Incretin-based Muscle-targeted Gene Therapy for Type 2 Diabetes

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Abstract

According to WHO (August 2011), 346 million people worldwide have diabetes and, of those, 90% have type 2 diabetes (T2DM). This equates to approximately $465 billion (USD) in healthcare costs. Current treatments, which include various different drug types, both oral and injectable, are somewhat effective (i.e. do not prevent associated complications) but also still heavily rely on patient and/or care-giver regimen adherence for the therapies to be effective.

One of the most recent classes of drugs is known as Glucagon-like peptide 1 receptor (GLP-1-R) agonists. Current treatment interval extends to only once weekly injections and, whilst this is far superior to daily or even twice daily injections, any possible extension would potentially provide an increased safety profile (by removing non-physiological peaks in hormone pharmacokinetics) and would generally be better received by the patient.

Gene therapy is one area of investigation for T2DM, which aims to provide longer-term therapeutic peptide delivery and therefore reduce the number injections required for satisfactory glucose homeostasis.

GLP-1 is a hormone within the group known as incretins. This group comprises hormones which enhance glucose-dependent insulin synthesis and secretion (from pancreatic β-cells) in response to glucose (and other nutrients) and subsequently suppresses post-prandial hyperglycaemia. One of the major pitfalls of GLP-1 is its incredibly short half-life (2-3 minutes) due to degradation by a ubiquitous dipeptidyl peptidase (DPP4). Current therapeutics are small compounds activating incretin functions, for example the GLP-1 analogue mimetics exenatide or liraglutide, or are DPP4 inhibitors, for example stigaliptin. However, due to the progressive nature of the disease and pathophysiological differences between individuals, these treatments may not be suitable for everyone and may not provide a long-term sustainable solution.

This thesis aimed to compare the efficacy and efficiency of utilising both plasmid and viral based (AAV) vectors for transgene delivery via skeletal muscle. Skeletal muscle is used mainly for its ease of access and its highly vascularised system, which is extremely capable of uptake and expression of foreign DNA.

The transgenes chosen for this study encoded unaltered human GLP-1 and the longer-acting GLP-1-R agonist, Exendin-4. Exendin-4 shares 53% homology with GLP-1 and exerts insulinotropic activity while lacking the amino acid cleavage site via which DPP4 degrades GLP-1. Enhanced green fluorescent protein (eGFP) was also used, as a control vector and an easy to visualise reporter that would aid in the determination of expression both during the study (for use in live animals) and in post-mortem analysis.
In vitro studies for proof of concept were performed in the C2C12 murine muscle cell line, and in a minor part, HEK293 fibroblasts, before being progressed into both normal (CD1) and diabetic (db/db) mouse models.
Transgene expression was attained in C2C12 cells, as determined via immunofluorescence staining and ELISA/EIA analysis of both the cells and the cell media. Standard transfection/infection protocols were utilised. The longest study concluded at 5 days post transfection/infection.
In vivo studies also attained expression of the three transgenes, eGFP, GLP-1 and Exendin-4 via direct hind limb skeletal muscle injection. *In situ p*lasmid uptake was augmented by pre-injection of the muscle with hyaluronidase and external calliper electroporation. Circulating peptide levels were confirmed via blood serum analysis using ELISA/EIA. Muscle peptide concentration was determined from muscle homogenates and by immunofluorescence staining together with circulating hormone concentration by ELISA/EIA. Pancreas samples were evaluated for β-cell mass. IPGTT data were obtained throughout the study. In addition, each mouse was subject to non-invasive imaging (IVIS), which allowed visualisation of the eGFP reporter gene. Gene expression was followed to a suitable end point, which was determined on a study-by-study basis.
Results showed the delivery system to be effective yielding promising circulating peptide concentrations in all animal models. The data obtained from IPGTT analyses in the diseased model showed the improved capability of glucose clearance when transfected with Exendin-4 transgenes. Expectedly, no differences were noted in the healthy model. The plasmid vectors appeared to give a greater efficacy profile than the AAV vectors, based on both eGFP expression analysed via IVIS and circulating peptide concentrations. In light of this, only the plasmid vector studies were performed in the diseased animal model.

In conclusion, a skeletal muscle based gene delivery system for GLP-1 and longer acting GLP-1-R agonists has proven to be tolerated by both normal and diseased animal models, Furthermore, the administration of the Exendin-4 to diseased animals suggests positive glucose lowering effects. The current work suggests that further studies are worthwhile, including those conducted in a range of animals with overt and perhaps spontaneous diabetes mellitus.

List of Abbreviations

AAV	Adeno-associated virus
ADA	Adenosine deaminase
AMD	Age related macular degeneration
ANOVA	Analysis of variance
ASGCT	American Society of Gene and Cell Therapy
ATP	Adenosine triphosphate
AV	Adenovirus
cAMP	Cyclic adenosine monophosphate
cfu	Colony forming unit
CMV	Cytomegalovirus
CNS	Central nervous system
CO2	Carbon dioxide
CVD	Cardio vascular disease
DAPI	4', 6-diaminidino-2-phenylindole
dH20	distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Dexoyribonucleic acid
DPP4	Dipeptidyl peptidase-4
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Ex-4	Exendin-4
FBS	Foetal bovine serum
FFPE	Formalin fixed paraffin embedded
GI	Gastro intestinal

GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide-2
HbA1c	Glycated haemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIV	Human immunodeficiency virus
HOMA	Homeostatic model assessment
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IM	Intra-muscular
IPGTT	Intra-peritoneal glucose tolerance test
IVIS	In vivo imaging system
KCl	Potassium chloride
LB	Luria-Bertani
MCK	Muscle creatine kinase
MLV	Moloney-murine leukaemia virus
MOI	Multiplicity of infection
Opti-MEM	Reduced serum minimal essential medium
PBS	Phosphate buffered saline
pDNA	plasmid DNA
PFA	Paraformaldehyde
PIL	Personal Home Office animal licence
PKA	Protein kinase A
PPAR?	Peroxisome proliferator-activated receptor gamma
PPL	Home Office animal Project licence
rANOVA	repeated measures ANOVA
RCF	Relative centrifugal force
ROI	Region of interest
RT	Room temperature
SCID	Severe combined immunodeficiency disease
SD	Standard deviation
siRINA	Small interfering RNA

SOC	Super optimal broth with catabolite repression
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TET	Tetracycline
UPV	Viral vector production unit, Barcelona
WHO	World Health Organisation

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1 Chapter 1 – Introduction

1.1 Diabetes Mellitus

There are many conflicting reports of who first described the disease and when. Some say that the observation of 'sweetened' urine dates back as far as ancient Greece. However, it is arguably not until the late 1800's that recognised medical records show the gradual observations and discoveries that have led to the current classifications of Type 1 and Type 2 Diabetes Mellitus.

