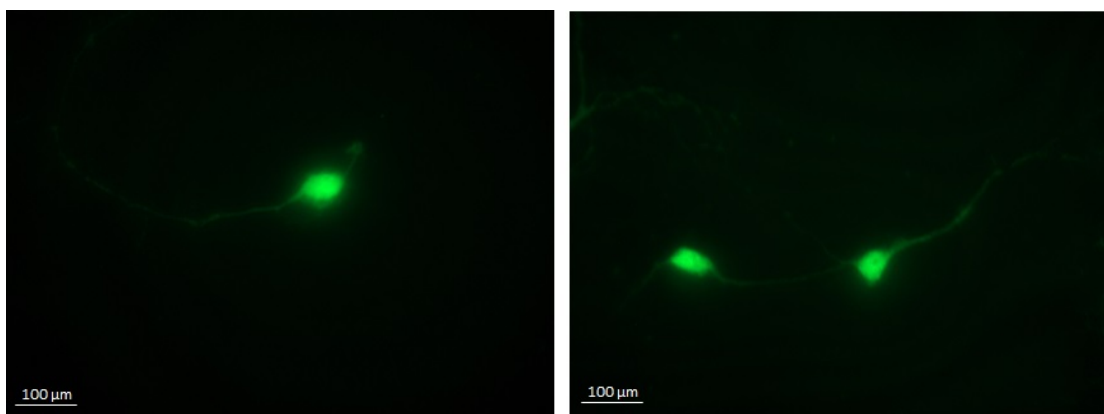
Figure 4.8: Identification of motor neuron cells in culture.

Cultured motor neurons express neuronal marker ChAT. Three days post-seeding, the cells were fixed and immunostained for a common motor neuronal marker (ChAT) to confirm motor neuron identity. Scale bars = 100 μm.

4.6.2 Lentiviral vectors transduce cultured purified primary motor neurons cultures

The experiment was designed to transduce primary motor neuron culture with produced HIV-1 based integrating and non- integrating lentiviral vectors, encoding either wild-type *hSMN1* or the novel *Co-hSMN1* downstream of either CMV or hSYN promoter. E15 primary motor neuronal cultures were transduced with vectors of interest 2 hours after seeding and incubated for 3 days. Q-PCR MOI 30, 60 and 100 were used to transduce the cells. Transduced cells were fixed and underwent immunostaining to quantify the SMN over expression, and to investigate the effect of different parameters on the expression of SMN protein in transduced cells (Figure 4.9, 4.11, 4.13 and 4. 15). The quantified SMN intensity was performed at day 3, post-vector transduction. The analysis of collected data showed that all generated vectors were transduced primary motor neuron cells and overexpression of SMN protein was detected in all transduced cells (Figure 4.10, 4.12, 4.14 and 4.16). Higher transgene expression was observed when cells were transduced using an IPLV and transgenes were inserted downstream from CMV promoter and the *Co-hSMN1* increased protein production relative to wild-type *hSMN1* (Table 4.3).
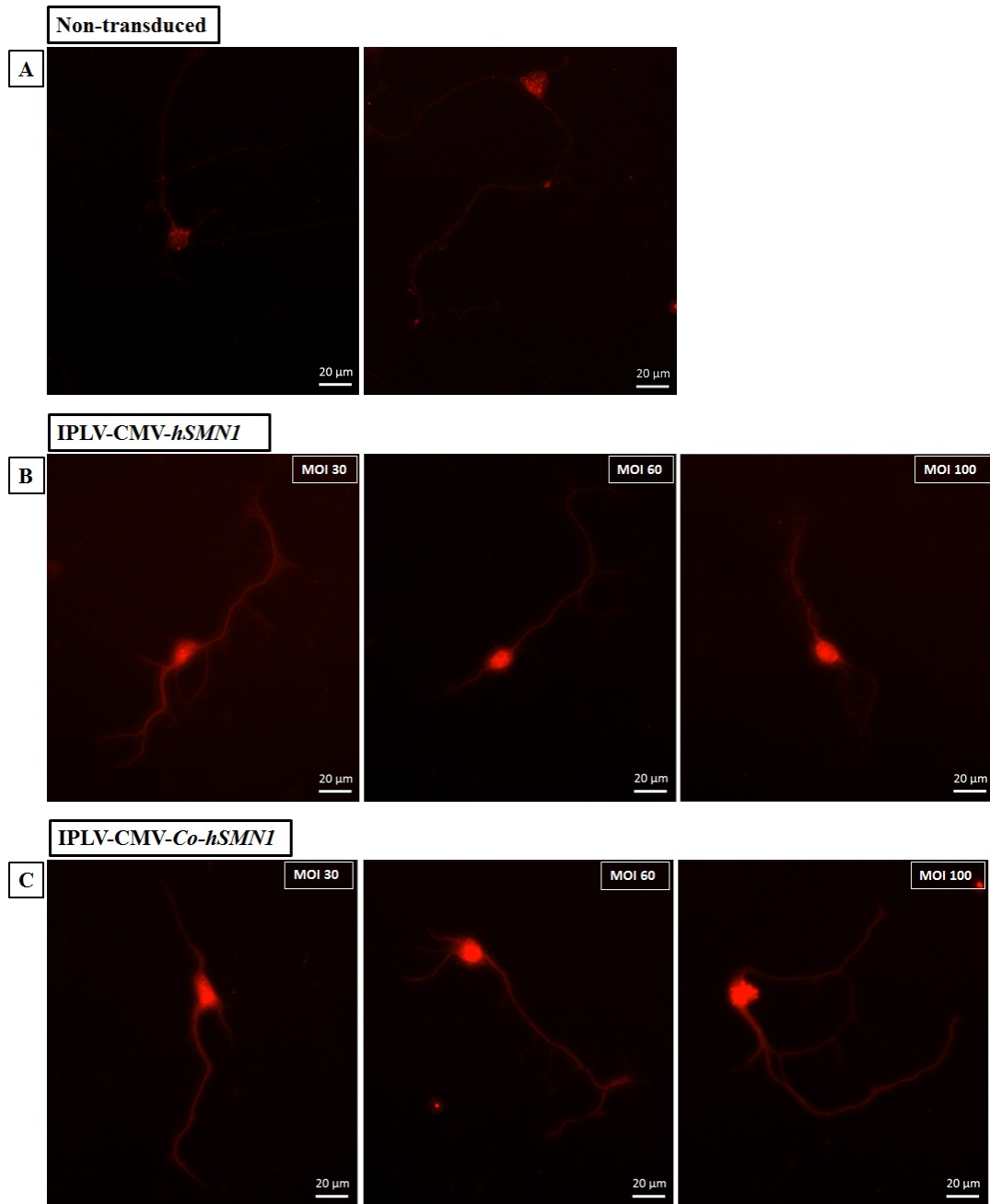
Figure 4.9: Overproduction of SMN protein in rat primary motor neurons cells transduced using IPLV driven by CMV promoter.

E15 rat primary motor neuron cultures were transduced with lentiviral vectors encoding either *hSMN1* or *Co-hSMN1* two hours after seeding the cells. Sample analysis by immunohistochemistry was performed three days post- vector transduction. (A) Images show a non-transduced motor neuron, which was treated according to the same protocol of transducing cells, however, it did not receive any vectors. (B) The representative images in panel B demonstrate overexpression of SMN protein in transduced primary motor neuron with IPLV-CMV-*hSMN1* at q-PCR MOI 30, 60 and 100. (C) The images in panel C are examples of SMN protein overexpression in transduced cell with integrating lentiviral vector expressing Co-hSMN1 driven by CMV promoter. Anti SMN antibody was used to detect in SMN protein in transduced or non-transduced neuronal culture. Scale bars = 20 μm**.**

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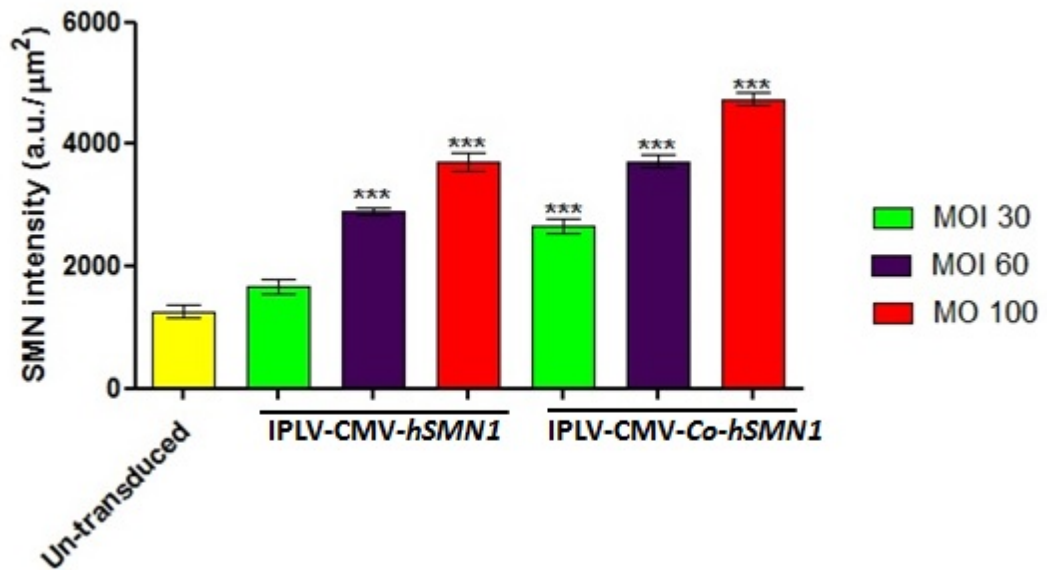
Figure 4.10: Quantification of SMN protein levels in transduced motor neurons *in vitro*.

Primary rat motor neuron cells were transduced at different doses of IPLVs expressing either *hSMN1* or *Co-hSMN1* under the control of CMV promoter. The above graph plots the intensity of SMN fluorescence in neuronal cell bodies. Statistical analysis was performed between transduced and un-transduced samples to determine significant differences between them. The significant differences were observed in all transduced samples except those samples which were transduced using MOI 30 of IPLV-CMV-*hSMN1*. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.
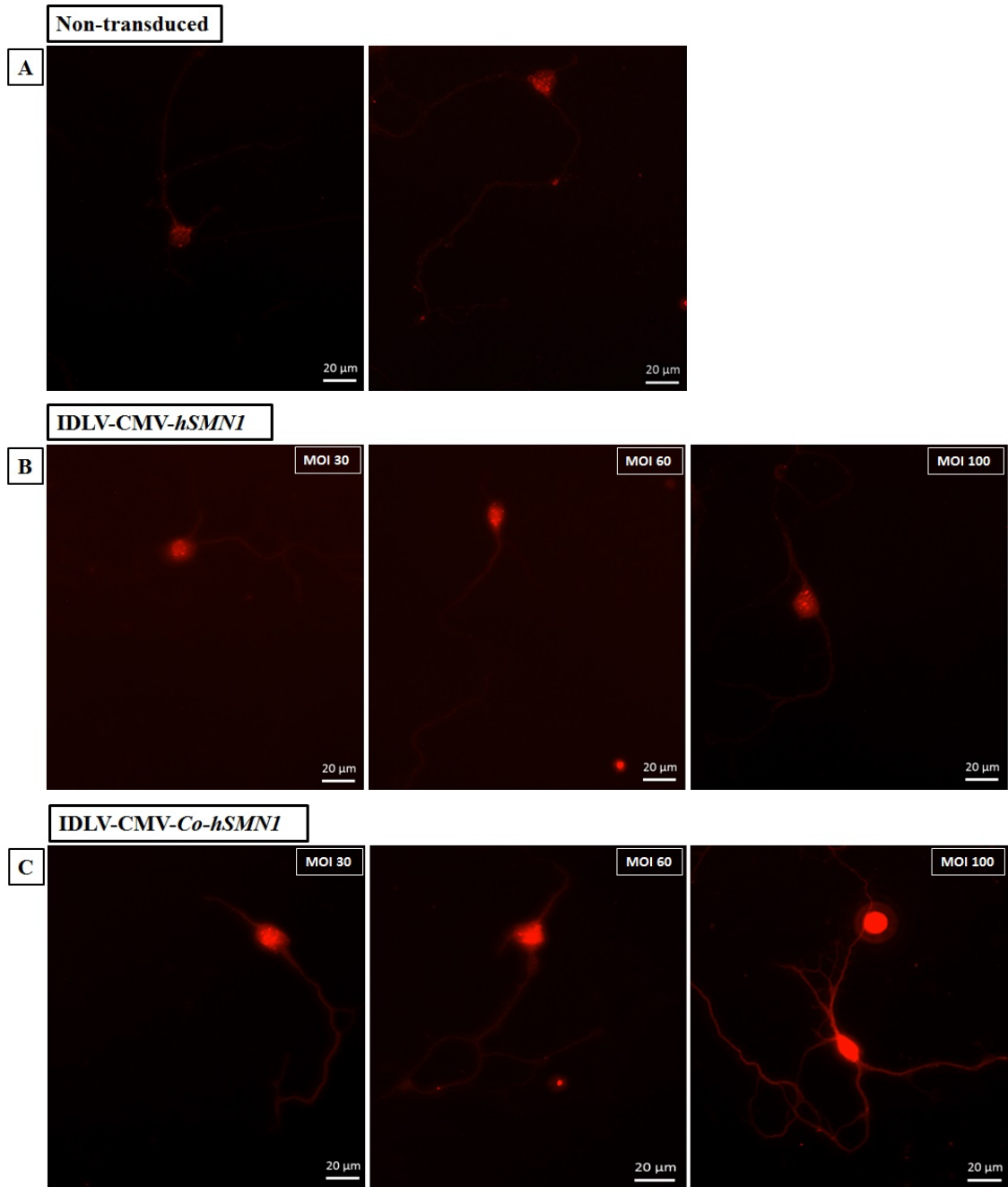
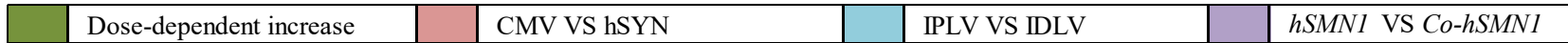**Figure 4.11: SMN protein production in E15 rat motor neurons transduced *in vitro* with IDLVs encoding *hSMN1* or *Co-hSMN1*.**

The above plates show SMN protein expression in three day old embryonic motor neuronal culture. Vectors were added to the cultures two hours after cell seeding, at q-PCR MOI 30, 60 and 100. (**A**) Motor neuron cells did not receive any lentiviral vectors and are representative of the SMN protein expression in non-transduced cell. (**B**) and (**C**) demonstrate SMN overexpression in transduced cell. (**B**) Motor neuron primary culture received different dose of IDLV-CMV-*hSMN1* while (**C**) demonstrates overexpression of SMN protein in transduced cells with non-integrating lentiviral vectors expressing *Co-hSMN1* under the control of CMV promoter. Cells were fixed and stained with anti-SMN antibody. Scale bars = 20 μm.
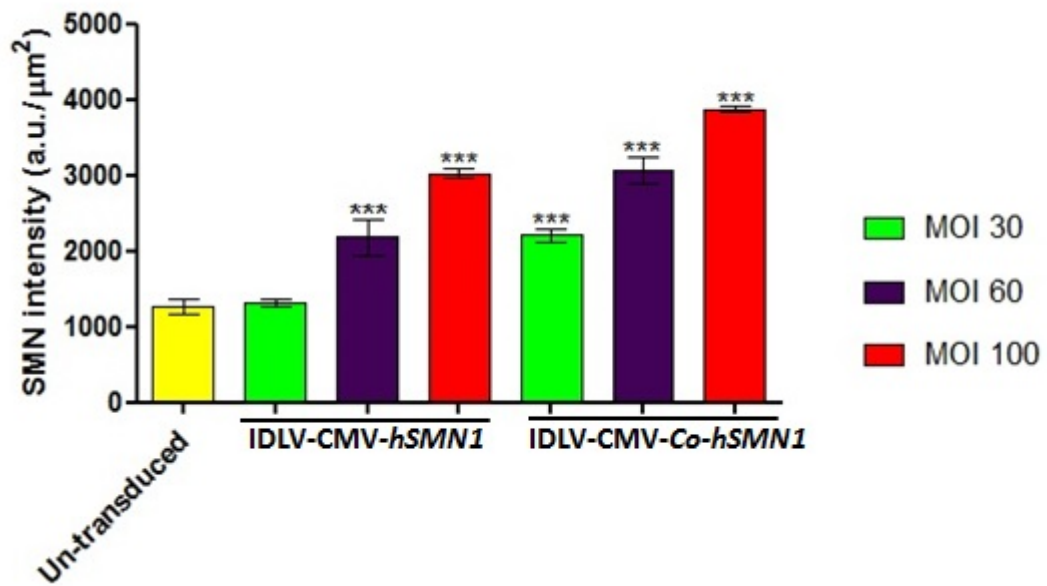
Figure 4.12: Quantification of SMN intensity in motor neurons transduced *in vitro* with IDLVs expressing *hSMN1* or *Co-hSMN1* under control of the CMV promoter.

Quantification of SMN fluorescent in cell bodies of transduced or un-transduced E14 rat primary motor neuron was scored and plotted. Each transduced set of data was compared to un- transduced motor neuron using one-way ANOVA and Dunnett's post-hoc test. Significant differences were observed in all samples except the samples that were transduced using MOI 30 of IDLV-CMV-*hSMN1*. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.
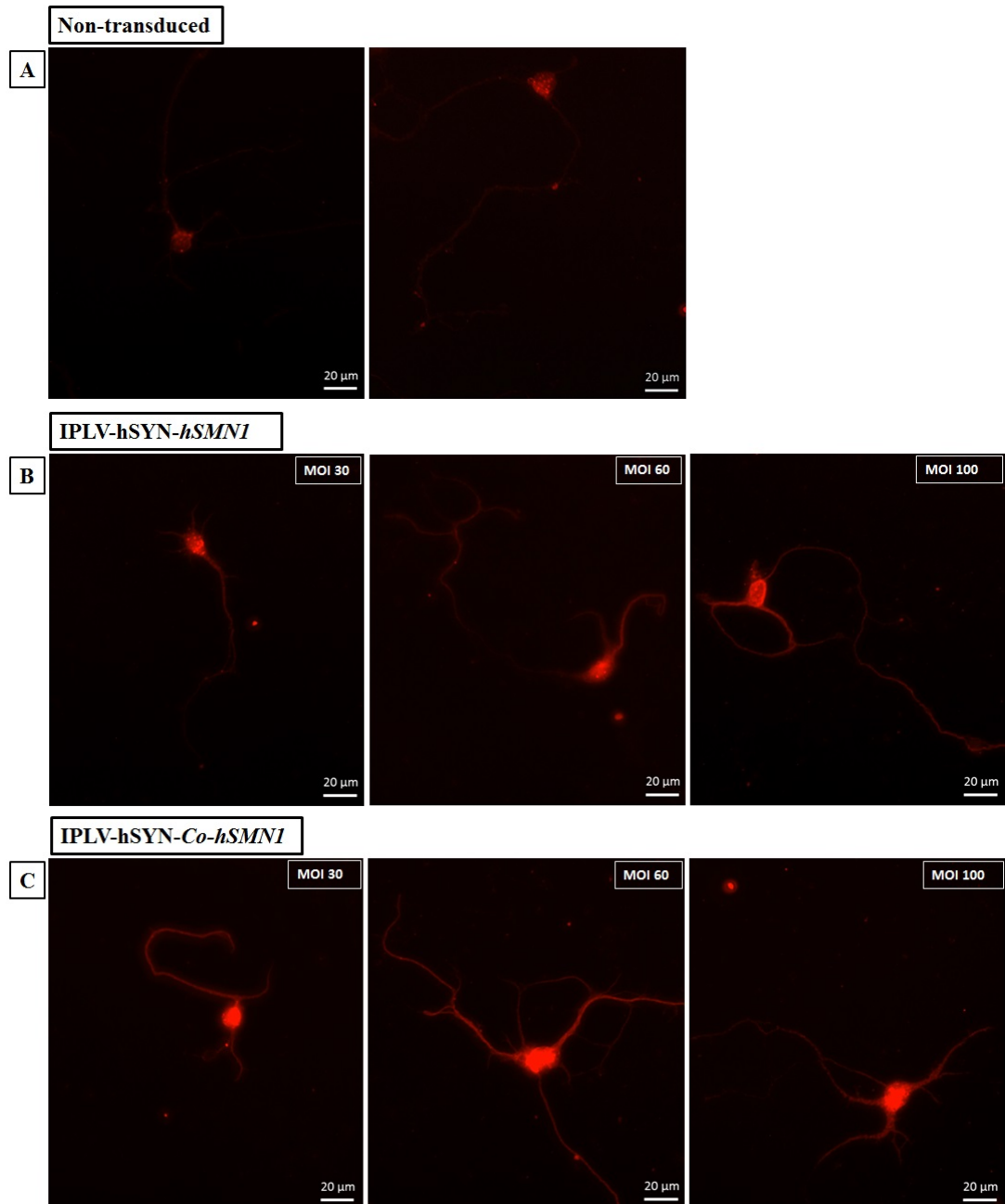
Figure 4.13: Primary motor neurons transduced with IPLV driven by hSYN promoter express *hSMN1* or *Co-hSMN1* transgenes.