The two main types of diabetes mellitus, now known as Type 1 (T1DM) and Type 2 (T2DM), were first fully characterised in 1951 by Lister, Nash and Ledingham, who differentiated between the two based on their different aetiologies (Lister *et al.*, 1951). Broadly, diabetes mellitus is defined as a group of metabolic disorders culminating in chronic hyperglycemia as a result of insulin deficiency or defectiveness.

In addition to the two major forms, intermediary stages have been determined, at which time the individuals suffering from impairment in glucose tolerance are at a higher risk of developing frank T2DM but this is not a certainty and in some cases the condition can be reversed. States such as Metabolic syndrome and Gestational Diabetes can be reversed with diet and lifestyle changes and in the case of gestational diabetes, resolve at least in the short term post-pregnancy (UK, 2012).

According to WHO, 346 million people worldwide have diabetes mellitus and of those, 90% have T2DM ((WHO), 2011a). The focus of the research within this thesis is T2DM.

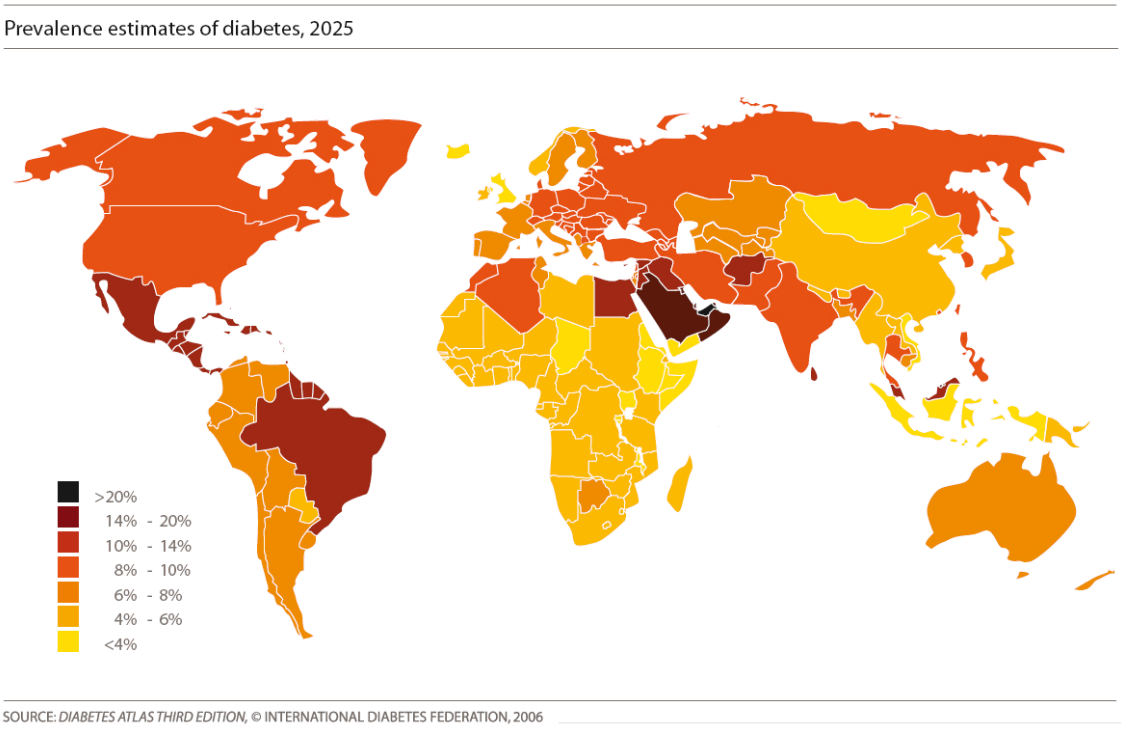


Figure 1-1- Estimated prevalence of T2DM worldwide in 2025. (*Downloaded 18.06.12 http://ocpstore.com/store/wp-content/uploads/2012/05/prevalence_diabetes_map_2025.jpg)***

1.1.1 Economic impact

As mentioned previously, T2DM is one of the most common non-communicable diseases and is the fifth most common cause of death worldwide. Diabetes UK reports that 1 in 10 people admitted to hospital (in the UK alone) have some form of diabetes and 15% of all deaths per year can be attributed to diabetes or a diabetes related complication (DiabetesUK, 2011).

Figure 1-1 refers to the estimated global distribution of T2DM in the year 2025. As can be seen, the distribution really is global and the health and economic consequences of this could be catastrophic to some health care providers and their recipients, especially in poorer areas of the world.

According to Hex *et al* (2012) (Hex *et al.*, 2012), diabetes cost the NHS approximately £23.7billion in the fiscal 2010/2011, which equates to roughly 10% of the entire NHS budget. Of this, £8.8billion is a directly attributable to T2DM. Projections for costs in the year 2035/2036, assuming current procedures are still adhered to and population

demographic assumptions are correct, are that a total of £39.8billion (17% of total NHS budget) will be spent on diabetes, with a £15.1billion spend on T2DM. Furthermore, Kanavos *et al* (Kanavos *et al.*, 2012) estimate the EU cost (across the 5 major EU countries of Spain, Italy, Germany, France and UK) to be €90billion.

1.1.2 Type 1 Diabetes Mellitus (T1DM)

T1DM is thought to be caused by an auto-immune attack on the body's own pancreatic islets, resulting in loss of β-cell function. It can occur at any age, but due to the strong link with genetic predisposition, it is most commonly detected in children and young adults (Lernmark, 1999) . Patients suffering from this produce little or no insulin of their own and are therefore completely reliant on exogenous insulin, normally via injection(Gale, 2002).