In order to investigate transduction efficiency of generated lentiviral vectors, isolated motor neurons from E15 rat embryo were transduced with IPLVs encoding either wild-type -*hSMN1* or *Co-hSMN1* under the control of hSYN promoter. (**A**) Demonstrates SMN protein in non-transduced motor neuron. (**B**) Images are representative of overexpression of SMN protein in transduced motor neuron cells by IPLV-hSYN-h*SMN*1 at different q-PCR MOI 30, 60 and 100. (**C**) Images are examples of transduce cells using IPLV-hSYN- *Co-hSMN1* at different q-PCR MOI 30, 60 and 100. Transduced and non-transduced motor neurons were in culture for three days and then were subjected to immunohistochemistry to stain the SMN protein, using primary anti-SMN antibody. Scale bars = 20 μm.
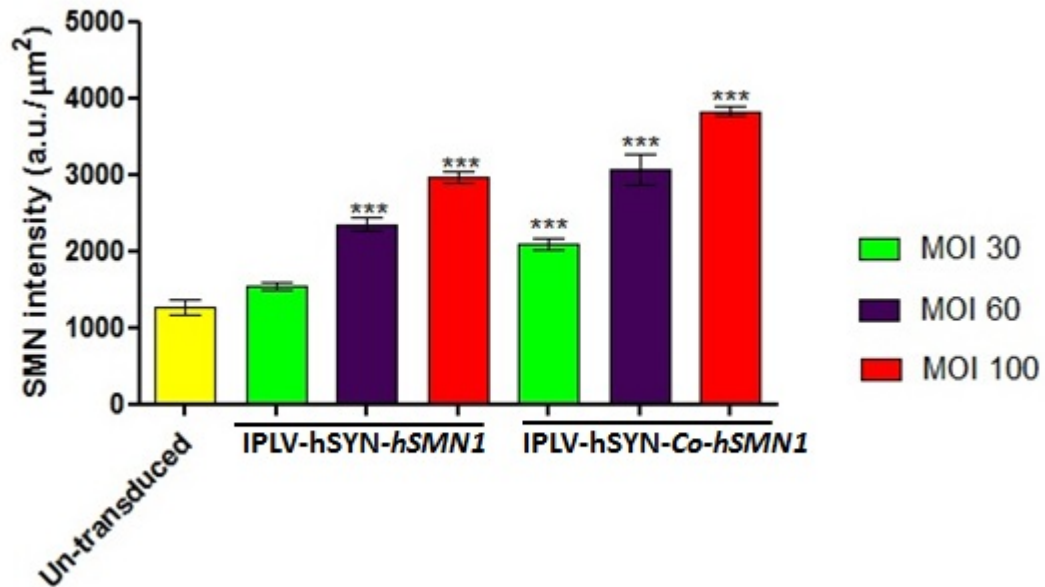
Figure 4.14: Analysis of SMN intensity following *in vitro* transduction of motor neurons with IPLVs expressing *hSMN1* or *Co-hSMN1* under control of hSYN promoter.

Isolated primary motor neurons were transduced at different doses of IPLVs expressing either *hSMN1* or *Co-hSMN1* under the control of hSYN promoter. The above graph plots the intensity of SMN fluorescence within the cell body. Statistical analysis was performed between individual transduced and un-transduced motor neurons to determine significant differences between them. Significant differences were observed in all transductions except the sample that was transduced using MOI 30 of IPLV-hSYN-*hSMN1*. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.
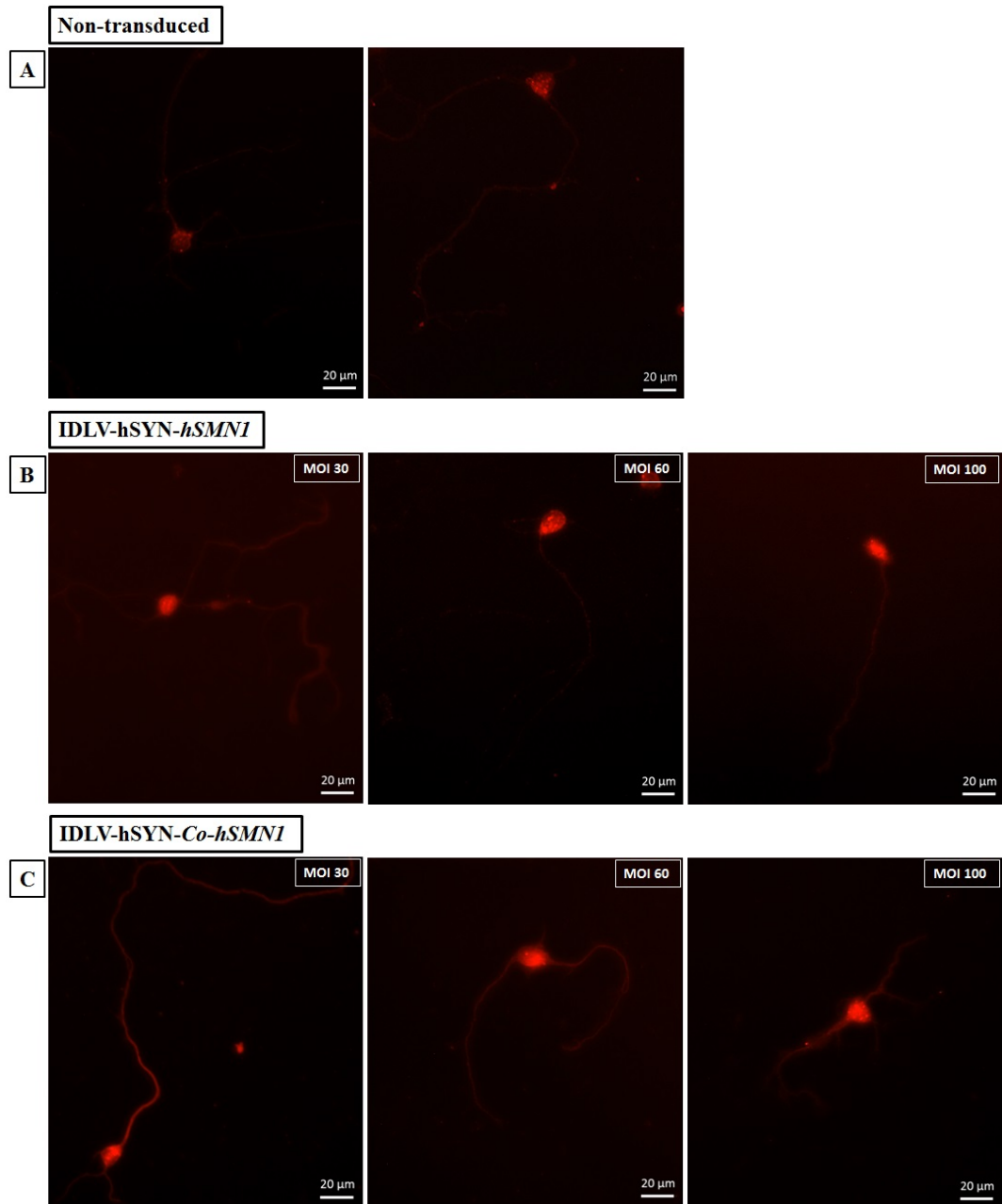
Figure 4.15: Overproduction of SMN protein in E15 rat motor neurons transduced with IDLVs driven by hSYN promoter and encoding *hSMN1* or *Co-hSMN1*.

E15 rat primary motor neuron cultures were transduced at 2 hours *in vitro*. (**A**) Motor neuron cells did not receive any lentiviral vectors and are representative of the SMN protein expression in non-transduced cell. (**B**) and (**C**) Photomicrographs are examples of overexpression of SMN protein in transduced cell by viral vectors. (**B**) Different dose (qPCR MOI 30, 60 and 100) of non-integrating lentiviral vectors encode wild-type *hSMN*1 gene under control hSYN promoter were used to transduce motor neuron primary cells. (**C**) Here, cells were transduced with the same multiple dose of non-integrating lentiviral vectors, but the vectors expressed a codon optimised version of the *hSMN1* transgene. 3 day old primary motor neuron cells in culture were fixed using 4% PFA and then exposed to primary antibodies directed against SMN protein. Scale bars = 20 μm.
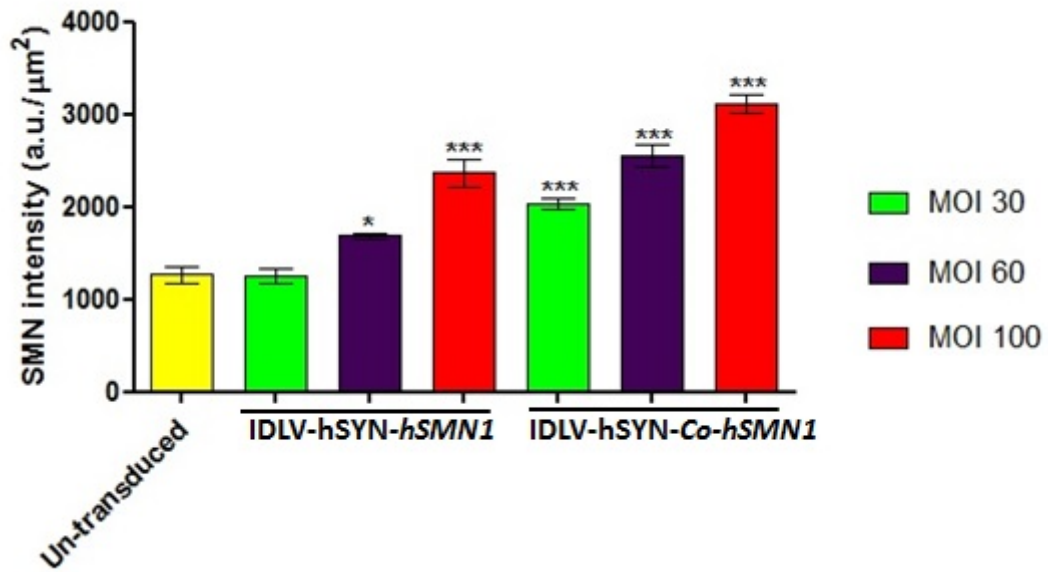
Figure 4.16: Quantification of SMN intensity in motor neurons transduced *in vitro* with IDLVs expressing *hSMN1* or *Co-hSMN1* under the control of the hSYN promoter.

Intensity of SMN fluorescence was evaluated 3 days post-transduction. Between all transduced samples, only the sample transduced with IDLV-hSYN-*hSMN1* at MOI 30 did not show any significant differences compared to un-transduced. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.

Table 4.3: Comparison of SMN protein production from all vectors using rat motor neurons *in vitro*.

One-way ANOVA and Bonferroni's post-hoc test were used to determine significant differences between obtained statistics from transducing rat motor neurons. The analysed data presented the effect of different parameters, such as type vectors, transgenes and promoters, on protein production. Additionally, the data was analysed to determine whether there were dose-dependent increases among each group. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, NS stand for significant different, n=3.

| Transgene	Pomoter	Vector	Tangene Promoter / Vector / MOI	hSMN1 CMV IPLV 30	60	100	CMV IDLV 30	60	100	hSYN IPLV 30	60	100	hSYN IDLV 30	60	100	Co-hSMN1 CMV IPLV 30	60	100	CMV IDLV 30	60	100	hSYN IPLV 30	60	100	hSYN IDLV 30	60	100
hSMN1	CVM	IPLV	30	***	***	ns				ns						***											
			60		**			***			*						**											
			100						***			***						***										
		IDLV	30					*	***				ns						**									
			60						*					*						**								
			100												**						**							
	hSYN	IPLV	30							***	***	ns										*						
			60								**			**									**					
			100												**									**				
		IDLV	30											*	***											**		
			60												**												***	
			100																									**
Co-hSMN1	CVM	IPLV	30													**	***	ns				*					
			60														**			**			**					
			100															***						***				
		IDLV	30																**	***					ns			
			60																	**						*		
			100																		***							
	hSYN	IPLV	30																			**	***	ns			
			60																				*			*		
			100																					***				
		IDLV	30																							*	***	
			60																								*	
			100																									

Legend: ■ Dose-dependent increase ■ CMV VS hSYN ■ IPLV VS IDLV ■ *hSMN1* VS *Co-hSMN1*

4.7 Discussion

Fast onset and high-level expression from transgenes *in vivo* is critical for designing a therapeutic vector to target a disease, and many factors can have an effect on transgene expression pattern, such as delivery system, promoter and transgene sequence. The level of SMN protein is a key factor in rescuing SMA disease, and the aim of this project is to optimise and develop a possible therapeutic vector for gene therapy of spinal muscular atrophy. Therefore, this project set out to investigate the effects of several parameters on SMN protein expression.

Vectors can vary in many different aspects, such as packaging capacity, transduction efficiency, the ability to transduce dividing or non-dividing cells and integration into genetic material of the targeted host. Lentiviral vectors are promising tools for gene-delivery vehicles. They possess several attractive properties, such as sustained gene expression through stable vector integration into the host genome, they have a broad tissue tropism and are able to deliver DNA into a broad range of cell types, they can transduce both dividing and non-dividing cells, have a higher transduction efficiency to adeno-associated virus vectors (at same MOI), do not express viral proteins after vector transduction and a fairly easy production process (Sakuma *et al*.,2012; Wanisch and Yáñez-Muñoz, 2009). Integration is an important feature of lentiviral vectors, but integration from the vectors may raise the insertional mutagenesis risk. Non-integrating vectors typically have mutations in the IN gene that prevent integration of the virus genome into the host genome, so they do not disrupt any of the cell's own genes (Yáñez-Muñoz *et al*., 2006). IDLV's have a reduced rate of integration (up to 10^4 –fold), and thereby avoid the problems associated with integration but still preserve some benefits of integrating lentiviral vectors (Nightingale *et al*., 2006). All non- integrating vectors

used in this project were produced by class I mutation via replacing aspartic acid at position 64 with valine (Yáñez-Muñoz *et al*., 2006).

An aim of this chapter was to compare transgene expression from IPLVs and IDLVs. In order to achieve this aim, a range of IPLVs and IDLVs had to be made and titrated (chapter 3). The titred vectors were tested in a variety of *in vitro* models - growth-arrested CHO, primary cortical neurons and embryonic motor neurons. Transgene expression of integrating and non-integrating lentiviral vectors was quantified by measuring the mean fluorescent intensity of SMN expression. Transduction results revealed that both IPLV and IDLV efficiently transduce employed quiescent cell culture models at different multiplicities of infection and expression of gene of interest.

Cells were transduced at multiple different MOI to maximize transgene expression and also to investigate a MOI-dependent manner. The level of transgene expression increased for both vector forms in a dose-dependent manner. The results demonstrate that transgene expression from both IPLV and IDLV is enhanced with increasing MOI (Table 4.1, 4.2 and 4.3).

Comparing mean fluorescence intensity of SMN-expressing demonstrated that the level of transgene expression in cells transduced using integrating vectors is higher than the cells that were transduced using non-integrating vectors (Table 4.1, 4.2 and 4.3). The result was the same in all tested *in vitro* models. The outcome of this experiment demonstrated that integrating vectors result in more transgene protein production per vector copy than non-integrating. When comparing transgene expression in both type of vectors, there is a 1.22-fold difference between integrating and non-integrating vectors in transduced motor neuron at MOI 100. Transduction in growth-arrested CHO and primary cortical neuron demonstrated that the lowest transgene expression from IPLV is at least 1 fold more than the highest transgene expression achieving using IDLV.

Considering transduction of various target cells, the results of this chapter reveal that, regardless of whether the promoter used was CMV or hSYN, non-integrating vectors express less transgene than the integrating vector. This finding is in line with other studies by Apolonia, 2009. Apolonia, 2009 suggested that the non-integrated vectors were less transcriptionally active than their integrating counterparts, so the non-integrating express less transgene that their corresponded IPLV. Schröder *et al*., 2002 and Aranyi *et al*., 2016 might offer two possible explanations as to why IDLVs have a lower transcription activity than IPLVs, however this subject needs further investigation. Schröder *et al*., 2002 reported lentiviral vectors do not integrate into the host genome based of a random site. The vectors integrate anywhere along transcriptional units, particularly in highly active genes and regional hotspots. Therefore, the integration of vectors into active genes might have evolved to facilitate efficient transgene expression from IPLVs. Another possible explanation for lower transcription activity in non-integrating vectors could be explained by a new finding by Aranyi *et al*., 2016. This study suggested that the presence of episomal genomes in cells transduced by non-integrating lentiviral vectors may trigger DNA methylation. This change might prevent high promoter activity over time, however, the authors mentioned this finding needs further investigation.

It is important to emphasise that it was observed that the IPLVs transduced more cells than IDLV, even when the transduction was carried out in non-dividing cultures. Taken together, higher transcription activity and transduction efficiency of IPLVs results in IPLVs expressing more transgene protein than IDLVs.

Another aim of this chapter was to have a comparison between CMV and hSYN to evaluate the efficiency of these promoters for producing high levels of transgene protein expression using lentiviral vector.

CMV immediate early region enhancer-promoter is one of the strongest promoters used in gene therapy, and has been used to achieve high levels of constitutive expression of transgenes in a wide variety of mammalian cell lines. Previous studies have shown that the CMV promoter led to a faster rate of onset of detectable transgene expression and higher level of transgene expression (Montesinos, Chen, & Young, 2011 and Williams *et al*., 2005). While the hSYN promoter is a strong and relatively neuron-specific promoter, this promoter has been extensively used for neuron specific transgene expression (Kügler *et al*., 2001; Kügler, Kilic *et al.*2003 and Kügler, Lingor *et al*., 2003). It should be mentioned that there are a number of studies claiming that hSYN is a highly neuronal-specific promoter, and that the expression of this promoter occurs exclusively in neuronal cells (Dittgen *et al*., 2004 and Kügler, Kilic, *et al*., 2003). According to the results obtained in this study, the transgene of interest can be expressed using the hSYN promoter in non-neuronal cells as well as in neuronal cells. This finding aligns with other studies which used hSYN promoter to drive transgene expression in non-neuronal cells (Holzmann *et al*., 1998 and Yaguchi *et al*., 2013). Holzmann *et al*., 1998 compared the level of transgene expression controlled by hSYN in neurons and non-neuronal cells. The result of this comparison showed low transgene expression in non-neuronal cells, while hSYN promoter drove strong transgene expression in neurons, confirming the relative neuron specificity of hSYN.

Moreover, cell lines in culture are more permissive to transgenic expression than cells in their natural environment. It is not unusual that tissue specific promoter show a degree of transgenic expression in common cell lines. As previously stated, the comparison between hSYN and CMV was carried out in this study to evaluate which of these two promoters drives the strongest transgene expression in a variety of cell culture systems.

To assess the promoter efficiency, the expression of transgenes controlled by either CMV or hSYN promoters carried by lentiviral vectors in growth-arrested CHO cells and primary cortical and motor neurons were compared. Different MOIs of each vector were used to transduce the cell to maximise the protein production. The results revealed that the two promoters under investigation express the transgene in transduced cell cultures. Using the growth-arrested CHO cells clearly demonstrates that CMV is a much stronger promoter than hSYN (Table 4.1), and using CMV promoter led to high-level transgene expression. This finding was not surprising, as CMV is a generic promoter, and resulted in stronger expression in CHO cells compared to hSYN, which is a relatively neuron-specific promoter.

Transducing primary cortical and motor neurons revealed that both these promoters can efficiently express targeted transgenes in transduced primary cortical and motor neurons. When comparing hSYN with CMV, it was revealed that utilising CMV promoter led to the most robust transgene expression using primary neuronal cultures (Table 4.2 and 4.3).

Significant differences were observed between both promoters in terms of transgene expression at each transduction of the primary cortical neuron and growth-arrested CHO. This significant difference was not observed in three out of 12 transductions (Table 4.3). These occasions were when the primary motor neurons were transduced with MOI 30. This could be because qPCR MOI 30 is not high enough to distinguish the significant difference between these two promoters, as the significant difference was clearly observed in all other cases that used MOI for transduction.

Taken together, the results of a comparison between of CMV and hSYN promoters in this study demonstrate the following: transduction of target cells with lentiviral vectors carrying CMV promoter resulted in more robust transgene expression compared to

corresponding lentiviral vectors carry hSYN promoters. Therefore, CMV promoters are stronger, and this finding is in agreement with other studies (Gascón, *et al.*, 2008; Kuroda *et al.* 2008 and Scott & Lois, 2005).

However, it need to be mentioned, there are studies that report that hSYN led to stronger reporter gene expression and CMV is a weaker promoter in cells of the CNS. Moreover, hSYN promoter expresses exclusively in neuronal cells, (Dittgen *et al.*, 2004 and Kügler, Kilic, *et al.*, 2003). There are many factors that can affect expression, and two key factors here are the type of transgene, and vector stock titration. Many methods can be used to titre the vector stock and it is not clear how the vectors have been titred in these studies. In general, these studies use the *eGFP* as reporter gene and they titre the vector stock using FACS. The cell line used during the titration process is very important, as efficiency of chosen promoters may react differently among cell line and effect the titration result (Kuroda *et al.*, 2008). Moreover, different genes are transcribed in cells differently because of the needs of the cells. Thus, these results are difficult to interpret and will be explained in more details. Additionally, the method of transgene delivery can be another effective parameter that effects the expression of transgene.

Codon optimization is a novel technique that improves the expression of a transgene to maximise the protein production in the host by increasing the translational efficiency of interested gene. This is achieved by replacing rarely used codons (at the point of protein translation) in the genome of the host with the ones that are frequently used (Condon & Thachuk, 2012). Codon optimized on human protein expression has been widely used by many scientists and they reported higher protein expression. The increase could be up to 15 fold more (Fath *et al.*, 2011 and Graf *et al.*, 2004).

Therefore, this technique was used to optimise *hSMN1* transgene in this project in order to increase the level of SMN protein, which is in a critical factor for SMA therapy. The

codon optimisation was carried out using GENEART's GeneOptimizer software. GENEART optimised *hSMN1* transgene using a specific algorithm. This algorithm considers optimising a variety of critical relevant factors involved in the different stages of protein expression, such as codon adaptation, mRNA stability, and various *cis*-elements in transcription and translation to achieve the most efficient expression of gene of interest.

Wild-type and codon optimised *hSMN1* transgenes were inserted into the integrating and non-integrating lentiviral vectors. The efficacy of these transgenes for SMN expression was evaluated using a variety of quiescent cell culture models: growth-arrested CHO, E18 primary cortical neuron and E15 primary motor neuron.