1.1.2.1 T1DM Associated Complications

There are some major and potentially devastating microvascular complications, which are common to both T1DM and T2DM, which occur when an increased concentration of glucose is present in the blood and causes some of the smaller blood vessels/capillaries to become chronically damaged. These include retinopathy (damage to retinal cells, which can ultimately lead to blindness), nephropathy (kidney damage) and neuropathy (nerve damage)(Fowler, 2008) .

1.1.2.2 T1DM Treatment

There is no 'cure' for T1DM and insulin replacement is needed daily for life. This is why T1DM is also often known as insulin-dependent diabetes. The amount of insulin needed is calculated depending on what food has been ingested. As insulin therapy is a non-

disease modifying agent, progressive β-cell failure is a certainty. Current research is focusing on approaches beyond insulin injection or insulin pump therapy.

A relatively new method of treatment for T1DM is islet transplantation. There are two main types of transplant; auto- and allo-transplantation. Auto-transplantation is not suitable for T1DM patients but can be used for those who are undergoing a complete pancreatectomy for reasons such as chronic pancreatitis, and involves removing islets from the patient's own pancreas and re-implanting them into their liver. In allo-transplantation, islets are removed from a deceased donor, purified and then implanted into T1DM patients. The Edmonton protocol, developed by J. Shapiro (2000), is considered the standard and most successful method and has resulted in several patients becoming insulin free for at least 1 year (Shapiro *et al.*, 2000)

1.1.3 Type 2 Diabetes Mellitus (T2DM)

T2DM tends to have a later age of onset in comparison to T1DM and is characterised by a decreased sensitivity to insulin (also known as insulin resistance) together with decreased insulin secretion rather than the complete loss of β-cell function seen in T1DM.

Impaired glucose tolerance often progresses to T2DM. This and T2DM are associated with progressively increased risk of cardiovascular disease. Once glucose levels are diagnostically high enough for T2DM, there is an increased risk of diabetic microvascular complications.

Other symptoms can include, but are not limited to, an increase in the volume of urination, known as polyuria which, in turn, increases thirst, known as polydipsia, and leads to weight loss through loss of glucose in the urine. Although blood plasma glucose level is still the formal method of diagnosis for T2DM, a new method utilising glycosylated haemoglobin (known as HbA1c) has been acknowledged as a diagnostic tool. WHO guidelines suggest a cut-off point of 48mmol/l (6.5%) glycosylated haemoglobin in the sample, but concentrations lower than this do not necessarily rule out diabetes mellitus in that individual, and there are several situations where this test may not be appropriate(International Diabetes Federation; (WHO), 2011b).

1.1.3.1 T2DM Risk Factors

There are several risk factors that have been identified as a diabetes-risk precursor, including ageing and genetics. However the major risk factor linked to the onset of T2DM is obesity((WHO), 2011a). A sedentary lifestyle coupled with the easy availability of cheap, poor quality processed food items, which have a high fat and high carb content, and a lack of understanding of the nutritional value of different food choices can be implicated in increases in obesity, which as described before, is a major contributor to the onset of T2DM, which is occurring at an ever increasing younger age. Furthermore, genetic factors which have been shown to run in families and a predisposition to 'weak' β-cells which cannot overcome the stress of glucolipotoxicity are also to blame (Campbell, 2011). Campbell (2011) demonstrates this in an extremely easy to read diagram, shown in Figure 1-2.

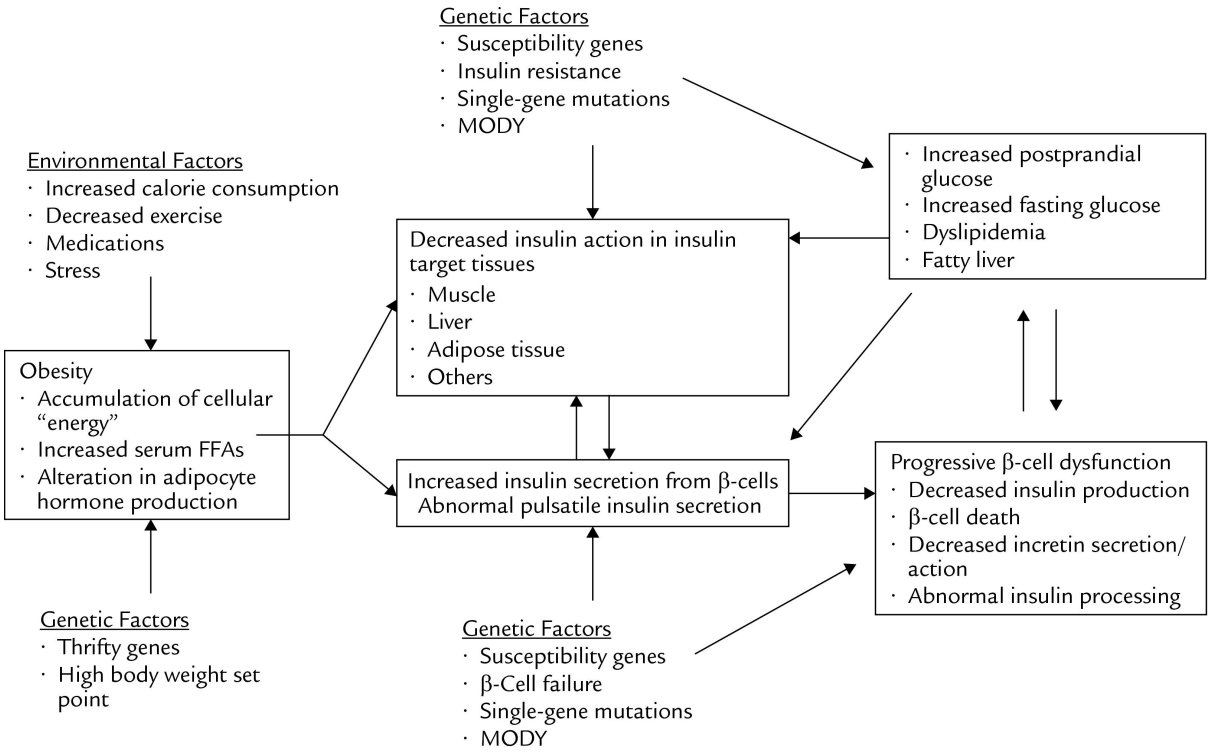


Figure 1-2 – Pictorial representation of T2DM risk factors. *This diagram, taken directly from Campbell (2011), shows the multifactorial risk factors associated with T2DM (Campbell, 2011).*

1.1.3.2 T2DM Associated Complications

Elevated blood glucose, as seen in T2DM, has strong links to both microvascular and macrovascular complications, with cardiovascular disease (CVD) being the major macrovascular risk and microvascular conditions including retinopathy, nephropathy and neuropathy

Diabetic retinopathy can be classified as background or proliferative and involves disturbances in the blood flow to the retina, with background involving reducing the blood flow and proliferative expanding the blood flow, often resulting in micro-haemorrhages. Both ultimately result in deterioration and even loss of vision.