In a bid to maximize the transgene expression, the cells were transduced at three different MOIs. Transgene expression was evaluated in transduced cell culture three days after transduction, and the result of transduction revealed *Co-hSMN1* transgene and its corresponding wild-type transgene (*hSMN1*) are encoding SMN protein. Quantified transduction results demonstrated that *Co-hSMN* dramatically increases SMN expression, compared with *hSMN1*.

Significant differences were observed between *Co-hSMN1* and *hSMN1*, in terms of SMN expression at each transduction of growth-arrested CHO (Table 4.1) and primary cortical and motor neuron (Table 4.2 and 4.3). This finding is in agreement with other studies (Dominguez et al., 2011; Fath et al., 2011 and Valori et al., 2010).

In summary, the results in this chapter demonstrate vector dose-dependent increases in level of SMN protein for both vector formers. Highest level of SMN protein production by using: CMV promoter, Co- *hSMN1* and integrating lentiviral vectors.

Chapter 5 Functional effect of *SMN* over-expression in SMA fibroblasts

5.1 Introduction:

Thus far, it has been demonstrated that the lentiviral vectors generated can successfully express the gene of interest and produce SMN protein, as demonstrated in the three different models of arrested CHO cells, cortical neurons and motor neuron primary cells. Now it is time to take the project further, and to investigate whether SMN protein produced using lentiviral vectors is functional or not.

SMN protein is found in the cytoplasm and nucleus (Coovert *et al*., 1997). Full length SMN protein is bound with a set of diverse proteins collectively known as gemins to form a macromolecular complex (Borg & Cauchi, 2014). These nuclear SMN complexes are local to specific structures called nuclear gems. Gems were introduced by Liu & Dreyfuss for the first time in 1996. Using immunostaining of Hela cells, they detected several intense dots in cell nuclei, and named them 'gems,' for 'Gemini of the coiled bodies'.

As previously stated, SMN oligomerises form a stable large multiple-protein complex with five additional proteins (gemins2-6). In addition to SMN protein, this large multi-protein complex also requires gemin2, which is a core protein, although the mechanism by which it drives formation is unknown yet. Binding between SMN and gemin2 forms a sub complex, which is essential for interaction between SMN and other gemins (gemin3-7), and for forming SMN complex (Ogawa et al., 2007).

SMN protein is required to form a large multi-protein complex, in order for it to be stable and functional. In addition, this multi-protein complex plays an essential role in the assembly of the spliceosomal snRNPs, which are essential components of the splicing machinery (Cauchi, 2014; Gubitz *et al*., 2004 and Ogawa *et al*., 2007).

In SMA patients, the number of gems is greatly reduced as a consequence of dramatically reduced levels of endogenous SMN. Reduced gem formation correlates

with SMN protein expression and disease severity in human SMA fibroblasts (Coovert *et al*., 1997). Coovert *et al*., 1997 reported that fibroblast cell lines derived from SMA type I patients, which is the most severe type of SMA disease, have few or no gems. A feature of fibroblasts and motor neurons in SMA animal models is the low number of gems. In fibroblast cells, the number of gems per cell can be used as a prognostic indicator of SMA type and disease severity (DiDonato *et al*., 2003).

5.2 Aims of the chapter

The aim of this chapter is to investigate the functional effect of SMN protein produced in human type I SMA fibroblasts by determining whether nuclear gems can be restored in transduced cells as an indicator of SMN functionality.

For cells to assemble gems efficiently, it is first necessary for them to produce sufficient amounts of the correct SMN isoform. Gemins bind the full-length isoform of SMN protein. The SMN isoform lacking exon 7 is unable to be efficiently transported across the nuclear member to form gems. Linked to this, the previous chapter demonstrated that different parameters (integration proficiency, promoter and transgene) can affect the production of SMN protein from lentiviral vectors. In this chapter, the effect of those parameters on the restoration of nuclear gems in transduced type I SMA fibroblasts is explored.

The aim of this chapter can be broken down into five specific goals, studied using type I SMA fibroblasts:

(**A**) to determine the efficiency of various lentiviral vectors producing SMN to restore gems in SMA patient–derived fibroblasts.

(**B**) to study whether restoration of gems by lentiviral vectors is MOI-dependent.

(**C**) to analyse the effect of *hSMN1* transgene codon-optimisation on the restoration of gems in transduced cells.

(**D**) to study the effect of using different promoters on the restoration of gems in transduced cells.

(**E**) to analyse the impact of lentiviral integration efficiency on the production of gems in transduced cultures.

5.3   Summary of experiments and methods

Low-passage human fibroblast cell lines were grown in DMEM with high glucose that had been supplemented with 21% M-199, 10% FBS, 10 ng/mL Fibroblast growth factor 2 (FGF2), 25 ng/mL Epidermal growth factor (EGF) (Miltenyi, Germany), 1μg/mL gentamicine. All cultured cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere.

Control, type I, II and III fibroblast cells were harvested in Santa Creu i Sant Pau Hospital (Barcelona) from skin biopsies of either a healthy adult or patients with SMA type I, II and III, respectively. Cells were obtained with informed consent by our collaborator Dr. Eduardo Tizzano. SMA type I, II and III patients are dependent on SMN protein produced from the *SMN2* gene. The variability in the level of SMN protein among different types of SMA fibroblast is due to differences in *SMN2* copy number. An individual affected by SMA type III normally has higher level of SMN protein and hence a less-severe disease phenotype than a patient affected by SMA type II, (and I), as they have more copies of *SMN2*.

Prior to transduction of the human fibroblasts, I tried to provide a baseline for my study by counting gems in different types of SMA fibroblast cells. All types of SMA human fibroblast (type I, II, III SMA) and control human fibroblast were seeded at a density of 1.0×10^5 cells/well in a 12 well plate. Seeded cells were subject to immunostaining seven days after seeding.

For the transduction stage, among different type of SMA patient fibroblast cells, type I SMA fibroblast were chosen for transduction as these cells produced very few or no gems. The type I human fibroblast were seeded as per the stated density and conditions. The seeded cells were transduced at varying MOI (qPCR MOI 30, 60 and

100) of either IPLVs-CMV / hSYN or IDLVs-CMV / hSYN vectors encoding either *hSMN1* or *Co-hSMN1* transgene twenty-four hours after seeding.

To detect gems in the nuclei of the cells, immunofluorescence labelling was performed, and cells were visualized under fluorescence microscope at 72 hours post-transduction. The process involved washing, fixing, and blocking the cells, and then human fibroblast cells were incubated with diluted fluorescein isothiocyanate-conjugated gemin2 antibody overnight in a cool, dark place. The cells were visible under a fluorescent microscope, following the addition of a secondary antibody and DAPI. To avoid unintentional bias, the investigator was blinded to the type of vector, transgene, promoter and MOI that were used to transduced cells. 100 randomly selected nuclei were scored for counting the number of gems, and then the number of gems per 100 nuclei was calculated.

5.4 Result

5.4.1 Gems in different types of SMA fibroblasts

Before transducing the cells, the experiment was set up to demonstrate the changes in the number of gems among various SMA types and control fibroblasts. This experiment forms the basis of this chapter. It has been reported that there is a noticeable difference between the amount of endogenous SMN and the number of gems in various types of SMA (Coovert *et al*., 1997 and DiDonato *et al*., 2003). The results of this chapter demonstrated that the number of gems per cell inversely correlates with disease severity. Very few or no gems were detected in SMA type I, and while the number of gems increased in type II and III SMA, it was still fewer than the number of gems detected in control fibroblasts (Figure 5.1).
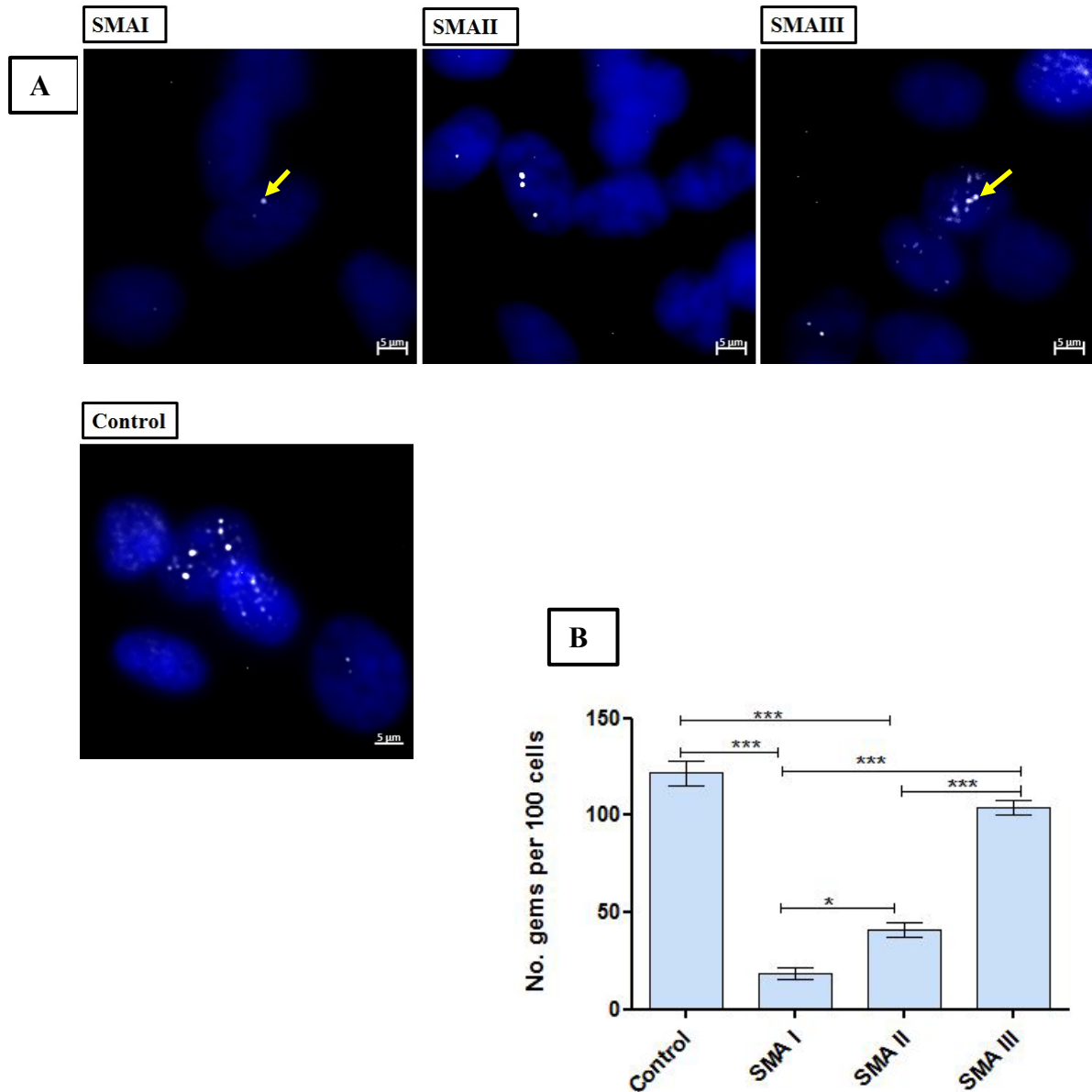
Figure 5.1: Immunostaining of gems in human SMA fibroblasts

Cultures were immunostained at day seven post seeding. The goat anti gemin2 polyclonal antibody was used to detect gems in the nuclei of seeded fibroblast cells and DAPI was used to stain the nucleus of the cell.

(**A**) Representative number of gems in SMA I, II, III and control human fibroblasts. Scale bars = 5 μm. (**B**) Presenting the analysis of the result of counting gems in 100 cells in each type of fibroblast cell line. One-way ANOVA and Bonferroni's post-hoc test was used to determine significant differences between all different types of fibroblast. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.

5.4.2 Increase in number of gems in transduced SMA fibroblasts

As previously stated, the focus of this study is to investigate and optimise the possible configuration of lentiviral vectors to target SMA disease. In order to achieve the goal of this study, a variety of IDLVs and IPLVs were produced during this project. These vectors expressed either wild type *hSMN1* or novel *Co-hSMN1* transgene under the control of either CMV or hSYN promoter. The type I SMA fibroblast cell line was transduced with produced vectors to investigate the effect of different type of vectors, promoter and transgenes on restoring gems. The results revealed that all produced vectors successfully restored gems in transduced cells, and that increasing the vector concentrations resulted in the detection of a higher number of gems (Figure 5.2, 5.4, 5.6 and 5.8). It was observed that the highest number of gems was achieved when cells were transduced using MOI 100 of both vector, IPLVs or IDLVs (Figure 5.3, 5.5, 5.7 and 5.9). As indicated by the graphs, in some cases, the number of detected gem structures in the transduced cells were higher than in healthy fibroblast cells. A statistical comparison was performed to investigate the effect of different parameters on restoring gems. The result of this comparison revealed that the number of gems in transduced cell cultures with integrating lentiviral vectors is higher than the transduced cells with non-integrating lentiviral vectors (Table 5.1). When comparing CMV with hSYN, the CMV restored more gems in transduced type I SMA fibroblasts (Table 5.1). Codon optimised versions of *hSMN1* led to a noticeable increase in the number of gems in transduced cells, compared to cells that were transduced using wild-type *hSMN1* (Figure 5.3, 5.5, 5.7, 5.9 and Table 5.1).
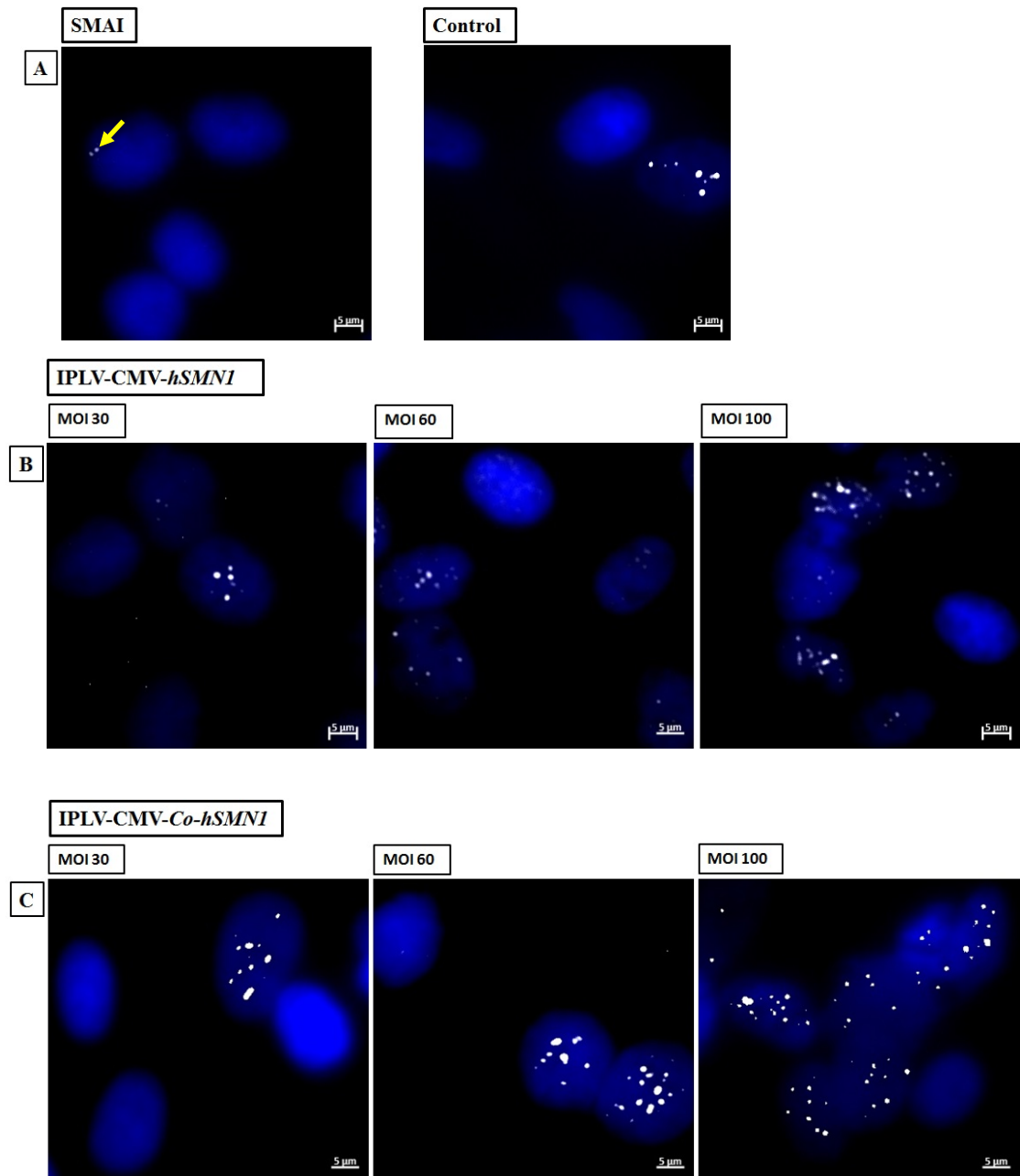
Figure 5.2: Restoration of gems in type I SMA fibroblasts transduced with IPLV driven by CMV promoter and encoding *hSMN1* or *Co-hSMN1*.

Cultured human SMA I fibroblasts were transduced with either IPLV-CMV-*hSMN1* or IPLV-CMV-*Co-hSMN1* at qPCR MOI 30, 60 and 100. The number of gems was quantified 3 days post- transduction. (**A**) Representative images of detecting gems in non-transduced SMA I and control human fibroblasts which have been treated following the same protocol of transducing cells, except they did not receive any lentiviral vectors. (**B**) Gems in transduced SMA I fibroblast cells using different doses of integrating lentiviral vector encoded with *hSMN1* under the control of CMV promoter. (**C**)The images in this panel demonstrate the number of gems in transduced cells with integrating lentiviral vectors expressing *Co-hSMN1*. Scale bars = 5 μm.
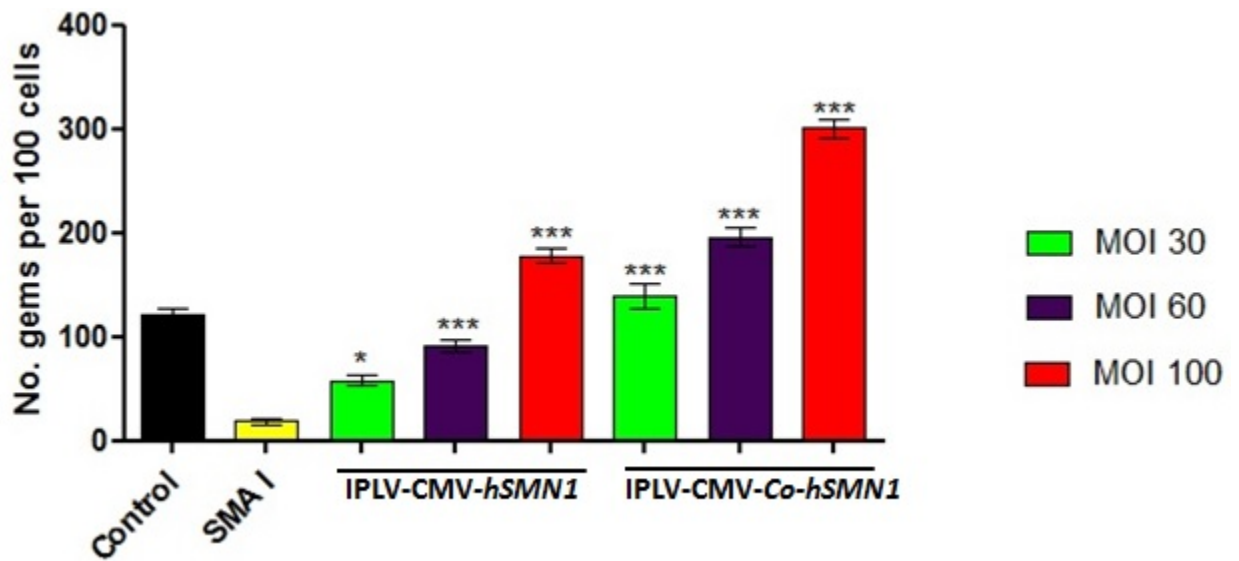
Figure 5.3: Quantification of the number of nuclear gems in human type I SMA fibroblasts transduced with IPLV-CMV-*hSMN1* or *Co-hSMN1*.