Nephropathy is an increase in protein in the urine, which can ultimately lead to kidney failure. Neuropathy is any evidence of nerve alteration, after any other causes have been ruled out. A classic symptom of neuropathy is 'diabetic foot', in which an ulcer forms after a small injury which is persistent and hard to heal. In severe cases, injuries such as these result in amputation.

Macrovascular CVD is essentially increased atherosclerosis, although the precise mechanism by which diabetes (both T1 and T2) increase this risk is unknown, many studies have shown the link is true (Fowler, 2008; Peterson, 2012).

In addition to the common micro/macrovascular conditions, there is currently a strong belief that increased blood glucose is also linked to some forms of cancer, although published literature describes conflicting results. Shih *et al* conducted a literature review investigating the link between thyroid cancer and diabetes, and concluded there was no statistical evidence to support the theory of a link (Shih *et al.*, 2012) whereas a paper published the previous year by Aschebrook-Kilfoy et al concluded there was a link between diabetes and thyroid cancer (Aschebrook-Kilfoy *et al.*, 2011). However, on close inspection of the paper, they do state the adjuvant health states that are often found in people with diabetes, such as increased BMI (with associated poor lifestyle choices), may in itself be a risk factor for thyroid cancer, and additionally, thyroid cancer is the sixth most common cancer in American women.

An alternative area of study on the link between cancer and diabetes is thought to be the diabetic treatments rather than the conditions itself. Gallagher and LeRoith have

published findings that Metformin, commonly used as the first treatment methodology for T2DM, has been shown to supress breast cancer stem cells (Gallagher and LeRoith, 2011).

1.1.3.3 T2DM Treatment

As discussed previously, 90%, or 346 million people, equates to healthcare costs of approximately $465 billion (USD)(International Diabetes Federation; (WHO), 2011a). Current treatments are somewhat effective but still rely heavily on patient and/or carer regimen adherence for the therapies to be effective and none of these medications address the underlying problem of decreasing β-cell function (S.Niessen, 2011).
Current treatment options for T2DM vary from patient to patient. There is not one 'treat all' medication available. If caught early, exercise and modified (calorie controlled) diet has been proven to lower blood glucose, however, as T2DM is a progressive disease, treatment options may have to be altered without any specific time frame, to address current glycaemic control in the individual.
There are several medications which can be considered singly or in combination, with various actions.

1.1.3.4 Conventional Therapy

The current normal primary treatment for T2DM involves diet and lifestyle modification, which is essentially used to lower BMI, which has been shown to aid in improving glucose homeostasis (Group, 2002). If this is not effective, treatment moves on to **Metformin,** which is the first line of medication for early T2DM (Peterson, 2012). Metformin is a biguanide which allows enhanced glucose uptake by various tissue types, including skeletal muscle, therefore reducing the concentration of circulating glucose. It does not stimulate insulin secretion so there is no risk of hypoglycaemia (Viollet *et al.*, 2012). If metformin fails to control glucose homeostasis, a **Sulphonylurea** may be

considered**.** These work in a complementary fashion to metformin and stimulate insulin release by triggering cascade pathways within the β-cells.
These are both non-disease modifying treatments and, due to the progressive nature of the disease, will ultimately fail to provide adequate glucose control and the patient may still end up on insulin therapy.

1.1.3.5 Newer and/or novel therapies

Various other agents are available which are less commonly prescribed. These include, **Meglitinides** which act in a similar fashion to sulphonylureas but on different receptors within the β-cell but still triggering cascade pathways. These also result in an increase in insulin secretion. **Glucosidase inhibitors** don't have a direct influence on control of insulin but inhibit disaccharide digestion in the gut, which in turn slows entry of glucose into the bloodstream therefore reducing the release of insulin.
However, some effective treatments are proving to be cause for concern due to generating unwanted side effects which may outweigh the benefits of glucose homeostasis. One such drug was the **Thiazolidinedione (or glitazone)** group, containing, amongst others, Rosiglitazone (associated with increased CVD), Troglitazone (withdrawn after reports of severe liver damage) and Pioglitazone. Pioglitazone is the only drug that is available in the UK. This group of drugs act directly on gene transcription factors involved in glucose and lipid metabolism, by activating the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR). The resulting effect is the reduction in insulin resistance in fatty tissue, muscle and liver (Hauner, 2002). This would be an ideal treatment for long term glucose homeostasis if it were not for the adverse side effects associated with this drug, such as weight gain (somewhat of an oxymoron in diabetes care), oedema, decreased bone density and again, CVD (Shah and Mudaliar, 2010).
A relatively new class of drugs known as **Gliflozins** have been developed which act by inhibiting sodium-glucose transport protein 2 (SGLT2) (and are therefore also known as SGLT2 inhibitors). These drugs work by preventing the reabsorpbtion of glucose in the kidney, resulting in any excess glucose being expelled via urine.