The graph plots the number of gems per 100 nuclei and indicates statistical significance above each column obtained by comparing individual transduced samples with non-transduced type I SMA fibroblasts. Significant differences were detected between all transduced samples and the untransduced sample. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, stars represent level of significance, n=3.
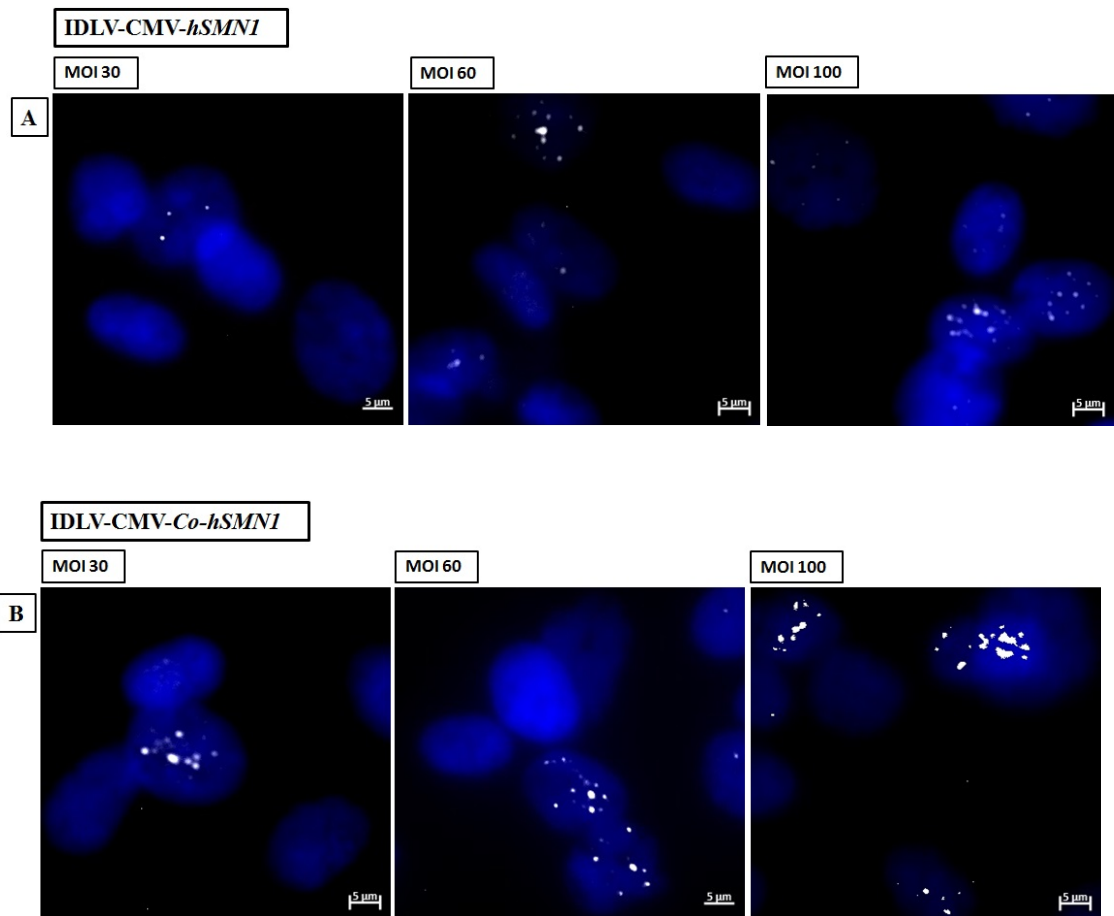
Figure 5.4: Increase in number of gems in transduced SMA type I fibroblasts using IDLV-CMV h*SMN1* or Co-h*SMN1*.

SMA I human fibroblast cultures were transduced with non-integrating lentiviral vectors encoded with either h*SMN*1or *Co*-h*SMN*1 one day after seeding the cells. Analysis of the samples was performed three days post- vector transduction by immunohistochemistry. **(A)** Representative images in this panel demonstrated an enhanced number of gems in transduced cells with IDLV-CMV-h*SMN*1 at qPCR MOI 30, 60 and 100. **(B)** Images in this panel are examples of increasing the number of gems in transduced cell with non-integrating lentiviral vectors, expressing *Co*-h*SMN1* driven by CMV promoter. Anti gemin2 antibody was detected in gems in transduced or non-transduced cells. Scale bars = 5 μm.
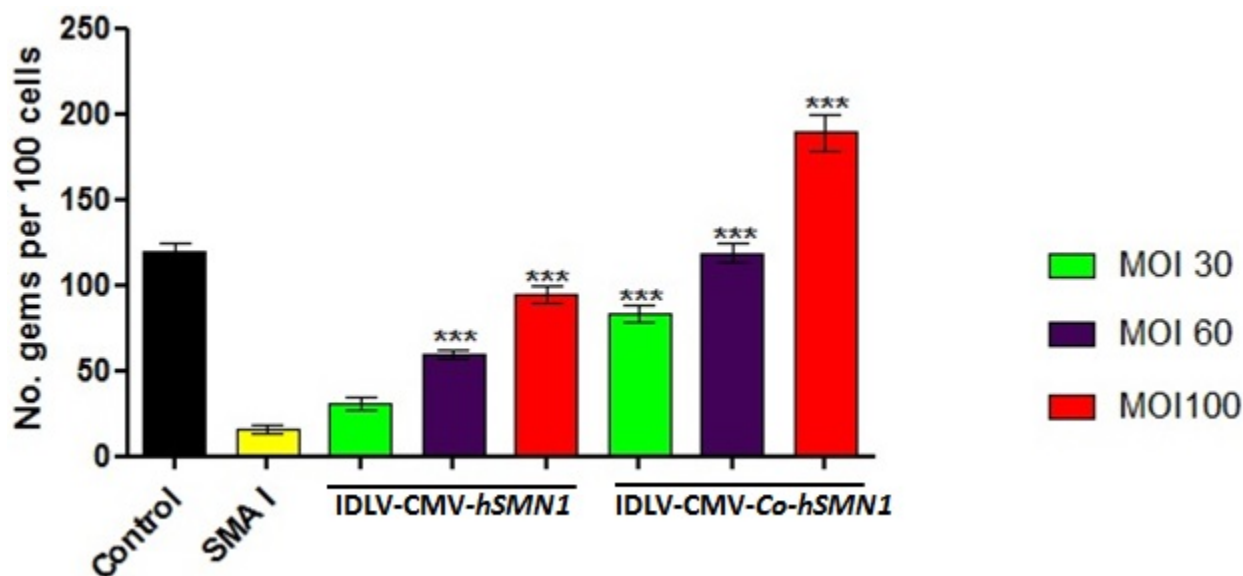
Figure 5.5: Quantification of gems in SMA type I fibroblasts after transduction with IDLVs expressing *hSMN1* or *Co-hSMN*1 under control of CMV promoter.

Nuclear gems were counted and plotted as the number of gems in 100 cells. Each treated set of data was compared to untreated SMA I using one-way ANOVA and Dunnett's post-hoc test. Significant differences were observed in all treated samples, except the samples that were treated with MOI 30 of IDLV-CMV-*hSMN1*. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.
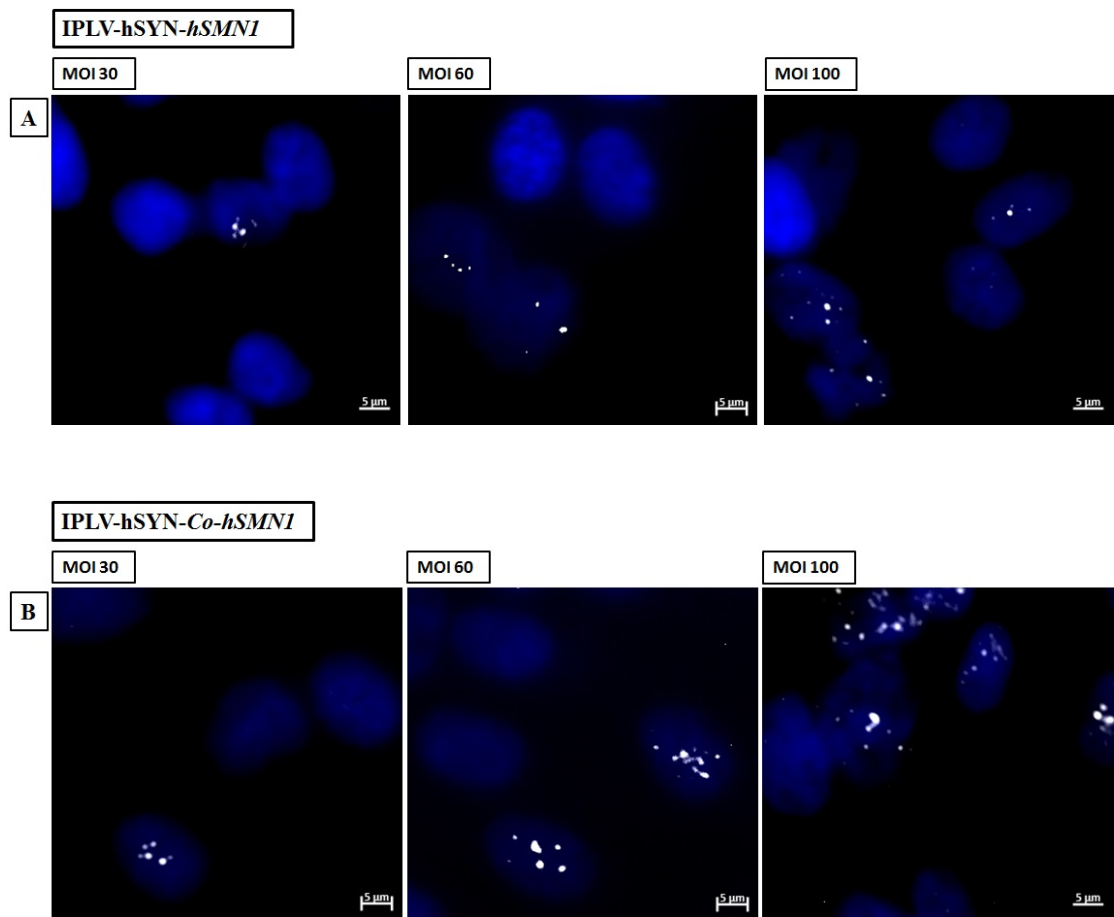
Figure 5.6: Increased number of nuclear gems in transduced type I SMA fibroblasts using IPLV- hSYN *hSMN1* or Co-h*SMN*1.

SMAI human fibroblast cultures were transduced one day after seeding the cells. (**A**) Different doses (qPCR MOI 30, 60 and 100) of integrating lentiviral vectors encoded with wild type h*SMN*1 gene under the control of hSYN promoter were used to transduce SMA I human fibroblast cells. (**B**) Here, cells were transduced with the same multiple dose of integrating lentiviral vectors, but the vectors expressed a codon optimised version of *hSMN*1 gene. 3 days after transducing, the SMAI human fibroblast cells were fixed using 4% PFA and then exposed to primary antibodies directed gems. Scale bars = 5 µm.
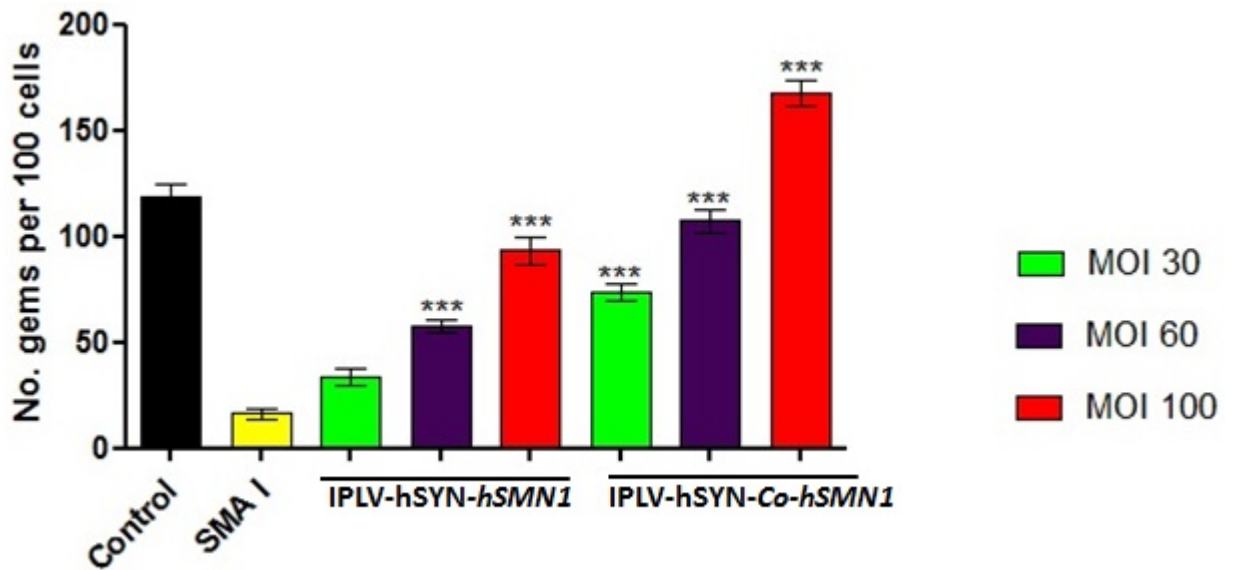
Figure 5.7: Analysis of gems in SMA fibroblasts treated with IPLVs expressing *hSMN1* or *Co-hSMN1* under the control of hSYN promoter.

SMA patient fibroblasts were transduced at different doses of IPLVs expressing either *hSMN1* or *Co-hSMN1* under the control of hSYN promoter. The graph above plots the number of gems per 100 nuclei, and a statistical analysis was performed between each individual treated and untreated SMAI to determine significant differences between them. The significant differences were observed in all treatments except the sample, which was transduced using MOI 30 of IPLV-hSYN-*hSMN1*. Data presented in this graph was statistically analysed by one-way ANOVA and Dunnett's post-hoc test. Values represent mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, stars represent level of significance, n=3.
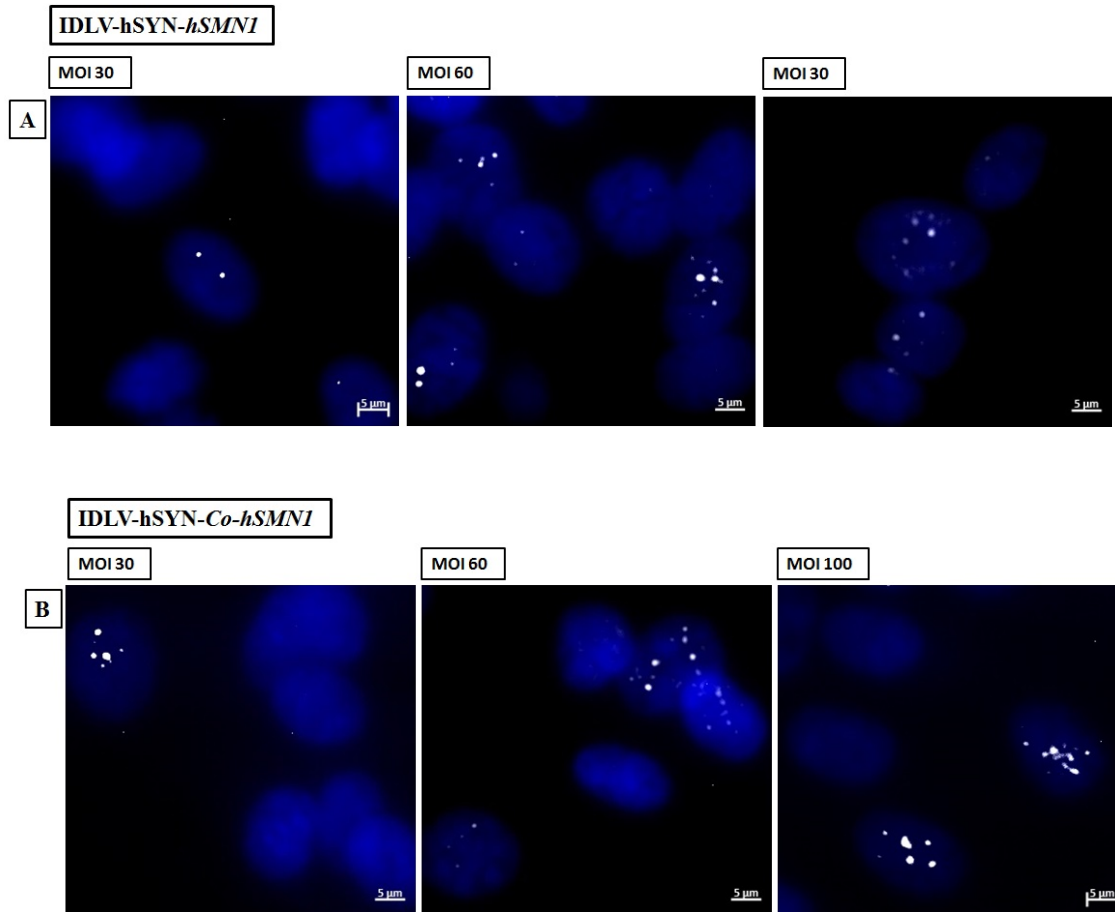
Figure 5.8: Immunofluorescence staining showing gem restoration in type I SMA fibroblasts transduced using IDLV-hSYN *hSMN1* or *Co-hSMN1*.

Lentiviral Vectors were added to the SMAI human fibroblast cultures twenty-four hours after cell seeding, at qPCR MOI 30, 60 or 100. (**A**) and (**B**) Representative photomicrographs are examples of increasing the number of gems in transduced cell by viral vectors. Images in panel (**A**) are representative of the cell culture that received different doses of IDLV-hSYN-*hSMN1*. Images in panel (**B**) show transduced cell cultures by IDLV-hSYN-*Co-hSMN1*. Scale bars = 5 µm.
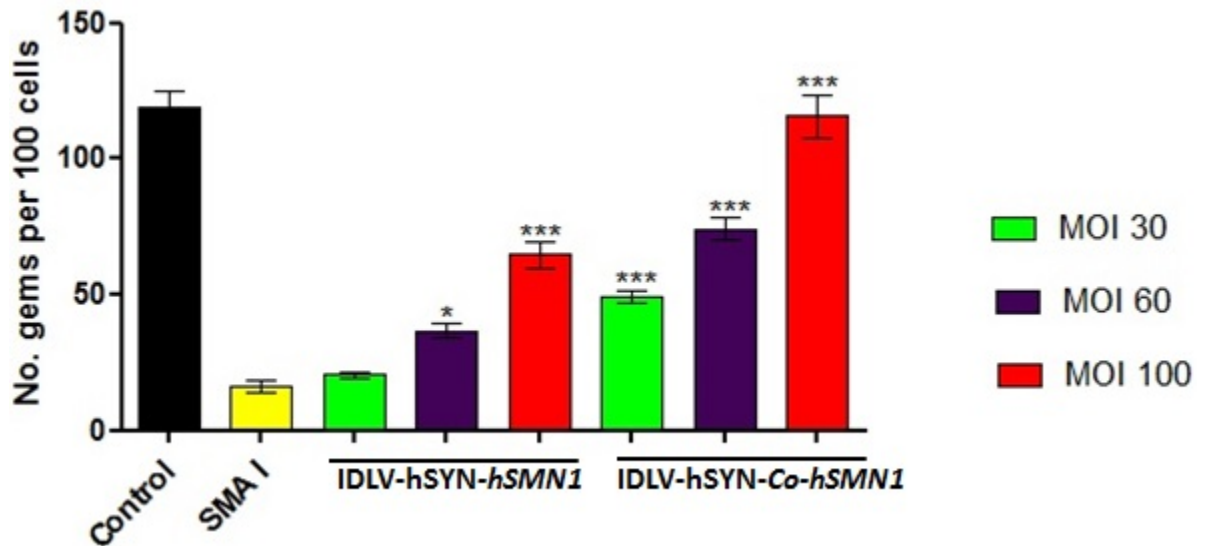
Figure 5.9: Quantification of the number of nuclear gems in human type I SMA fibroblasts transduced with IDLV-hSYN *hSMN1* or *Co-hSMN1*.

Cultures of SMA patient fibroblasts were transduced with IDLV- hSYN expressing either *hSMN1* or *Co-hSMN1* at different MOIs (30, 60 and 100). Nuclear gems were counted and plotted as the number of gems in 100 cells. One-way ANOVA and Dunnett's post-hoc test was run to determine whether there were any significant differences between transduced samples and untransduced SMAI. Significant differences were observed in each transduced sample, except transduced samples using MOI 30 IDLV-hSYN-*hSMN1*. Values represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, stars represent level of significance, n=3.

Table 5.1: Comparison of gem restoration by all vectors in type I SMA fibroblasts.

One-way ANOVA and Bonferroni's post-hoc test was used to determine significant differences between obtained statistics from transducing type I SMA patient fibroblasts. The analysed data presents the effect of different parameters such as lentiviral vector configurations, transgenes and promoters, on restoring gems. In addition, the data was analysed to determine whether there were dose-dependent increases among each group. Values represent mean $\pm$ SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, stars represent level of significance, ns stands for no significant difference, n=3.

| Transgene	Pomoter	Vector	Tansgene Promoter Vector / MOI	hSMN1												Co-hSMN1N											
				CMV						hSYN						CMV						hSYN					
				IPLV			IDLV			IPLV			IDLV			IPLV			IDLV			IPLV			IDLV		
			MOI	30	60	100	30	60	100	30	60	100	30	60	100	30	60	100	30	60	100	30	60	100	30	60	100
hSMN1	CVM	IPLV	30		*	***	*			ns						***											
			60			***		**			**						***										
			100						***			***					***										
		IDLV	30					**	***				ns					***									
			60						**					ns					***								
			100												*						***						
	hSYN	IPLV	30							*	***	ns										**					
			60								**		ns										***				
			100												*									***			
		IDLV	30											*	***										*		
			60												**											**	
			100																								***
Co-hSMN1	CVM	IPLV	30														*	***	***				***				
			60															***		***			***				
			100																		***			***			
		IDLV	30																*	***					**		
			60																	**						***	
			100																								***
	hSYN	IPLV	30																			*	***	ns			
			60																				***			**	
			100																					***			
		IDLV	30																						*	***	
			60																							**	
			100																								

Legend: 🟩 Dose-dependent increase   🟥 CMV VS hSYN   🟦 IPLV VS IDLV   🟪 *hSMN1* VS *Co-hSMN1*

5.5 Discussion:

Endogenous SMN proteins are present in both the nucleus and cytoplasm of normal cells. Full length SMN does not appear to exist in an isolated form within the cells, and it is always in interaction with its partner proteins (Kolb *et al.,* 2007). SMN protein is a component of a large multiprotein complex called a Gem within the nucleus. The gems are found in a size range of ~ 0.1–1μm and they reside in the nucleus of the cell, due to concentrating SMN complex. The SMN complex consists of the SMN protein and seven other proteins, designated as gemin2, gemin3, gemin4, gemin5 and gemin6, gemin7 and gemin8 (Carissimi *et al*., 2006). Forming SMN complex is essential for the biological activity of SMN protein and its interaction with other proteins (Liu & Dreyfuss, 1996 and Ogawa *et al*., 2007). The forming of SMN complex is indispensible for the role of SMN protein in the assembly of snRNPs, which constitute the building blocks of spliceosomes (Cauchi, 2014). There is still a lack of clear evidence about the exact function of gems, however, Coovert *et al*., 1997 have suggested that gems have a role in RNA metabolism. Great progress has been made toward determining the correlation between gems and SMA phenotypes. In addition, it is very well established that the number of gems in a cell nucleus are a faithful indicator of full length SMN protein and the severity of SMA disease (Coovert *et al*., 1997 and DiDonato *et al*., 2003).