However, arguably the most promising forms of treatment lie in being able to harness what is referred to as the 'incretin effect' (see section 1.2.1). Again, this is an example of how this 'effect' can control glucose homeostasis. This will be discussed in more detail in section 1.4, but briefly, comprises agents such as **DPP4 inhibitors** (sitagliptin, vildagliptin, saxagliptin, linagliptin) which increase endogenous GLP-1 concentrations by preventing the enzyme dipeptidyl peptidase 4 (DPP4) degrading GLP-1 and **GLP-1 receptor agonists** (GLP-1-R) (exenatide, liraglutide) which bind to receptors on β-cells and elsewhere, in place of endogenous GLP-1, triggering insulin release. An example of a GLP-1 receptor agonist is **Exenatide.** Exenatide is a synthetic version of Exendin-4. There are currently two versions of exenatide, a twice daily 10µg injection or a once weekly 2mg injection (NHS; UK; DeFronzo *et al.*, 2010; Riedel and Kieffer, 2010; Derosa *et al.*, 2012; Peterson, 2012)

Exendin-4 is one of the compounds of interest for this study and will be discussed in more detail later (see 1.2.3).

1.2 The pancreas and the β-cell

The endocrine pancreas is mainly comprised of islets (of Langerhans), which inturn, are comprised of three main cell types. Briefly, Alpha (α) cells secrete glucagon to increase blood glucose, Beta (β) cells secrete insulin to decrease blood glucose and Delta (Δ) cells secret somatostatin to aid regulation of the α and β cells. All of which are polypeptide hormones which play a role in glucose homeostasis.

The trigger of the pathways that instigate the stimulation of these cells and the subsequent secretion of peptide are a group of hormones known commonly as incretins. These are gastrointestinal hormones comprising mainly of GLP-1 and GIP, which play a key role in what is known as the 'gastroenteropancreatic-brain axis', maintaining glucose homeostasis (Brubaker and Drucker, 2004). The effect that these hormones exert is known as the incretin effect.

1.2.1 *The incretin effect*

The incretin effect is a term used to describe the increased insulin response that occurs post oral glucose ingestion, when compared to the response seen after intravenous glucose loading (Bagger *et al.*, 2011b). The incretin effect is controlled by incretin hormones, these are a group of circulating, gut derived hormones which, amongst other functions, enhance glucose-dependent insulin production and secretion, from pancreatic β-cells, in response to glucose (and other nutrients in food) and subsequently suppress postprandial hyperglycaemia by playing a key role in what is known as the 'gastro-enteropancreatic-brain axis' (Brubaker and Drucker, 2004). It is estimated that 60-70% of insulin secretion after the intake of food is brought about by the incretin effect (Drucker, 2006; Ahren, 2012; Kinalska *et al.*, 2012).

The major hormones involved in the incretin effect are glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2) and gastric inhibitory peptide (GIP). The discovery of GIP first occurred in the 1970's and was the first incretin to be identified. GIP is a 42 amino acid peptide and is secreted from K cells located in the intestinal wall. GLP-1 was not identified until the late 1980's (Drucker, 2006) and, along with GLP-2 and glicentin, is secreted from L cells located in the ileum, colon and rectum. GLP-1 and GLP-2 have previously been studied in conjunction with diabetes control due to the fact that both hormones are encoded by the preproglucagon gene, and additionally share a significant homology with glucagon, however, only GLP-1 was determined to have any insulinotropic activity (Mojsov *et al.*, 1987).

1.2.2 *Incretin structure, function and action*

As previously mentioned, GLP-1 and GLP-2 are synthesised and secreted from intestinal L cells. The process by which this happens involves translation of the proglucagon gene by the prohormone convertase PC 1/3, in response to oral nutrient intake. Both glucagon-like peptides have approximately 50% homology with glucagon, but only GLP-1 exhibits insulinotropic properties. The secretion of GLP-1 is in response to nutrient intake, with some studies showing the macronutrient content of the meal and the time

of day the meal is consumed affects the levels of GLP-1 released. Circulating levels of GLP-1 drop shortly after secretion (within 2 minutes) due to the endogenous enzyme dipeptidyl peptidase 4 (DPP4), which cleaves the alanine at position 2, resulting in GLP-1 (7-36) turning into the inactive forms GLP-1 (9-36), GLP-1 (9-37) and GLP-2 (3-33). Circulating GLP-1 (7-36) forms at least 80% of the biologically active GLP-1 in humans. (Drucker, 2006; Drucker and Nauck, 2006)

The action of GLP-1, which includes increased insulin secretion, reduced β-cell apoptosis, stimulated β-cell proliferation, slowing gastric emptying, regulating appetite and increasing insulin sensitivity in several tissue types, starts when it binds to cell surface G-coupled receptors (GLP-1-R) located on pancreatic islet cell membrane (beta, alpha and delta cells), along with other locations in the GI tract, heart, lung and CNS and adipose tissue, which goes to somewhat explain the action of GLP-1 on other tissue types (Davidson, 2011). It has also been to exhibit some form of protective effect on the β -cells themselves, shielding from apoptosis and stimulating β-cell proliferation via up regulation of PDX-1 (a β-cell transcription factor; pancreatic duodenal homeobox-1) which has been shown to augment insulin gene transcription and up-regulate glucokinase and glucose transporter-2. However, the main action of GLP-1 is on the β-cell to promote postprandial insulin secretion, dependent on glucose levels. This has been confirmed via many studies not only in rodent and cell line models, but also in human studies. Willms et al (1996) showed exogenous GLP-1 administration inhibited gastric emptying and stimulated insulin secretion whilst simultaneously inhibiting glucagon secretion, thus contributing to blood glucose homeostasis, in 8 T2DM patients (Willms *et al.*, 1996). In a high glucose environment, GLP-1-R stimulation leads to the activation of adenylate cyclase, which increases intracellular cyclic AMP (cAMP) levels and calcium activation. An increase in cAMP activates protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII). These two proteins are linked to various routes of GLP-1 based insulin secretion, including the closing of ATP-sensitive potassium (K_{ATP}) channels which influence membrane depolarization and other electrical activity and increased calcium activity resulting in increased mass/volume of insulin containing granules available for release (insulin exocytosis) (Holst and Gromada, 2004). This is shown concisely in Figure 1-3, taken from Drucker, 2006 (Drucker, 2006).

cAMP, via GLP-1 activation, has also been proposed to play a role in β cell proliferation and reduced apoptosis (Farilla 2002, Drucker 2006).

Studies conducted in both human islets and Zucker diabetic rats have shown that administration of GLP-1 resulted in a decreased level of pre-apoptosis factors and reduced levels of cell death in freshly prepared human islets and decreased apoptotic β-cells and increased β-cell mass in Zucker diabetic rats (Farilla *et al.*, 2002; Farilla *et al.*, 2003; Li *et al.*, 2003)

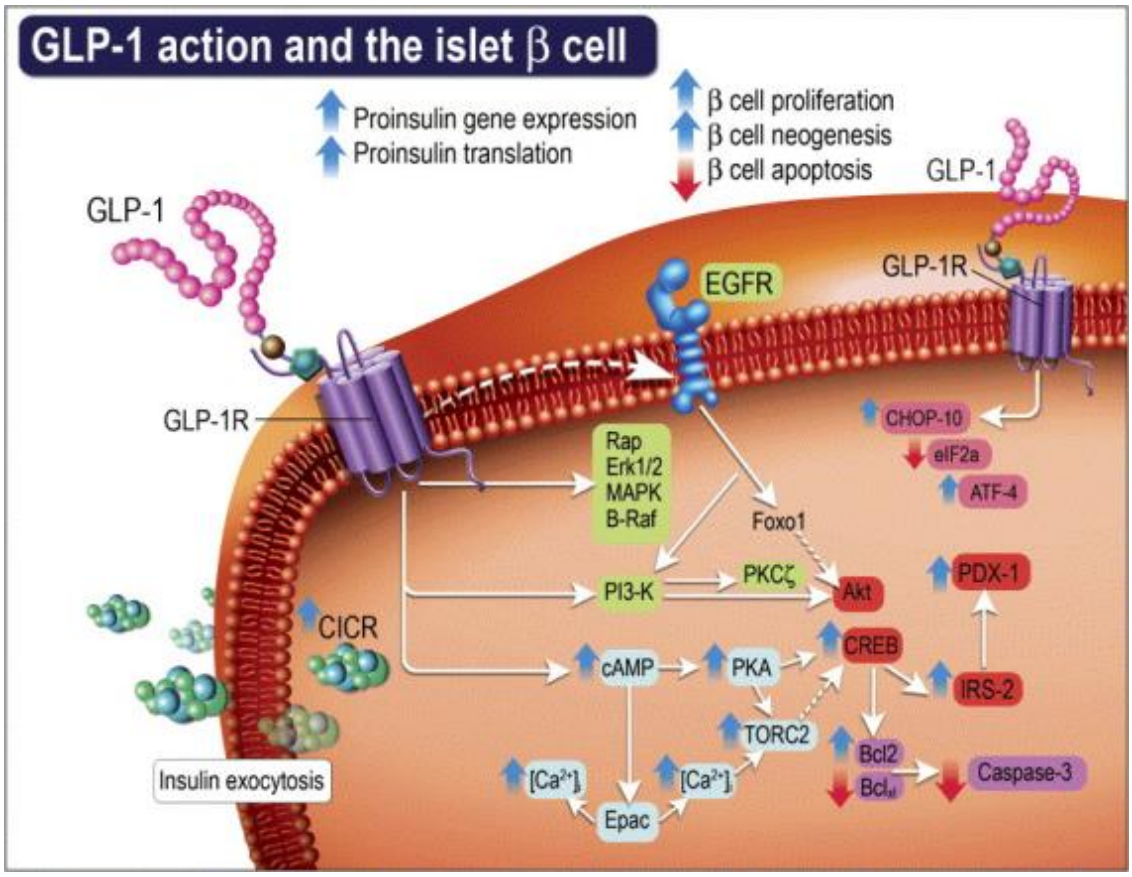


Figure 1-3 - GLP-1 receptor signalling pathways in the pancreatic β-cell. *GLP-1 receptor activation leads to insulin release via the stimulation of exocytotic pathways. Some of these pathways also lead to cell proliferation and survival (Drucker, 2006).*

In T2DM, the ability of GLP-1 to stimulate insulin secretion is reduced. GLP-1 sensitivity remains and external administration of GLP-1 shows insulinotropic activity (Kinalska *et al.*, 2012).

However, even exogenous GLP-1 is still subject to DPP4 degradation. Due to this greatly reduced half-life, GLP-1-R agonists have been developed which are resistant to DPP4 degradation.

1.2.3 GLP-1-R Agonists

GLP-1-R agonists have been developed that are structurally resistant to DPP4 degradation, which increases the circulating half-life of the protein.
Several iterations of this approach are currently available, and in use, as therapeutic agents. Commonly known as incretin mimetics, Exenatide is one such agent (trademark Byetta for twice daily administration or Bydureon for once weekly).
Exenatide is a synthetic version of a hormone called Exendin-4. Exendin-4 was originally isolated from the saliva of the lizard *Heloderma suspectum*. It is a 39 amino acid peptide which has 53% homology with human GLP-1 and binds to GLP-1-R on β-cells with equal efficacy and efficiency. It also shows the same therapeutic effects as GLP-1 on reducing blood glucose levels, increasing insulin sensitivity and secretion and slowing gastric emptying and regulating appetite. However, Exendin-4 contains the amino acid glycine at position 2 rather than the alanine which is seen in GLP-1, and is therefore not subject to DPP4 degradation (Baggio *et al.*, 2006; Drucker, 2006). Figure 1-4 shows the amino acid sequence of both endogenous GLP-1 and Exendin-4, and indicates the position of the alanine substitution with glycine, which removes the cleavage site and increases the half-life.
Several studies have been conducted to examine the effects of Exendin-4 and the potential of Exendin-4 as a therapeutic agent. As with GLP-1, Exendin-4 has been shown to improve β-cell function by reducing apoptosis and promoting proliferation. Derosa et al used Homeostatic Model Assessment (HOMA) to assess β-cell function (HOMA-β) in a trial which assessed how much exenatide added to metformin improved β-cell function when compared to metformin alone. Their results showed that the combination of exenatide and metformin decreased body weight, increased glycaemic control and increased HOMA-β (Derosa *et al.*, 2012).

There are additional therapeutics which have been developed that whilst being DPP4 resistant, do still contain the DPP4 degradation site. For example, Liraglutide is a daily injectable GLP-1-R agonist that has been formulated by replacing a lysine amino acid with an arginine and adding a fatty acid chain, which provides protection to the cleavage site and consequently provides DPP4 resistance and an increased half-life up to 13 hours (hence once daily injection)(Campbell, 2011).