Determining whether lentiviral vector transduced cells with encoding SMN protein can lead to the restoration of gems is an essential step in optimising and developing potential therapeutic vectors for SMA. Employing SMA human fibroblast to assess the functionality of SMN protein in response to restoring SMN protein is a very common and well established practice (Azzouz *et al*., 2004; Coovert *et al*., 1997; DiDonato *et al*., 2003; Nizzardo *et al*., 2015 and Valori *et al*., 2010). Immunofluorescence method

was utilised to label the gems in the nuclei of cells, as this method makes it possible to score the number of gems on a cell by cell basis, and focuses on the more stable gem associated pools of SMN protein. Other methods, such as western blot, are used to determine the total level of gems in a protein mixture (Cherry *et al*., 2013).

Prior to testing the functional efficiency of produced protein encoding by lentiviral vectors, the number of gems in all different types of fibroblast were examined. The results revealed that the lowest number of gems were detected in the most severe type of SMA (type I SMA). A higher number of gems was detected in SMA type II and III (Figure 5.1). In addition, the results demonstrated the level of SMN expression in each type of cell line correlates positively with the number of gems, while there is an inverse correlation between the number of gems and clinical severity of SMA disease. This result is supported by other studies (Coovert *et al*., 1997; DiDonato *et a*l., 2003 and Patrizi *et al*., 1999).

It has been established that transgenic production of SMN protein in patient fibroblasts leads to the restoration of nuclear gems and phenotypic rescue of this aspect of the SMA model. Moreover, ectopic expression of SMN also causes relocalisation of gemin2, an SMN-interacting protein, to gems (DiDonato *et al*., 2003). Therefore, in this study, antibody against SMN-reacting protein (gemin2) was used to detect the gems. The immunofluorescence results of the transduction of type I SMA fibroblasts using produced lentiviral vectors revealed that the delivery of *hSMN1* transgene into patient cells led to a restoration of the number of gems in all cases (Figure 5.2, 5.4, 5.6 and 5.8). Thus, the produced SMN protein is functional and is able to interact with its normal cellular binding patterns.

In a bid to maximize the restoration efficiency of nuclear gems and to investigate a MOI-dependent manner, the patient fibroblast cells were transduced with different

MOIs. This ascertained that more vectors could be transduced into cell cultures to produce more SMN protein, which led to restoring gems in the nucleus of transduced cells. Therefore, the results demonstrate that the number of gems was enhanced by increasing the MOI of transduction. A high number of gems was detected when cells were transduced at MOI 100 (Figure 5.3, 5.5, 5.7, 5.9 and Table 5.1), and this was achieved without causing any toxicity or changes in cell morphology due to overdose of the vectors.

It should be noted that, as previously stated, SMA I type fibroblast were chosen for transduction. In figures 5.3, 5.5, 5.7, 5.9 there are nuclei showing absence of gems, which most likely indicate these cells have not been transduced; the number of non-transduced cells was reduced by increasing MOI of transduction. Treating SMA fibroblast cells with MOI 30, 60 and 100 results in the transduction of approximately 20%, 40% and 80% of cells, respectively.

In addition to testing the functional effects of encoding SMN from produced lentiviral vectors on SMA fibroblasts, the effect of different parameters (type of vector, transgene and promoter) on gem restoration was also tested.

Both types of vectors (IPLVs (Figure 5.2 and 5.6) and IDLVs (Figure 5.4 and 5.8)) successfully restored gems in transduced cell nucleus. However, when comparing the number of detected gems, it was revealed that more gems were restored in cells when they were transduced using integration vectors (Table 5.1). The number of gems in IDLV transduced cell culture was not as high as the cell culture transduced with IPLV. Comparing the quantified number of gems in transduced cells using both type of vectors revealed that the number of gems in transduced cells using IPLV is 1.6 times greater than the number of gems detected in transduced cells using IDLV, regardless type of promoters and transgene.

There are two explanations for the lower number of gems in cells transduced using IDLV. The first is that circular vector episomes generated in transduced cells by non-integrated vectors do not have any origin of replication, so they are progressively diluted out through cell division in proliferating cells (Wanisch & Yáñez-Muñoz, 2009). Therefore, these lentiviral episomes are gradually reduced in number by every cell division (Nordin *et al*., 2014). The second reason is non-integrating vectors may have lower transcription activity than IPLV (Apolonia, 2009 and Sakuma *et al*., 2012). Following transduction of lentiviral vectors expressing either wild-type *hSMN1* or novel *Co-hSMN1* into type I SMA human fibroblasts, the results demonstrated that both types of transgene are efficient to restore gems and rescue this aspect of the SMA phenotype (Figure 5.2, 5.4, 5.6 and 5.8). DiDonato *et al*., 2003 believed the very high level of SMN protein leads to an increase in the number of gems. Comparison between wild-type *hSMN1* and *Co-hSMN1*, demonstrated that using novel *Co-hSMN1* led to the restoration of a significantly higher number of gems than wild-type *hSMN1* (Table 5.1). The quantification of detected gems demonstrated that using a codon optimised version of the *hSMN1* transgene resulted in the detection of 1.7 times more gems than wild type *hSMN1*.

This finding indicates that the level of produced full length SMN protein using *Co-hSMN1* is higher than wild-type. It is further confirmation of the results of the previous chapter, and is an expression pattern consistent with that previously found by (Valori *et al*., 2010). It is unsurprising that the high level of SMN protein production restored more gems in the nuclei of cells, as there is direct link between the expression of SMN protein and the formation of gems. The high level of endogenous SMN protein did not interfere with normal cell function, and had no effect on growth, morphology or death of cells.

Regarding the effect of different promoters on restoring gems through their control of expression of transgene in SMA human fibroblast, the transduction results demonstrated that CMV (Figure 5.2 and 5.6) and hSYN (Figure 5.4 and 5.8) are efficient promoters, and are able to restore gems in transduced fibroblast cells. The number of detected gems in transduced fibroblasts using CMV were significantly higher than hSYN (Table 5.1). Regardless of lentiviral vector configurations and type of *hSMN1* transgene, CMV promoter formed 1.8 times more gems, versus hSYN promoter.

This finding correlates with the results of the previous chapter. Chapter 4 demonstrated that CMV showed stronger expression and resulted in higher protein production, so this rationalises the use of CMV to detect more gems in transduced cells. In addition, this finding further supports evidence that CMV is a stronger promoter than hSYN promoter, as it restores more gems in transduced cells than hSYN.

Considered together, the results of this chapter demonstrate that lentiviral vector-mediated expression of h*SMN1*/ *Co*-h*SMN1* can lead to the formation of gem-like structures in the nucleus of type I SMA fibroblast. It can likely rescue the SMA phenotype, as there is a relationship between the number of gems and the severity of SMA disease. Therefore, produced endogenous SMN protein is functional, and can interact with its normal cellular binding partners. Moreover, the results demonstrated an increase in the number of detected gems following a dose-dependent manner in all cases. Another finding of this chapter was the direct correlation between the expression of SMN protein and the number of gems. A high level of SMN protein is required to restore gems. Therefore, any relevant factors for efficient SMN protein expression will also effect restoration of gems and the clinical severity of SMA disease.

Chapter 6   *In vivo*

6.1 Introduction

The *SMN1* gene produces the fully functional, full-length SMN protein that is essential for the survival of nearly all cell types. Genetic defects in the *SMN1* gene and resulting depletion of SMN protein cause SMA. Increasing the levels of the full-length SMN protein through the replacement of the *SMN1* gene or through the regulation of *SMN2* expression are two possible strategies for treating SMA disease. Recent evidence has demonstrated that SMA is a multi-system disorder. Liver, brain, blood vessels, heart, lung, bone, muscle, pancreas, motor neurons, and Schwann cells are organs and cells currently known to be affected by SMA (Hamilton & Gillingwater, 2013 and Hunter *et al.*, 2016). Motor neurons are predominantly affected by SMA, however, the reason for motor neurons' specific vulnerability to low levels of full length SMN protein is still unknown (Mulcahy *et al.*, 2014). Thus, motor neurons are the main target in the majority of therapeutic studies.

Lentiviral vectors are highly successful and efficient candidates for the delivery of the gene of interest for clinical application. Different studies have reported amelioration of several genetic diseases when lentiviral vectors were used to deliver the therapeutic genes (Philippe *et al.*, 2006 and Yáñez-Muñoz *et al.*, 2006). Transducing the spinal cord is a key factor to rescue the SMA phenotype, as motor neurons are most profoundly affected by SMA. To this end, one published study (Peluffo *et al.*, 2013) and one unpublished study from the Prof. Yáñez's lab have reported transduction of the spinal cord using lentiviral vectors. The unpublished study was carried out by Dr. Sherif Ahmed in Prof. Rafael J. Yáñez-Muñoz's lab, and is currently being prepared for publication. It reported the complete transduction of the spinal cord for the first time. In this study, E16 mouse embryos had an intraspinal injection of IDLV expressing a marker gene (*eGFP*). Bio-distribution of delivered *eGFP* was assessed by

immunostaining of either whole spinal cord, or different sections of the spinal cord, and the results demonstrated strong *eGFP* expression up to 7 months after *in utero* administration. Expression of *eGFP* was sufficiently strong for fluorescence to be visible to the naked eye in whole harvested cords.

Animal models of human disease can significantly clarify confusing issues of pathology and are useful tools for testing any potential treatments before applying them to human subjects. Mice have anatomical and physiological similarities to the human neuromuscular system, and it is fairly easy to manipulate the mouse genome, so these features make the mouse a great candidate to design an animal model for SMA (Sleigh *et al*., 2011). Mice have a single *Smn* gene, which is 82% identical to *SMN1* at the amino-acid level. In addition, mouse and human *SMN* genes show a similar expression pattern (DiDonato *et al*., 1997). One of the most commonly used SMA animal models are so-called Taiwanese SMA mice. This model is very well established and is used by other researchers for preclinical studies (Glascock *et al*., 2012; Hunter *et al*., 2016; Lin *et al*., 2016; Passini *et al*., 2010; Powis *et al*., 2016; Tsai *et al*., 2008 and Valori *et al*., 2010). The Taiwanese-SMA mouse model was introduced in 2000 by Hsieh-Li *et al*. Each Taiwanese-SMA mouse carries two *SMN2* copies per allele on *Smn* null background ($Smn^{-/-} SMN^{tg/tg}$) and pure FVB/N background.

In the previous chapters, it was demonstrated that generated vectors are infectious and the transgene is efficiently expressed in the transduced, targeted cells. Moreover, the SMN protein produced from lentiviral vectors is fully functional and can restore gems in SMA type I fibroblasts. Thus, testing the developed lentiviral vectors *in vivo* was the natural extension to this work.

6.2 Aims of the chapter

The experiments in this chapter were performed:

(A) To establish the *in utero* delivery technology in the host laboratory

(B) To study the transduction efficiency of generated vectors *in vivo* after intrauterine injection.

(C) To perform a very preliminary investigation testing whether *in vivo* administration of viral vectors expressing either *hSMN1* or *Co-hSMN1* into the foetuses of an SMA mouse model could prevent or delay the disease onset.

In utero SMN1 gene therapy work was carried out in Professor Thomas Gillingwater's lab at the University of Edinburgh, in a collaboration with *in utero* expert Dr. Simon Waddington (University College London). This was the initial experiment of a long-term collaboration, undertaken to: (i) provide initial training in *in utero* technology and behavioural studies for me and several Edinburgh colleagues; (b) perform an initial comparison of previously produced AAV vectors and the lentiviral vectors I manufactured; (c) compare the new transduction results with those previously obtained by former PhD student, now Dr. Sherif Ahmed, using *eGFP* lentivectors. The results that I present in this chapter are therefore the results of a pilot study, which did not allow for complete optimisation of the procedure. I would like to stress that this pilot experiment was carried out in close collaboration with several members of Prof. Thomas Gillingwater group, namely Dr. Ewout Groen, Dr. Rachael Powis and Miss Hannah Shorrock, who carried out a significant part of and taught me animal handling, behavioural studies and immunostaining of *in vivo* samples.

6.3 Summary of experiment and method

Following the testing of produced lentiviral vectors in different *in vitro* models, a very brief pilot study was performed to assess the efficiency of delivery systems and delivered transgenes to rescue SMA pups or improve the SMA phenotype.

The breeding strategy utilised in this study was developed by (Riessland *et al.*, 2010). In this strategy, Taiwanese-SMA mouse model (Li *et al.*, 2000), a homozygous *SMN2* transgenic *Smn* knockout mouse ($Smn^{-/-}$; $SMN2^{tg/tg}$) was crossbred with a heterozygous *Smn* knockout mouse ($Smn^{+/-}$). 50% of the offspring were homozygous knockout for endogenous *Smn* ($Smn^{-/-}$; $SMN2^{tg/-}$) and the other 50% were heterozygous for *Smn* knockout ($Smn^{+/-}$; $SMN2^{tg/-}$). The $Smn^{-/-}$; $SMN2^{tg/-}$ littermates were used as the SMA model in this study. These animals carry two copies of *SMN2* gene on one allele on a null murine *Smn* background. Mice with a $Smn^{+/-}$; $SMN2^{tg/-}$ phenotype were used as a control group: they have a copy of *mSmn* and two copies of *SMN2*. All animal procedures were performed under appropriate licences from the UK Home Office and were approved by an internal ethical review committee at the University of Edinburgh. Either 2 μL of lentiviral vectors (intraspinally) or 10-20 μL of adeno associated virus (intraperitoneally) were injected into E14-16 mouse embryos using a 33G needle and Hamilton syringe (for more details please refer to 2.6.4). A list of candidate vectors for the *in vivo* experiment is provided on the table below.

Table 6.1: The list of used viral vectors for *in vivo* experiment.

Viral vectors	qPCR titre TU/mL	Source
IPLV-CMV- *Co-hSMN1*	3.20E+09	These vectors were developed during this study.
IDLV-CMV- *Co-hSMN1*	1.0E+09	
IDLV-CMV- *eGFP*	3.0E+09	
AAV9-CAG-*hSMN1*	7.61E+9	These two vectors were produced by Dr. María Gabriela Boza, former PhD student in Prof. Rafael J. Yáñez-Muñoz's laboratory.
AAV9-CAG- *eGFP*	6.42E+10	
AAV9-CMV- *eGFP*	1.0E+13	This vector was provided by Prof. Tom Gillingwater's group.

To investigate the therapeutic effect of the delivered transgene on injected SMA pups, a set of measurements was performed daily to closely monitor therapeutic improvement. Pups were weighed daily and Kaplan-Meier survival analyses carried out as previously described (2.6.5.2). Righting reflex tests were performed to assess changes in neuromuscular function (for more details please refer to 2.6.5.1).

Two weeks following vector transduction, the whole spinal cord, and the liver, heart and muscle (Gastrocnemius) were collected from mice killed by an intraperitoneal injection of sodium pentobarbital. Sequentially, one half of each tissue was fixed in 4% PFA and underwent immunohistochemical staining (for more details please refer to 2.6.6 and 2.6.7) and the other half was stored in a -80°C freezer for future testing. Proteins from frozen tissues were extracted in RIPA buffer with 10% protease inhibitor cocktail for quantitative western blotting (for more details please refer to 2.6.6 and 2.6.8).

6.4 Results

6.4.1 Assessment of SMA rescue mice

In this part of the study, a group of E14-16 mouse embryos was injected with either IPLV-CMV-*Co-hSMN1*, IDLV-CMV-*Co-hSMN1* or IDLV-CMV-*eGFP* vectors via the intraspinal route. Another group of mouse embryos received intraperitoneal injections of AVV9-CAG-*eGFP* or AVV9-CAG-*hSMN1*.

To examine potential physiological changes in SMA mice, each mouse pup was inspected daily and a record of animal weight and survival was made from the day of birth (P1) until the ending point of the experiment (two weeks) for further analysis.

A common behaviour test to investigate therapeutic effects on neuromuscular function is the righting reflex test. This test was carried out from the day after birth (P2) and was performed every day by placing each pup in a supine position and measuring the time it took them to turn over and stably place all four paws on a flat surface (cut off time of 60s). The tester was blinded to the genotype of each mouse pup and type of injection. All analyses were conducted during the light period between 09:00 and 11:00.

The body weight in SMN- or GFP-treated animals was compared to control animals (Figure 6.1A, 6.2A and 6.3A). The result of this comparison did not show an increase in body weight or an improvement in weight gain patterns of animals treated with SMN, versus those animals treated with GFP. The survival rate of SMN-treated SMA animals was compared with GFP- treated SMA animals and the control group (Figure 6.1B, 6.2B and 6.3B)). The result of this comparison demonstrated that survival in SMN–treated animals was not greater than survival in the untreated animals. Furthermore, analysis of the righting ability of animals revealed that SMN/ GFP- treated SMA animals cannot right themselves as quickly as control group animals

(Figure 6.1C, 6.2C and 6.3C)). Moreover, righting ability in SMN-treated SMA animals did not improve over GFP-treated SMA animals.
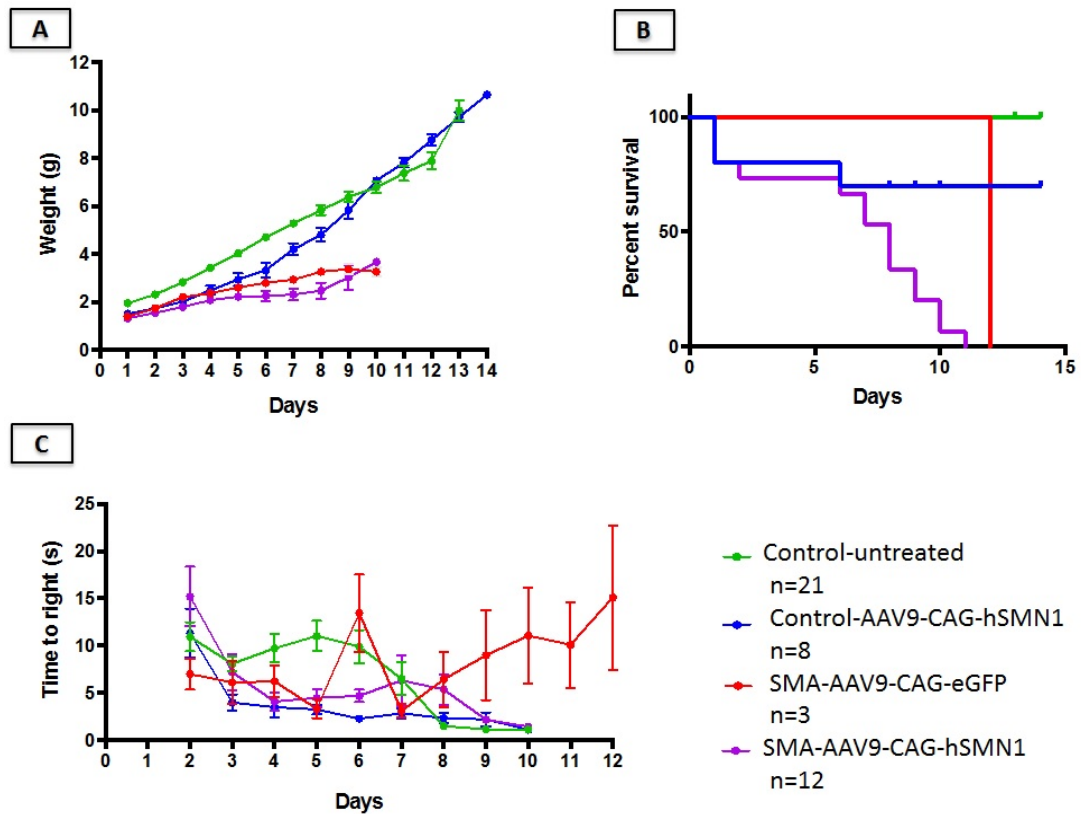
Figure 6.1: Effect of a single intraperitoneal injection of AAV expressing either *hSMN1/eGFP* on life span, body weight and righting reflex in SMA mice model.

E14-16 SMA mice embryos were injected with AAV9-CAG-*hSMN1/eGFP* via intraperitoneal route. Analysis of body mass, survival and righting reflex assay was performed to assess the therapeutic effect of injection.

(**A**) Growth curve demonstrated control pups have a continuous growth curve while treated SMA mice do not continue to gain body mass.

(**B**) Kaplan Meier analysis reveals that lifespan did not improve in treated-SMA animals.