The only current method of GLP-1 R agonist treatment still currently involves regular injections by the patient or carer. This can cause distress and other psychological influences to the patient and/or caretaker. Other physical issues associated with multiple injections at the same site (such as bruising and scar tissue) can also occur with prolonged insulin use. Any method of providing prolonged exposure to a therapeutic agent would be beneficial to the patient, and also help reduce costs. One such treatment modality under extensive investigation for many disease types is gene therapy.

Figure 1-4 – Diagrammatic representation of the homology between GLP-1 and Exendin-4 sequences and the absence of the DPP4 cleavage site in Exendin-4 . *One of the most important*

reasons Exendin-4 is used as a GLP-1 R agonist is the absence of the DPP4 cleavage site. This allows the Exendin-4 to have a much longer half-life than the endogenous GLP-1.

1.3 Gene Therapy

There are many sources which provide a definition for gene therapy, the American Society of Gene and Cell Therapy (ASGCT) describe gene therapy as being 'the approach to treating disease by either modifying the expressions of an individual's genes or correction of abnormal genes, by administration of DNA rather than a drug' (ASGCT, 2015). The concept of gene therapy as a whole has a 20+ year history, with the first clinical trials conducted in the late 1980's. Since then, there have been many documented success stories. A move to a gene therapy treatment, with the genes of interest introduced into appropriate target cells for sustained therapeutic expression, provides a potential logical next step in treatment advance for T2DM.

Successful gene transfer has several considerations, not least the delivery methods. Considerations must include vector uptake and persistence, sustained gene expression and favourable host immune response or ideally, no immune response at all, and all with limited side effects.

For any successful translation to a commercial setting, practical considerations must also include the ease of large scale manufacturing and the cost of the resulting treatment, again, taking into consideration delivery material and any adjuvant treatment that may be necessary. There is also many discussions taking place around the ethical and social impact of gene therapy, including questions such as those posed in a review by Misra (2013) – which described issues such 'what is the difference between a disease and disability?', 'should disabilities be cured?', 'is somatic gene therapy more or less ethical than germ line gene therapy?' (Misra, 2013).

Gene therapy has been used successfully in a number of trials for several other disorders;

- Gene therapy trials for haemophilia B are arguably one of the most publicised and well known disorders for which gene therapy is a success. Trials have been performed using both plasmid and viral vectors; however viral vectors (mainly

Adeno-associated vectors (AAV)) have definitely conferred most successful results (Buchlis *et al.*, 2012; Cancio *et al.*, 2013).

- Severe combined immunodeficiency (SCID) has been the subject of gene therapy trials for many years. This is a disorder whereby a defect in the adenosine deaminase (ADA) enzyme encoding gene results in the failure of the deamination of deoxydenosine which then triggers failures of other cascade pathways and results in a build-up of many intra-cellular bio-molecules. This ultimately inhibits lymphocyte activation. This results not only in a severe lack of immunity but also a 'failure to thrive', as ADA is expressed, at some level, in all cells. Trials using autologous transplantation of hematopoietic stem cells transduced with a recombinant viral vector (Gammaretrovirus) encoding a functional ADA gene has been successfully used to treat patients suffering from SCID-ADA. Trials conducted in both the US and in Europe have results in more than 30 patients having 100% survival post treatment (at time of publication- 2012) (Montiel-Equihua *et al.*, 2012).

- Clinical trials using AAV vectors have been undertaken for Age Related Macular Degeneration (AMD), a disease in which vision loss is caused by leaking blood vessels in the retinal layer. This is most commonly seen in patients over the age of 70 and current treatment involves regular injections directly into the eye, which is clearly invasive and potentially distressing. Gene therapy treatments also need injections into the eye, but at reduced frequency with sustained expression of therapeutic agents (Scholl and Sahel, 2014)

To date (July 2015), The Journal of Gene Medicine reported that 2255 gene therapy clinical trials have been approved worldwide. Of these, only 6.2% employed AAV vectors whereas plasmid vectors and/or naked DNA made up 17.5% (the split between plasmid mediated and naked DNA was unavailable). The remaining 76.3% were from 46 different vector or gene delivery systems. The top 4 most popular systems were adenovirus (21.7%), retrovirus (18.9%) with naked/plasmid DNA 3rd and AAV 4th most frequent. 18% of all trials have used skeletal muscle injection as the delivery method.

1.3.1 Gene delivery methods

There are many methods which can be utilised to achieve clinically successful gene delivery and expression. They can be, broadly, broken down into two main categories, viral and non-viral. Potential limitations exist with both approaches, with concerns mainly surrounding safety and efficacy. Traditionally, viruses were seen as the less safe option but conferred good expression, whereas non-viral methods had fewer concerns around safety but the transgene expression was not as good as that seen in the viral vectors (Wells, 2010; Zhou *et al.*, 2010).

1.3.1.1 Non-Viral Vectors

Non-viral vector methods can include a variety of vector backbones, different types of vector modification and vector delivery system, all mechanisms which are designed to achieve the best levels of transgene expression without instigating any negative reactions, particularly related to immune response. These methods can include, but are not limited to, direct 'naked' DNA delivery, DNA plasmid-mediated gene transfection, with gene delivery potentially augmented by electroporation, chemical agents or transposons.

Apart from the reduced safety concerns, non-viral methods also have the additional appeal of being less restrictive regarding size of insert, which can be quite limiting in a viral system.

1.3.1.1.1 Plasmids for non-viral delivery

There are many plasmids currently available for gene transfer. The initial plasmid proposed for the current studies is the Vical (CA, USA) plasmid pVR1012, with human cytomegalovirus (CMV) promoter and kanamycin resistance.

Plasmids are circular molecules of DNA which can replicate independently of the host chromosomes. They are non-essential for normal cell function. An area of selectivity, which is often based on antibiotic resistance, is an advantage in cloning and bacterial growth, as only the cells that show this antibiotic resistance can grow. For example, the plasmid chosen for study in this thesis, pVR1012, contains a region of kanamycin resistance, so when grown in media containing the antibiotic kanamycin, as part of the transformation process, only the bacteria containing the vector, and therefore resistant to this antibiotic, will survive, therefore producing a batch of bacteria all of which should contain the plasmid.