(**C**) Righting ability shows that injected SMA-pups cannot right themselves similarly to wild type animals (control). SMA-treated animals did not show any therapeutic effects.
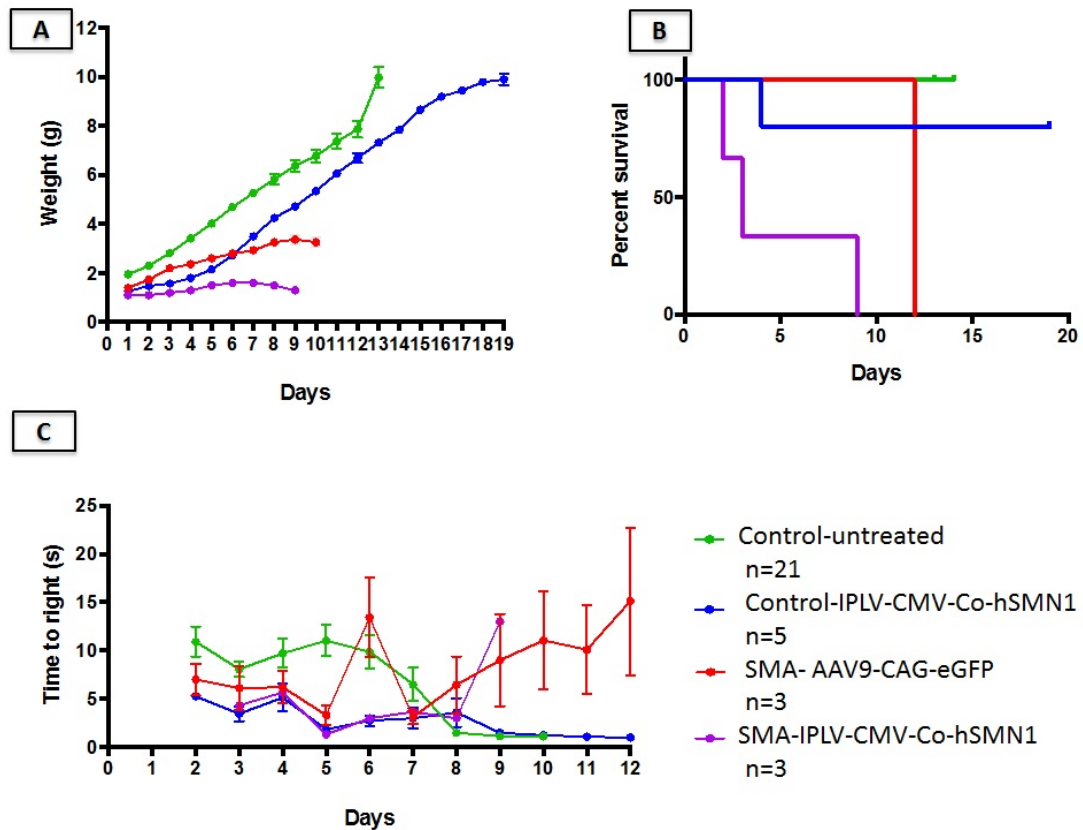
Figure 6.2: Effect of a single intraspinal injection of IPLV-*Co-hSMN1* on life span, body weight and righting reflex in SMA mouse model.

In order to assess the therapeutic effect of novel codon-optimised *hSMN1* transgene, mice were injected *in utero* with IPLVs carrying *Co-hSMN1* via intraspinal route. To determine a potential change in phenotype of these mice, different analyses were performed on wild type animal (control) and SMA animal.

(A) No increase in body weight was observed in SMA-mice treated with therapeutic integrating vectors.

(B) IPLV-*Co-hSMN1* delivery in SMA animals does not increase life span for these animals.

(C) In agreement with two other tests, SMN-treatment did not improve the performance of mice on the righting test. The survival, pattern of weight gain and righting ability in the SMA- animal group that received therapeutic transgene was similar to the SMA pups with no therapeutic injection (SMA-AAV9-CAG-*eGFP*).
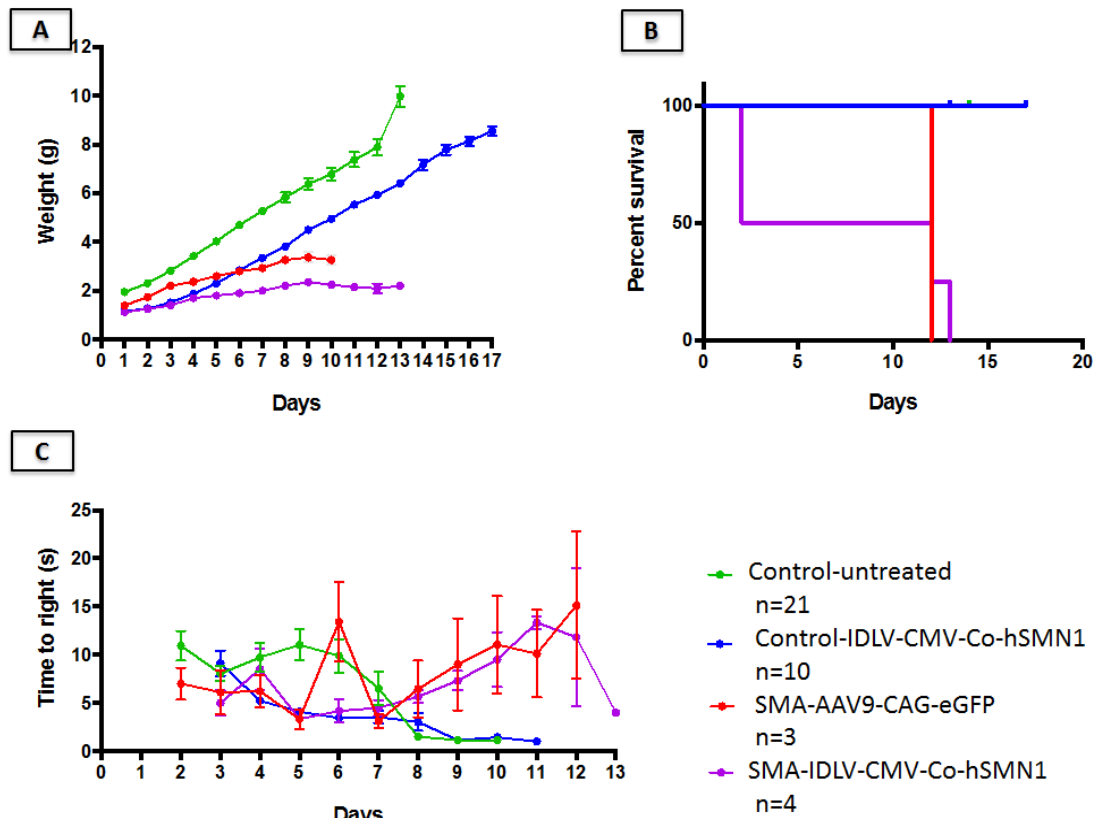
Figure 6.3: Effect of a single intraspinal injection of IDLV-*Co-hSMN1* on life span, body weight and righting reflex in SMA mice model.

Daily inspection of the weight, survival and righting ability of animals that had an *in utero* injection of IDLV-CMV-*Co-hSMN1* determined the existence and extent of any therapeutic benefits. The collected data was plotted for analysis, and the results were compared between the different groups.

(A) Weight curves of the four groups of mice highlight the weight gain pattern of SMA-AAV9-CAG-*hSMN1*, which follows the same pattern as the SMA group that was not injected with therapeutic vector.

(B) A comparison of lifespans determined that *in utero* injections of therapeutic vector (AAV9-CAG-*hSMN1*) did not extend the lifespan of SMA animals.

(C) Righting time was assessed by measuring the required time for a pup to turn over after being placed on its back. Therapeutic injection did not show any improvement in the righting ability of SMA- animals.

6.4.2 Gene expression pattern

Unfortunately, there was no sign of therapeutic effect on treated mice, or even a trend to improvement in SMA phenotype. Therefore, the mice were killed at the clinical end point when they reached 20% weight loss or they showed a clear downward trend. Heart, liver, muscle (Gastrocnemius) and spinal cord of the animals were harvested to investigate whether there was any transgenic expression from delivered vectors. The bio-distribution of the transgene product was studied using immunostaining or western blotting. A full description of tissue harvesting, immunostaining or western blotting procedure was provided in section 2.6.6, 2.6.7 and 2.6.8 respectively.

Figure 6.4 and 6.5 show the detection of SMN protein in the spinal cord and muscle of both SMA and control group animals that had been injected with IPLV/ IDLV-*Co-hSMN1* or AAV9-*eGFP*, respectively. The corresponding Western blot results (Figure 6.6 and 6.7) detected SMN protein in animals that had been treated with AAV9- *SMN/ eGFP*. SMN protein was detected in all samples, however, quantification of western blot results demonstrated that there was no noticeable difference of SMN levels between SMA animals regardless of the vector received; the same was true for control animals. Figure 6.8 presents *eGFP* expression in the spinal cord and muscles of the control group and SMA animals that had been injected with AAV9-eGFP. *eGFP* expression was detected only in muscle tissue.

Immunostaining (Figure 6.9) was performed to study the presence of eGFP and SMN in the spinal cord, heart and liver of SMA animals. To test *eGFP* expression, the animals were injected with either AAV9-CAG-*eGFP* or AAV9-CMV-*eGFP*. The expression of *eGFP* was only detected in the heart and liver of SMA animals that had been treated with AAV9-CMV-*eGFP*. *SMN* expression was observed in the spinal cord, heart and liver of SMA animals that had been treated with AAV9-CMV-*hSMN1*.

eGFP expression was also observed in the transduced spinal cord of SMA animals that had been injected with IDLV-CMV-*eGFP* (Figure 6.10). *SMN* expression was detected in the spinal cord of animals that had been injected with IPLV/IDLV-CMV-*Co-hSMN1* (Figure 6.10).
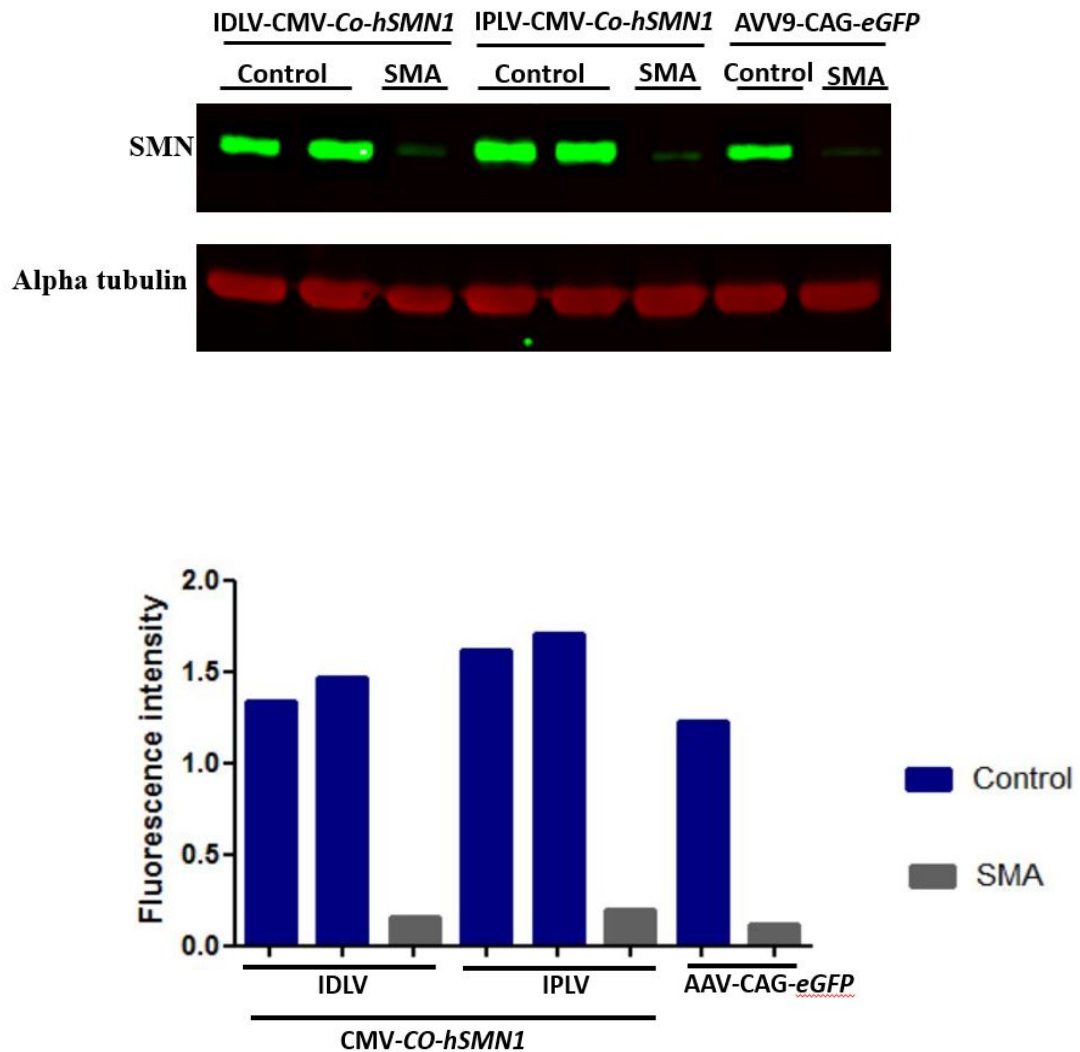
Figure 6.4: Transgene expression in the spinal cord following *in vivo* viral vector administration.

Western blot analysis showing the presence and quantification of SMN protein levels in spinal cord of injected wildtype control and SMA mice killed two weeks after birth. These animals had an *in utero* injection of IPLV/ IDLV- CMV-*Co-hSMN1* (intraspinal injection) or of AAV9-CAG-*eGFP* (intraperitoneal injection). Spinal cord tissue proteins were solubilised and run on a 12% polyacrylamide gel and blotted. The blots were then washed and probed with anti SMN antibody to detect SMN protein (~ 35 kDa) and α-tubulin antibody to detect α tubulin protein (~ 55 kDa). The α tubulin protein was used as loading control. The animals injected with AAV-CAG-*eGFP* were used as a control and displayed the expected level of SMN protein in wild type and SMA mice.
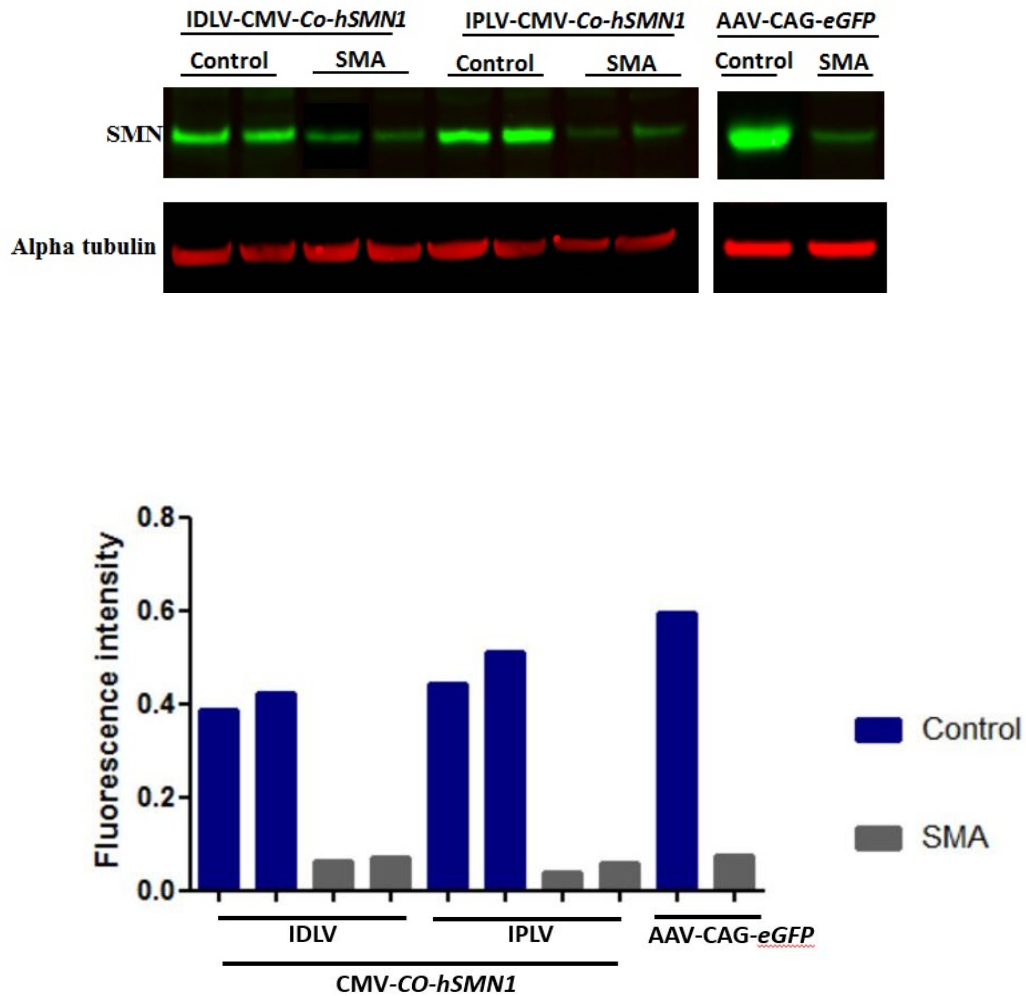
Figure 6.5: SMN expression in muscle following *in vivo* viral vector administration.

SMN expression in the muscle of two week-old CD1 mice. These animals received an *in utero* injection of either IPLV/ IDLV- CMV-*Co-hSMN1* (intraspinal injection) or of AAV9-CAG-*eGFP* (intraperitoneal injection). Muscle tissue proteins were solubilised and run on a 12% polyacrylamide gel and blotted. The nitrocellulose-membrane was incubated with anti SMN antibody to detect SMN protein (~ 35 kDa) and α-tubulin antibody to detect α tubulin protein (~ 55 kDa). Animals injected with nontherapeutic vectors (AAV9-CAG-*eGFP*) were used as control and displayed the expected SMN expression pattern for wild type and SMA mice.
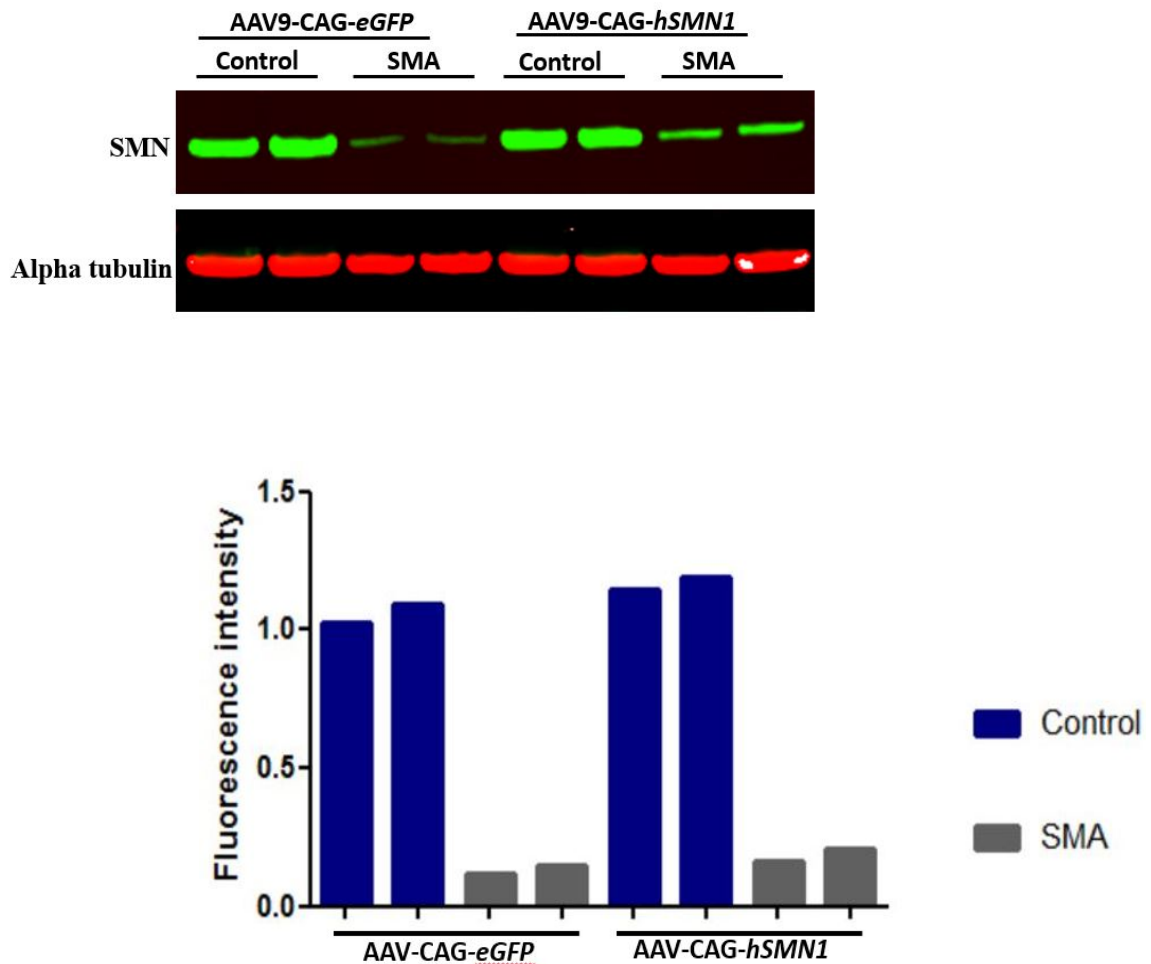
Figure 6.6: Identification of *SMN* expressing in spinal cord tissue by western blotting.

This figure shows representative western blotting from the spinal cord tissues of two-week-old mice. Control and SMA mice received an *in utero* injection of AAV9-CAG expressing either wild type *hSNM1* transgene or *eGFP* via intraperitoneal route. Protein lysates of harvested spinal cord tissues were resolved by 12% SDS-PAGE. The nitrocellulose-membrane was incubated sequentially with anti SMN antibody to detect SMN protein (~ 35 kDa) and α-tubulin antibody to detect α tubulin protein (~ 55 kDa). The animals injected with AAV-CAG-*eGFP* acted as a control and displayed the expected level of SMN protein in wild type and SMA mice.
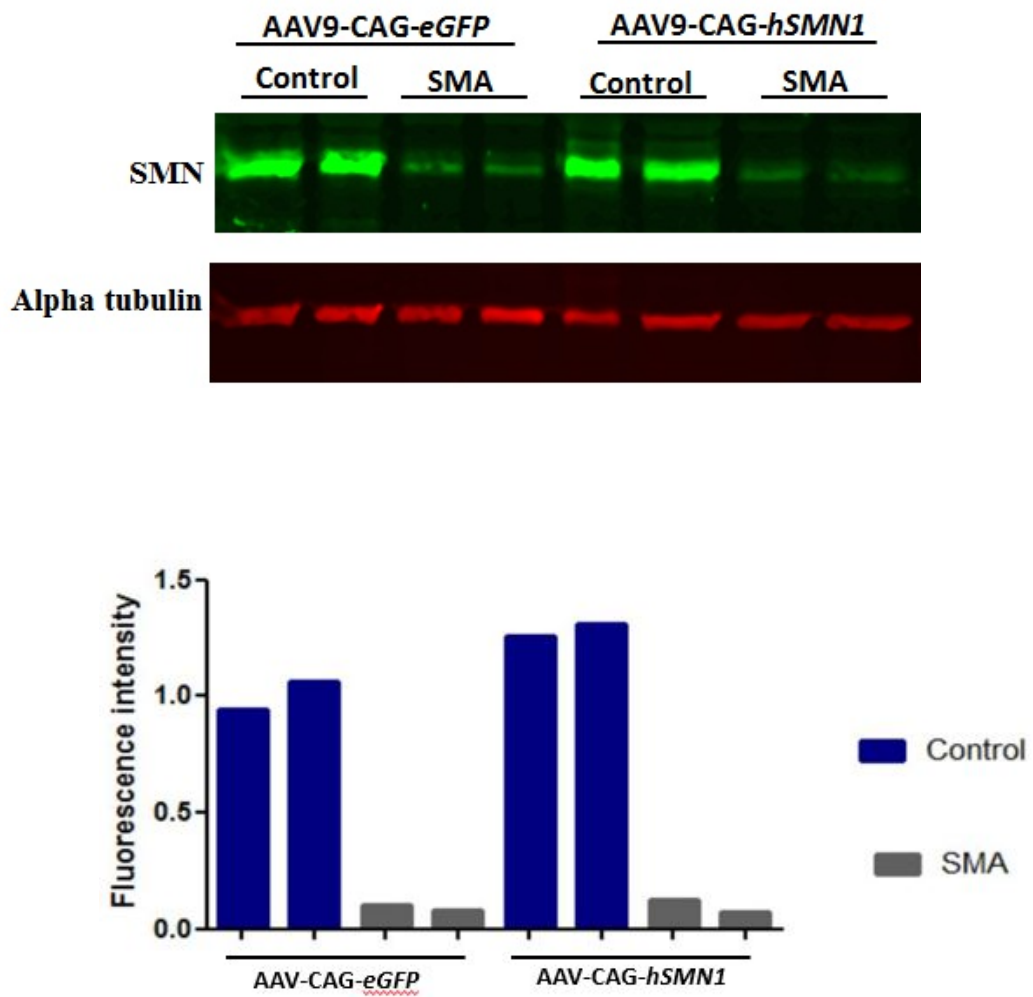
Figure 6.7: Western blot quantifying *SMN* expression from mice muscle.

Western blot analysis demonstrating the presence and quantification of SMN protein levels in muscles of injected wildtype (control) and SMA mice killed two weeks after birth. These animals had an *in utero* intraperitoneal injection of AAV9-CAG-*eGFP*/ *hSMN1*. Muscle tissue proteins were solubilised and run on a 12% polyacrylamide gel and blotted. The blots were then washed and probed with anti SMN antibody to detect SMN protein (~ 35 kDa) and α-tubulin antibody to detect α tubulin protein (~ 55 kDa). The α tubulin protein was used as a loading control. The animals injected with AAV-CAG-*eGFP* were used as a control and displayed the expected level of SMN protein in wild type and SMA mice.
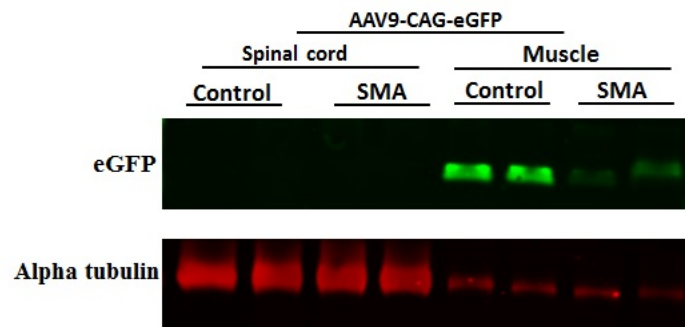
Figure 6.8: Western blot quantifying eGFP production from muscle and spinal cord.

Bio-distribution of *eGFP* expression in mice following an intraperitoneal injection of AAV9-CAG-*eGFP* at embryonic stages (E14-16). Western blot analysis showing the presence of eGFP protein in muscle and spinal cord of mice culled two weeks after birth. Proteins extracted from the harvested tissues were used to verify *eGFP* expression. 30 µg of extracted protein was loaded into each well. The membrane was incubated sequentially with the appropriate primary and secondary antibodies.
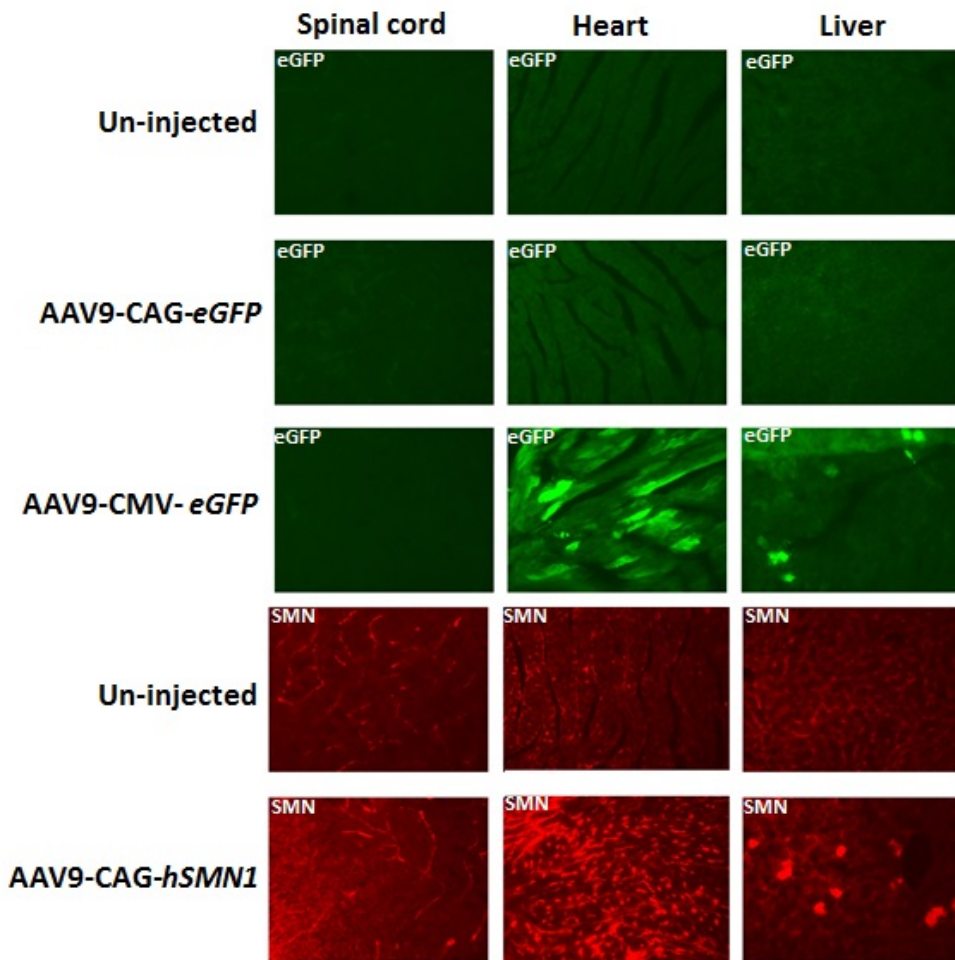
Figure 6.9: Detection of eGFP and SMN in lumbar spinal cord, heart and liver tissue in SMA mice following *in vivo* AAV9 administration.

Tissues from vector-recipient foetuses were harvested when animals were 14 days old. These animals received the viral vectors using *in utero* intraperitoneal injection. This figure shows the schematic representation of *eGFP* or *SMN* expression in the lumbar spinal cord, heart and liver of SMA mice. Tissue sections were stained by immunohistochemistry with antibodies against eGFP or SMN. Scale bars = 50 μm.
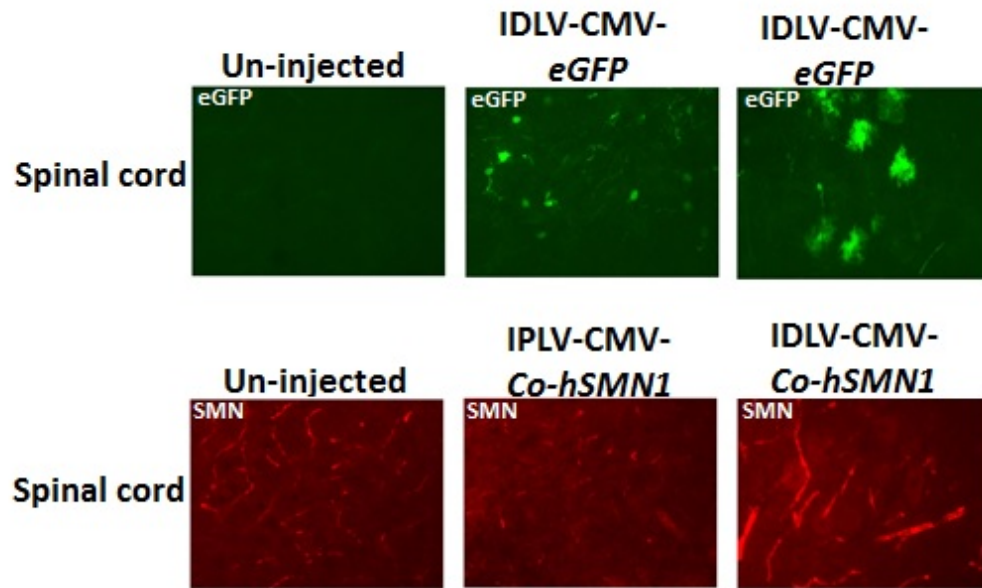
Figure 6.10: Lentiviral vector mediated *eGFP/Co-hSMN1* expression in mice lumbar spinal cord.

Harvested lumbar spinal cord from two-week old SMA mice were treated for eGFP or SMN immunofluorescence. These animals received the viral vector at embryonic stage via *in utero* intraspinal injection. Scale bars = 50 μm

6.5 Discussion

The majority of gene addition approaches published in the SMA therapy field rely on the use of AAV vectors to deliver *SMN1* gene to SMA animal models (Dominguez *et al*., 2011; Foust *et al*., 2010; Meyer *et al*., 2015 and Valori *et al*., 2010). The AAVs demonstrate a high potential in the gene therapy field, and they have been used intensively for pre-clinical research. One challenge of using AAV is pre-existing immunity against this viral vector. Manno *et al*., 2006 and Mingozzi *et al*., 2007 are two studies that reported an immune response against the AAV in humans. The only experiment that used lentiviral vectors to deliver the *SMN1* gene to an SMA mouse model was carried out by Azzouz and his collegues in 2004. Azzouz's study was a postnatal study, and the animals received the vectors using intramuscular injections.

The time of injection is a crucial factor, and is one of the limitations of current SMA strategies in achieving an effective treatment for SMA. Foust *et al*., 2010 demonstrated that intravenous administration of 5×10^{11} genomes of scAAV9-SMN on the first postnatal day led to partial amelioration of SMA symptoms. Therefore, there could be advantages to using prenatal gene therapy strategies to overcome limitations of ameliorated disease-related phenotypes in postnatal and current SMA therapies. Moreover, prenatal gene therapy has many benefits over postnatal delivery, such as the prevention of persistent and adequate transgene expression, pre-emption of the immune response, avoidance of the onset of disease and immune tolerance to the transgenic product (Waddington *et al*., 2005). Phenotypic correction of a number of genetic diseases using *in utero* gene delivery strategy has been reported (Dejneka *et al*., 2004b; Rucker *et al*., 2004; Seppen *et al*., 2003 and Waddington *et al*., 2004). Therefore, this brief *in vivo* experiment was designed to use *in utero* injection as an attractive technique to deliver the vectors at early stage of life.

Dr. Sherif Ahmed carried out a previous, related experiment in Dr. Yáñez's laboratory. In this study, Dr. Sherif Ahmed aimed to optimise the route of injection for prenatal delivery and he compared scAAV9 and IDLVs in terms of transduction efficiency of motor neurons. His result showed that out of the three different investigated routes, which were intraspinal, intracranial, and intravascular, intraspinal injection demonstrated robust e*GFP* expression along the entire spinal cord. In addition, IDLVs vectors were documented to be more efficient viral vectors over scAAV9 for transducing spinal cord motor neurons.

Further discussion with Dr. Simon Waddington, suggests that intraperitoneal injection of lentiviral vectors does not lead to transduction of motor neuron cells, which are the cells most profoundly affected by SMA disease. However, intravenous administration of lentiviral vector pseudotyped with rabies envelope enables transgene delivery to neurons. Intraperitoneal administration of AAV9 can efficiently deliver the gene of interest into the central nervous system, and the peripheral nervous system, possibly by transcytosis across the endothelium and astrocytic end-foot processes (personal communication from Dr Simon Waddington). Other studies demonstrate that intravenous delivery of AAV9 vectors mediates effective gene expression in the brain and spinal cord (Duque *et al*., 2009 and Foust *et al*., 2009).

The lentiviral vectors that were used in the previous prenatal experiment expressed *eGFP* as a transgene. Therefore, in order to produce a potential therapeutic vector for SMA (the main aim of this study), it was indispensable to change reporter transgenes (*eGFP*) into the clinically relevant *hSMN1* transgene. A large variety of lentiviral vectors were developed and tested in different *in vitro* models during this study. The outcomes of *in vitro* experiments revealed that produced vectors are able to transduce growth arrested CHO cells, primary cortical and motor neurons and transduced cells

produced full length SMN protein. Moreover, the SMN protein produced was fully functional and, as it could restore gems in SMA fibroblasts, was able to bind with its binding partners. Thus, this research was then extended by testing the produced vectors in a very brief pilot study of gene therapy in SMA mouse model.

The most efficient lentiviral vectors based on the result of *in vitro* experiment (IPLV/IDLV-CMV-*Co-hSMN1*) were tested in this pilot *in vivo* experiment. The AAV-CAG-*hSMN1* was included in this experiment in order to compare the efficiency of AAV and lentiviral vectors in the rescue of SMA. In addition, two different vectors (IDLV/AAV-*eGFP*) carrying *eGFP* were used in this experiment as control vectors. The vector of interest was injected into mouse embryos (E14-16), and the injected animals were monitored over time and a record of survival and weight progression of each animal was made in order to investigate the improvement in SMA phenotype. Righting reflex was also performed to assess improvement of neuromuscular function.

There was no improvement in survival time, body weight or motor movements of injected Taiwanese SMA mice, regardless of the vector used. The results of assessment of treated SMA mice indicated that the vectors delivered failed to ameliorate the phenotype in the SMA mice model, although based on other publications, it was expected that the delivered transgene would improve the SMA phenotype in injected animals (Dominguez *et al*., 2011; Foust *et al*., 2010; Meyer *et al*., 2015 and Valori *et al*., 2010). Therefore, additional tests were carried out to investigate the expression pattern of the delivered transgenes. A very limited amount of harvested tissue was analysed using immunostaining and western blotting.

The expression pattern was investigated in animals injected with AAV-CMV-*eGFP* and the result demonstrated the detection of *eGFP* expression only in muscle, heart and liver. No eGFP was observed in spinal cord, although other studies reported the

intensive expression of *eGFP* in different organs such as the spinal cord, eye, liver, kidney and heart of animals that had an intravenous injection of AAV9 carrying the reporter gene (*eGFP*) (Mattar *et al*., 2015 and Rahim *et al*., 2011).

Immunostaining of the spinal cord apparently showed some over-expression of *SMN*, however it is unclear in these images whether the signal is due to a high level of blood vessel autofluorescence. Detection of SMN was investigated by western blotting and the result demonstrated a very small increase in the level of SMN protein production in some samples over control samples. However, based on the results of *in vitro* experiments performed during this study and other publications, it would have been expected to achieve a significant increase in the level of SMN protein production in the injected animal.

Despite the efforts taken to investigate the reason for experimental failure, it is not clear what caused the failure of this pilot, *in vivo* experiment. Of note, the outcomes of this pilot study do not reflect the results of previous experiments conducted in our laboratory by Dr. Sherif Ahmed, as the procedure was not fully optimised in the current experiments. However, the experiment was successful in terms of establishing the technology, lack of treatment-related adverse events in the injected animals and determining further optimisation and training requirement to carry out a fully optimised experiment, to investigate the therapeutic effect of the viral vectors on SMA mice.

A number of suggestions were made as to how this *in vivo* work could be used for further work, in order to optimise the experiment. The volume of viral vectors that can be injected into the spinal cord of the foetuses using intraspinal injection is small (2 µL), so having high titre vector stock should be beneficial, as more viral vector

particles carrying the gene of interest can be transferred to the host. Moreover, a conclusion on how forthcoming *in utero* experiments need to be carried out has been made with our collaborators, Prof. Gillingwater and Dr. Waddington. The agreement is to develop a standard vector, which is designed to express *Co-hSMN1* under the control of a hybrid CMV enhancer/chicken-β-actin promoter (CBA). This vector would be comparable to the AAV vectors that are used in the latest gene transfer clinical trial for SMA (Clinicaltrials.gov identifier NCT02122952). The production of a standard vector (AAV9-CBA-*Co-hSMN1*) and a control vector (AAV9-CBA-*eGFP*) has been carried out by Atlantic Gene Therapies and the vectors will be used in the near future.

Chapter 7   Final discussion and Conclusions

7.1 Discussion

Gene therapy is a technology that allows the modification of gene expression patterns for therapeutic purposes. One possible strategy is so-called gene addition, which involves the introduction of transgenes for therapeutic purposes. Gene therapy has considerable potential when treating diseases that are traceable to a single defective gene. The theoretical basis for gene addition therapy is that a normal, healthy copy of the defective gene transferred into the appropriate cells of the host should be therapeutic in the case of recessive disorders. Therefore, the efficient delivery of therapeutic genes and appropriate gene expression are critical requirements for the development of an effective treatment base of gene therapy (Campbell *et al*., 2008 and Walther & Stein, 2000). The study of different types of viruses has led scientists to use engineered versions of these viruses as gene delivery vehicles, because of their capacity to successfully transfer the gene of interest into recipient cells.

Over the last few decades, intense efforts have been made to understand how different viruses and viral vectors interact with the infected host on a molecular level, in order to develop efficient and safe vectors. The majority of clinical gene therapy trials worldwide are designed to deliver their transgene of interest using viral vectors derived from adenovirus, adeno-associated virus, poxvirus, retroviruses and herpesvirus (Mancheño-Corvo & Martín-Duque 2006 and Thomas *et al*., 2003).

Each vector system has its own properties, strengths and weaknesses. Thus, depending on the nature of different diseases, different therapies need to be developed. For instance, in some cases long or short-term gene transfer is required, and in others, regulated gene delivery might be necessary. In some cases, widespread or localised gene transfer is required to develop a particular treatment for a given disease. Therefore, different viral vectors have to be developed in order to design individual therapies according to the

nature of the disease (Stone, 2010). Among different viral vectors systems, lentiviruses, a genus of retroviruses that includes HIV, have been widely used as delivery system. Lentiviral vectors have interesting properties, such as the ability to transduce different types of cells, including quiescent cells, reduced immunogenicity upon *in vivo* administration, stable gene expression, a lack of prior immunity, and an ability to be pseudotyped with various envelope proteins, which means that alternative envelopes can alter vector tropism. Another feature of lentiviral vectors is that they can integrate into the genome of the host cell and can be duplicated along with the host DNA during the synthesis phase of the cell cycle. Unfortunately, integration carries a risk of insertional mutagenesis at the integration site (Sakuma *et al*., 2012 and Wanisch & Yáñez-Muñoz, 2009). The first reported insertional mutagenesis in humans, involving retroviral vectors, occurred in a SCID-X1 gene therapy trial (Hacein-Bey-Abina *et al*., 2003). Intensive study of the genome and analysis of integration strategies of lentiviral vectors led to the development of a number of strategies to minimise the risks posed by genomic integration. These included the use of viral vectors with a safer integration pattern, the use of self-inactivating vectors and finally the design of integration-deficient lentiviral vectors in an effort to combat the problems caused by integration of viral DNA into the host-cell genome. IDLVs, which were developed by using class I IN mutations, most commonly involving an amino acid change at position D64 within the catalytic core domain, are the most frequently used type of retroviral non-integrating vectors (Apolonia *et al*., 2007; Leavitt *et al*., 1996 and Yáñez-Muñoz *et al*., 2006). Lentiviral vectors were successfully employed for gene therapy purposes (Aiuti *et al*., 2013; Biffi *et al*., 2013; Cartier *et al*., 2009; Cavazzana-Calvo *et al*., 2010 and Palfi *et al*., 2014).

SMA is caused by mutation or deletion of the *SMN1* gene. Low levels of the ubiquitous protein SMN are associated with a range of systemic pathologies reported in affected

individuals (Lefebvre *et al*., 1995 and Szunyogova *et al*., 2016). The principle of developing therapies for SMA is based on four different strategies: *SMN1* gene replacement, modulation of *SMN2* encoded full length SMN protein levels, neuroprotection, and targeted improvements of muscle strength and function (Farrar *et al*., 2016). More than 20 years have been dedicated to understanding the disease pathogenesis, and developing an appropriate and effective therapy, and until very recently, no approved treatment options were available. However, on December 23, 2016, the U.S. Food &Drug Administration (FDA) approved Spinraza (nusinersen) as the first marketed drug for SMA. On June 1, 2017, the European Union granted Spinraza a marketing authorisation. This drug is an antisense oligonucleotide that increases the production of full-length SMN protein by modulating the splicing of SMN2 pre-mRNA transcripts to improve inclusion of exon 7. The results of clinical trials of this drug demonstrated impressive achievements in several treated cases, albeit not all, and showed significant improvements in motor function (Finkel *et al*., 2016). Additionally, it should be noted that an ongoing phase 2 clinical trial, is making promising progress towards developing a therapy for SMA (Clinicaltrials.gov identifier NCT02122952).

As previously stated, one of strategies for developing an effective treatment for SMA is to provide a functional copy of the *SMN1* gene to replace the defective gene and increase the level of full length SMN protein. This strategy has been used by other researchers, and while they demonstrated positive results toward achieving an effective treatment for SMA, none of them were able to successfully effect a complete rescue of the SMA animal model (Dominguez *et al*., 2011; Foust *et al*., 2010; Le *et al*., 2011; Mendell *et al*., 2016 and Valori *et al*., 2010).

Having an optimised system can significantly reduce the amount of injected vectors required to maintain transgene expression and lead to sufficient levels of protein

production. Therefore, this study is aimed at optimising lentiviral vectors, which may be useful in developing a therapeutic viral vector for SMA.

The level of SMN protein is a key factor in developing an effective treatment for SMA, thus in this study different factors were studied to optimise and develop a possible lentiviral vector. Different factors can play a role in optimising the expression system, such as type of vectors, promoter, transgene and other sequences affecting transcription or translation. The first chapter of this thesis was devoted to producing and titrating a wide variety of IPLVs and IDLVs. These viral vectors expressed the transgene of interest under the control of either CMV or hSYN promoters. Another relevant factor for efficient protein expression is the type of transgene, so a novel codon-optimised *Co-hSMN1* was developed in the context of this project to study the effect of a sequence-optimised transgene on protein production. Finally, a mutated mWPRE sequence was added to promote mRNA stability and nuclear export. The generated vectors were tested in a variety of quiescent cell culture models. The outcome of the experiments demonstrated that the lentiviral vectors generated efficiently transduce different cells and express the gene of interest.

The promoters chosen for this study were two strong viral promoters, CMV and hSYN, which additionally are effective in IDLVs (Yáñez-Muñoz *et al*., 2006 and Lu-Nguyen *et al*., 2014). CMV is one of the strongest promoters for recombinant protein expression in mammalian cells, and it has been widely used in the biomedical field (Andersen *et al*., 2011). The other promoter, hSYN, is another strong promoter in its own right (Kügler *et al*., 2003). The expression of different *hSMN1* transgenes was studied under control of these two promoters and the result of *in vitro* experiments revealed that the CMV promoter directed the most robust transgene expression from lentiviral vectors. This finding was in agreement with other studies (Gascón *et al*., 2008 and Palfi *et al*., 2014).

Another important subject that needed to be considered for developing a potential expression system to target SMA disease was that SMA is not only a motor neuron disease. Many other cells and organs are reported to be affected by low levels of SMN protein in SMA (Hamilton & Gillingwater, 2013 and Szunyogova *et al*., 2016). Therefore, it would make perfect sense to choose a promoter like CMV, which can mediate gene expression in a remarkably broad range of cells within its host, rather than a relatively specific promoter such as hSYN, whose strongest expression is limited to a particular lineage. hSYN would be a good candidate if the target of the disease is limited to the central nervous system, however, an effective treatment for SMA must transduce a broad range of organs and cells, as SMN protein is essential for nearly all cell types.

For viral-vector based gene therapy to be clinically applicable, the biosafety of viral elements should be pre-determined. WPRE is a hepadnavirus sequence, which is widely used in retroviral gene transfer vectors. This element contains the promoter for the woodchuck hepatitis virus X protein (WHX) and an open-reading frame encoding a truncated peptide of the WHX. In most gene transfer vectors, the wild-type WPRE has the potential for transcriptional and translational activity, but it has also been associated with expressing unnecessary viral components in host cells which could result in toxicity or certain mechanisms of immune recognition (Zanta-Boussif *et al*., 2009). For this reason, a mutated mWPRE (mWPRE) region is more commonly used, as it prevents these possibilities without having any other effect in its capacity to improve cell transduction. mWPRE region contains six-point mutations, five of which are in the putative promoter region, while one lies at the start codon of X protein ORF. Zanta-Boussif *et al*., 2009 established that mWPRE efficiently reduces undesirable possibility and translation of a WHX polypeptide while having an equivalent level of transgene expression *in vitro* and *in vivo* over longer periods of time, compared to the wild-type

WPRE. Moreover, the promoter activity is not compromised by the mutations (Zanta-Boussif *et al*., 2009 and Zufferey *et al*., 1999). Since mWPRE improves vector biosafety, it can facilitate the use of lentiviral vectors for clinical applications. From the outset, this project replaced wild-type WPRE from lentiviral backbone with mWPRE. All lentiviral vectors in this project were therefore designed to carry mWPRE sequences.

In order to improve expression from the developed lentiviral vectors, it is possible to optimise the transgene sequence. A so-called codon-optimised version of the *hSMN1* transgene that potentially increases expression has been reported (Valori *et al*., 2010). In the context of this thesis, a novel codon-optimised version of the *hSMN1* transgene was developed. Codon-optimisation originally referred to the use of optimum codons for each amino acid, but sequences detrimental for gene expression are also eliminated. Comparing the protein production values obtained with the vectors expressing the codon-optimised and the wild-type *hSMN1* transgene, demonstrated that regardless of vector and promoter type, the *Co-hSMN1* transgene expresses more full-length SMN protein than any other type of *hSMN1* transgene. Therefore, codon optimisation had a positive effect on the transgene expression system, and could be applied to improve expression from transduced cells. These results are in accordance with other studies (Fath *et al*., 2011; Graf *et al*., 2004 and Valori *et al*., 2010)

During this study, lentiviral vectors were produced in two forms; integrating and integration-deficient, in order to compare levels of transgene expression from integration vectors with non-integrating counterparts. To complete this task, each *in vitro* model was transduced with the same MOI of an integrating vector and its IDLV counterpart. In short, all elements such as promoters, MOI of transduction and transgene were kept the same, and the only difference was the integration proficiency of the lentiviral vector. Therefore, the result of these experiments determined whether transgene expression was

different between IPLV or IDLV or not. The results of different transductions in a variety of *in vitro* models was taken into account, and it was possible to conclude that regardless of the promoter and transgene used, IDLVs generally expressed at lower levels than the IPLVs in the models used in the current work. The production of protein is expected to be linked to transcriptional activity, so the conclusion is that IDLVs have lower transcription activity compared to IPLVs. This result correlates with other studies (Apolonia, 2009 and Sakuma *et al.*, 2012).

Although IDLVs demonstrated lower transgene expression than IPLVs, they still displayed significant expression levels and could potentially be a safer option for therapeutic applications. IDLVs preserve the transduction efficiency of the wild-type lentiviral vectors (IPLVs), while providing expression of the gene of interest without viral integration into the host genome, thus reducing the potential risk of insertional mutagenesis. This project demonstrated that different relevant factors could be optimised to increase the level of SMN protein. IDLVs could benefit greatly from the optimisation approach and could reach the therapeutic threshold while also being safer vectors. Whether optimised IPLVs or IDLVs have reached or express above the therapeutic threshold, needs to be determined by testing *in vivo* models.

The main aim of this project is to develop a potential therapeutic vector: therefore, demonstrating whether the virally expressed SMN protein interacts with established partners of SMN is vital. Thus, this project was designed to examine whether delivery of the gene encoding SMN protein to type I SMA fibroblasts derived from human patients can lead to restoration of gems, which are absent or nearly absent in patients with severe SMA (Coovert *et al.*, 1997 and DiDonato *et al.*, 2003). Transduced cells using IPLV/IDLV-mediated gene delivery revealed that full length SMN protein could be efficiently expressed in transduced fibroblasts, and could lead to restoration of gems

and thus rescue an aspect of the SMA phenotype. In some cases, the detected number of gems in a transduced cell was equal to or greater than that found in a control fibroblast cell. These results are in accordance with previous studies (Azzouz *et al*., 2004; DiDonato *et al*., 2003 and Valori *et al*., 2010). The number of detected gems indicated that lentiviral vectors express a high level of SMN protein, as there is a direct relation between the two. The highest number of gems was detected in the cells that received *Co-hSMN1*. This finding further proves that using a codon-optimised version of *hSMN1* transgene can produce higher levels of SMN protein than wild type *hSMN1*. The results of chapter 5 demonstrated that the SMN protein produced is fully functional and lentiviral vectors encoding the *SMN* cDNA can rescue this SMA phenotype in an *in vitro* model.

Moreover, the results of *in vitro* experiments have demonstrated that by increasing the vector dose, it is possible to produce more SMN protein, and to form more gems, as shown in Chapters 4 and 5 respectively. It is important to note that the enhanced transduction observed here, resulting from optimisations and increased MOIs, was achieved without any obvious toxicity or changes in cell morphology.

The successful results from the initial *in vitro* experiments led us to further test the most efficient vectors in an *in vivo* model, in order to assess their efficiency in rescuing a severe mouse SMA phenotype. The reported therapeutic impact on SMA mouse models has demonstrated that the time of delivery of the *SMN1* transgene is critical to develop an effective treatment. Early postnatal injection of viral vectors encoding *SMN1* cDNA demonstrated a limited capacity to ameliorate disease symptoms (Foust *et al*., 2010 and Kariya *et al*., 2014). Moreover, it has been suggested that *in utero* injection may have advantages over postnatal delivery, as certain diseases with early onset, such as SMA, could benefit greatly from a foetal gene therapy strategy to overcome the current

limitations of conventional gene therapy approaches (Waddington *et al*., 2005). In the last stages of the current work, a very brief pilot study was designed to test the viral vectors encoding either *hSMN1* or *Co-hSMN1* in the Taiwanese SMA mouse model. This was our first attempt at performing *in utero* gene therapy with our colleagues in Edinburgh University. Unexpectedly, the outcome of the study did not reveal any improvement of the SMA phenotype in *hSMN1*/*Co-hSMN1*-treated mice, using AAV9 with the former or IPLD /IDLV vectors with the latter. Analyses of tissue harvested *post-mortem*, particularly from control injections performed using *eGFP*-encoding vectors, demonstrated that expression of the delivered genes was low or absent in some cases. The failure to improve the phenotype was therefore subsequent to poor transgene expression results. As the same vector stocks had been effective in tissue culture studies, we conclude that our initial set of *in utero* experiments has been unsuccessful for technical reasons. *In utero* intraspinal and intraperitoneal injections are a notoriously difficult technique, involving complex surgery and the delivery of the vectors by injection through the uterine wall. Relatively low levels of transgenic expression of *eGFP* were observed in some cases, indicative of successful transduction but of limited extent. What the experiments did confirm was our ability to perform the surgical procedures, inject and maintain viability of the injected dams and fetuses. Further experiments will be undertaken in the near future, including the use of an AAV9 vector encoding a CBA-driven *Co-hSMN* transgene, which we expect to set a standard for transgenic expression of *SMN1* in our future work.

In conclusion, the development of the aforementioned optimised lentiviral vectors has significantly improved expression of the transgene and could possibly become an excellent system for SMN-targeted therapy. A combination of optimised vectors and *in utero* injection may result in improvements to the therapeutic impact of lentiviral vector-

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mediated SMA gene therapy. However, more studies are required to determine the therapeutic effect of the generated vectors in an SMA animal model.

7.2 Conclusions

The results of this thesis support the following conclusions:

- Novel lentiviral transfer plasmids were produced encoding the following transgenes: *hSMN1*, *hSMN1-CtF*, *hSMN1-NtF* and *Co-hSMN1*.

- IPLVs and IDLVs were produced, with qPCR titres in the range of 1.01×10^8 to 2.89×10^{11} vector genomes/mL.

- Both IPLV and IDLV configurations encoding *SMN1* variants are efficient at transducing various growth-arrested or quiescent cell types *in vitro*.

- Generally, IPLVs demonstrated higher expression levels compared with their IDLV counterparts.

- Codon (sequence)-optimisation had a clear positive effect on *SMN1* transgene expression and led to increase levels of full-length SMN protein in transduced cells with both IPLVs and IDLVs.

- The CMV promoter drove higher levels of transgene expression than the hSYN promoter for both *SMN1* transgenes and IPLV/IDLV integration configurations.

- Levels of full length SMN protein increase in a vector dose-dependent manner in quiescent cells in culture.

- Transgenic production of SMN protein in type I SMA fibroblasts leads to significantly increased, dose-dependent levels of gems with both IPLVs and IDLVs.

- A preliminary *in vivo* experiment showed that surgical *in utero* delivery of AAV9, IDLVs or IPLVs encoding either *eGFP* or *SMN1* variants is well-tolerated by Taiwanese SMA mice and wild-type littermates.

- Further experiments are required to determine the optimum viral vector for *SMN*1 delivery in SMA.

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List of appendix
Appendix 1: list of biochemical reagents and kits

1× Phusion HF Buffer (Thermo Fisher Scientific, Germany)

30% Acrylamide/Bis-acrylamide (Sigma, UK)

4',6-diamino-2'-phenylindole dihydroclhoride (DAPI) (Sigma, UK)

Acid hydrochloric (HCl) (Sigma, UK)

Agarose (Sigma, UK)

Ammonium persulfate (Sigma, UK)

Ampicilin (Sigma, UK)

Antarctic phosphatase and corresponding buffer (New England Biolabs, UK)

β- mercaptoetanol (Sigma, UK)

B27 supplement without vitamin A 50X (Invitrogen, UK)

Bacteriological agar (Sigma, UK)

Bromophenol blue (Sigma, UK)

Bovine serum albumin (BSA) (Sigma, UK)

Calcium chloride ($CaCl_2$) (Sigma, UK)

Dimethyl sulfoxide (DMSO) (Sigma, UK)

DMEM high glucose with stable glutamine (PAA, UK)

DMEM, high glucose, no glutamine, no methionine (Life Technologies, UK)

DNA ladder (New England Biolabs, UK)

DNAse I (Promega, UK)

dNTPs (Thermo Fisher Scientific, Germany)

Ethanol (Sigma, UK)

Ethidium bromide (Sigma, UK)

Ethylenediaminetetraacetic acid (EDTA) (Sigma, UK)

EthyleneGlycol (Sigma, UK)

FACs Flow solution (BD Biosciences, UK)

FACs Rinse solution (BD Biosciences, UK)

FACS shutdown solution (BD Biosciences, UK)

Fetal Bovine serum (FBS) (Life Technologies, UK)

Fibroblast growth factor 2 FGF2 (Miltenyi, UK)

Gel loading dye (Bioline, UK)

Gentamicine (Life Technologies, UK)

Glutamax (Life Technologies, UK)

Glycerol (Sigma, UK)

Glycine (Sigma, UK)

Hank's balanced salt solution (HBSS) without Ca & Mg with Phenol Red (PAA, Austria)

HEPES Buffer Solution (Life Technologies, UK)

Isopropanol (Sigma, UK)

L-Ascorbic acid (Sigma, UK)

Luria Broth (Sigma, UK)

Medium 199 with Earle's BSS, with L-glutamine, HEPES and 2.2 g/L NaHCO3 (Lonza, Belgium)

Methanol (Sigma, UK)

Magnesium chloride (MgCl$_2$) (Sigma, UK)

Nitrocellulose membrane (Amersham Hybond – ECL) (GE Healthcare, UK)

Sodium chloride (NaCl) (Sigma, UK)

Sodium hydroxide (NaOH) (Sigma, UK)

Paraformaldehyde (PFA) (Sigma, UK)

Penicillin and streptomycin (Pen&Strep) (PAA, UK)

Phenol Red solution (Sigma, UK)

Phosphate buffered saline (PBS) (PAA, Austria)

Pidermal growth factor EGF (Miltenyi, UK)

Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Germany)

RbC12 (Sigma, UK)

Prestained protein ladders (Fermentas, UK)

Proteinase K (Life Technologies, UK)

Polybrene (Sigma, UK)

Poly-D-lysine (Sigma, UK)

QIAgen DNeasy Blood and Tissue kit (QIAgen, UK)

QIAgen Endotoxin-free plasmid maxiprep/megaprep (QIAgen, UK)

QIAprep spin miniprep kit (QIAgen, UK)

QIAquick gel extraction kit (QIAgen, UK)

RC DC Protein assay kit (Bio-Rad, UK)

Restriction enzymes and corresponding buffers (New England Biolabs, UK)

Skimmed milk powder (Tesco, UK)

Sodium acetate (Sigma, UK)

Sodium Dodecyl Sulfate (SDS) (Sigma, UK)

SYBR Green qPCR master mix (Bioline, uk)

T4 DNA ligase and corresponding buffer (New England Biolabs, UK)

Tetramethylethylenediamine (TEMED) (Sigma, UK)

Tissue culture grade water (PAA, Austria)

Tris (Sigma, UK)

Triton X-100 (Sigma, UK)

Trypan blue (Sigma, UK)

Trypsin/EDTA (PAA, UK)

Tween-20 (Sigma, UK)

Appendix 2: Buffers and Solution

Buffer for western blot

<u>1X TBS-T buffer</u>: 0.25% Trizma base, 0.8% NaCl and 0.05% Tween-20 in dH_2O; adjusted to pH 7.6.

<u>5X Laemmli sample buffer</u>: 10% SDS, 25% 2-mercaptoethanol, 50% glycerol, 0.1% bromophenol blue, 0.25 M Tris-HCl; adjusted to pH 6.8.

<u>Western blot blocking buffer</u>: 5% skimmed in 1X TBS-T.

<u>5% stacking gel</u>: 5% polyacrylamide, 125 mM Tris (pH 6.8), 0.1% ammonium persulfate, 0.1% SDS, 0.1% TEMED.

<u>10X TBS buffer</u>: 0.5 M Tris-HCl, 1.5 M NaCl in dH_2O; adjusted pH 7.5.

<u>10X WB Migration buffer</u>: 0.25 M Tris-HCl, 1% SDS, 1.92 M glycine; adjusted to pH 8.3.

<u>12% SDS-PAGE gel</u>: 12% polyacrylamide, 375 mM Tris (pH 8.8), 0.1% ammonium persulfate, 0.1% SDS, 0.04% TEMED.

<u>Tris-HCl buffer</u>: Trizma in ddH2O, and adjusted to appropriate pH by 1M HCl.

<u>Red Ponceau solution</u>: 0.1% Ponceau S, 1% acetic acidz:

<u>Western blot transfer buffer:</u> 0.25% Trizma base and 0.9% glycine in dH_2O, 20% methanol just before use.

Buffer and solution for bacterial work

<u>Tfb I</u>: 100 mM KCL, 50 mM RbCL, 10 mM CaCL2, 30 mM KOAc, 15% glycerol; adjusted to pH 5.8.

<u>Tfb II</u>: 10 mM MOPS, 10 mM RbCL, 75 mM CaCL2, 15% glycerol; adjusted to pH 7.

<u>Luria Broth (LB)</u>: 2% Luria broth.

<u>LB agar medium</u>: 2% Luria broth, 1.5% bacteriological agar.

Buffer and solution for immunofluorescences

<u>1% BSA blocking buffer</u>: 1% BSA, 0.02% Na3N in 1X PBS-T.

<u>PFA solution</u>: PFA, 1X PBS; adjusted to pH 7.4.

<u>1× TBS-T</u>: 1 ×PBS, 0.25% Triton-×100.

<u>Mounting solution</u>: 1 part of PPD solution, 9 parts of Mowiol solution.

Buffer for lentivectors production

2X HBS: 100 mM HEPES, 281 mM NaCl and 1.5 mM Na2HPO4 in cell culture grade water, adjusted to pH 7.12. Filtered through a 0.22 μM pore filter.

Buffer for isolation primary cells

1% BSA blocking buffer (for cultured cells and brain sections): 1% BSA, 0.02% Na3N in 1X PBS-T.

Brain dissection buffer: 1X HBSS, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μM ascorbic acid (added just before use).

Complete growth medium: DMEM, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% FBS.

Cortical neuronal differentiation medium: Neurobasal medium, 2% B27, 0.25% GlutaMax, 1% FBS, 100 μM ascorbic acid (added just before use).

Human fibroblast medium: 64.8% DMEM high glucose with stable glutamine, 21% M-199, 10% FBS, 10 ng/ml FGF2, 25 ng/ml Epidermal growth factor (EGF), 1 μg/ml gentamicine.

CHO growth medium: DMEM, 10% FBS, 0.02 g/l L-proline, 100 unites/ml penicillin, 100 g/ml streptomycin.

CHO cell-arrest medium: DMEM without methionine supplementes with 2% FBS, 0.02 g/l- proline, 100 unites/ ml pencilin, 100 g/ml streptomycin.

Buffer for Agarose gel electrophoresis

1X Tris-acetate-EDTA (TAE): 40 mM Trizma base, 20 mM glacial acetic acid and 1 mM Na2EDTA in ddH2O; adjusted to pH 7.6.

IgG192 antibody production

Hybridoma cells were seeded in a 75cm^2 flask in 20 mL of RPMI supplemented with 15%FBS and incubated at 5% CO_2 and 37°C. 15 mL of medium was added to the cells every 48 hours for 10 days. After this, 3mM glutamine and 2,5mM glucose was added to flask and the cell were left in incubator for two weeks. The IgG192 antibody was harvested by filtering the medium through 0.22 μM filter and stored in 5 mL aliquot at -20°C.