The pVR1012 constructs, including those able to express Exendin-4 GLP-1 and eGFP, were already available within the group. The Exendin-4 and GLP-1 sequences, which both include an upstream human preproinsulin signal peptide sequence, were sub-cloned between BamH1 and EcoRV restriction sites whereas the eGFP sequence was sub-cloned between two Xba1 restriction sites (Mahmoud, 2010). Inserting the transgenes downstream of the human signal sequence enables continuous secretion via the constitutive secretory pathway. Using known restriction sites enables the bacterially grown and selected subsequent batches of plasmid preparations to be checked easily via restriction digests and standard gel electrophoresis.

Another region of interest in pVR1012 vector is the promoter region. In this case it is a constitutively active CMV promoter. Figure 1-4 is a diagrammatic representation of the pVR1012 plasmid map, and the site of insertion for each of the transgenes.

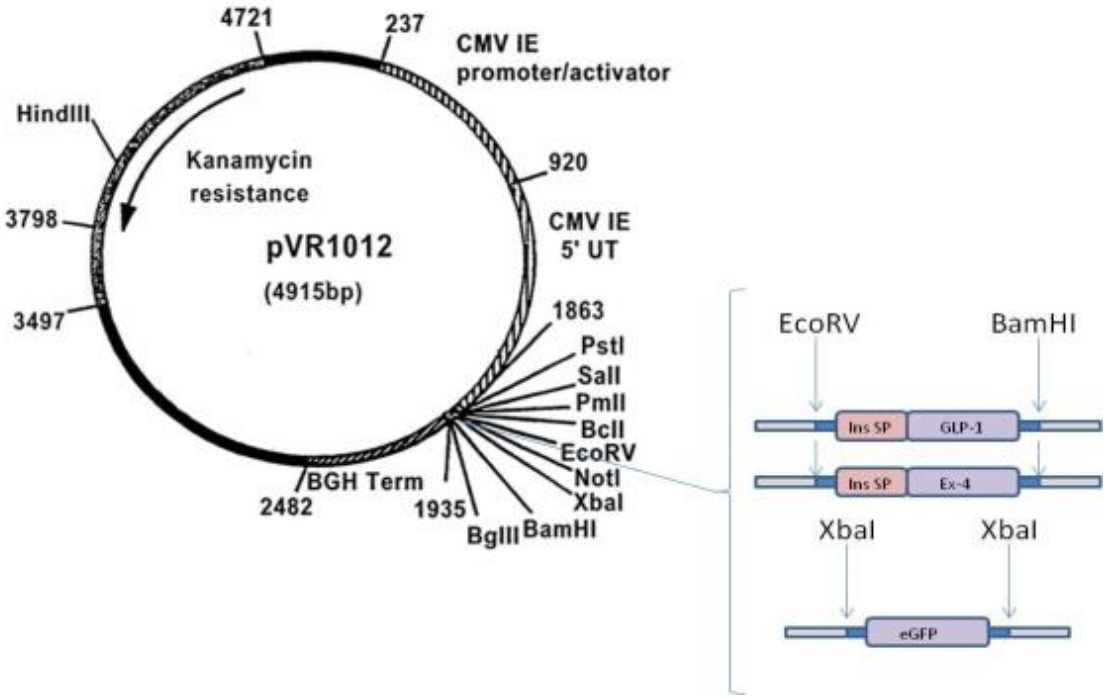


Figure 1-5 – Plasmid map of pVR1012 showing the points of insertion of the GLP-1, Ex4 and eGFP sequences (Audonnet et al., 2003; Mahmoud, 2010)

1.3.1.1.1.1 Plasmid promoter sequences

Promoters are an integral part of a plasmid system. They regulate the transcription process and determine the expression type of the transgene within the transfected cells. Transgene expression can be what is known as 'constitutive', which is constant expression, or inducible, which is regulated by transcription factors. Inducible promotors can also be used to 'dial' up or down the amount of expression generated.

Constitutive systems aim to provide continuous expression of the gene of interest by allowing continuous transcription. Most constitutive promoters are viral based, due to providing stable expression in many tissue type. The most common viral based promoters include human cytomegalovirus (CMV), Rous sarcoma virus (RSV) and simian virus 40 (SV40). Wells (1998) showed CMV gave greater transgene expression efficiency when used via skeletal muscle injection over RSV or SV40 promoters (Wells *et al.*, 1998).

In pVR1012 the CMV promoter DNA sequence initiates transcription of not only the transgene sequence but also the antibiotic resistance gene. Other vectors may also have other regions that need to be expressed along with the transgene of interest, such as a reporter gene like GFP. This allows, once validated, a direct correlation to be made between expression of the reporter gene and expression of the transgene, meaning non-invasive monitoring of expression can be made throughout the study. This is particularly advantageous when completing long term *in vivo* investigations.

In addition to basic function of promoters, there have been many studies conducted on the effect of different promoter types when used as a target for specific tissues. For example, Wang *et al* (2008) showed that modified variants of the muscle-specific promoter muscle creatine kinase (MCK), gave high efficiency expression in muscle cells (*in vitro* in C2C12 and *in vivo*), equal to or greater than CMV, but showed no activity in other cell types (Wang *et al.*, 2008)

Inducible or regulatable systems have been developed to control the level of transgene expression to a clinically beneficial level. Too little or too much and the protein levels would remain ineffective or could generate potentially unsafe levels. Systems that control expression include, but are not limited to;

- Rapamycin induced
- GAL4
- Tetracycline transcriptional systems

An example of one of the first inducible systems is tetracycline (Tet). This chemical based induction allows the expression to be tightly controlled. Expression is controlled by managing the transcription of the transgene via Tet regulation. This can be an on or off system, and the amount of Tet can be used to control the amount of expression (Scougall *et al.*, 2003; Wilson *et al.*, 2005).

1.3.1.2 Viral Vectors

In viral vectors, the DNA of the gene of interest (therapeutic or otherwise) is introduced into the virus and packaged in such a way in which the gene of interest is expressed. Expression can be constitutive or regulated. Viral vectors can be further subdivided into those vectors which integrate into the host chromosome (chromosomal) and those which do not (episomal)(Kootstra and Verma, 2003). Table 1-1 outlines the pros and cons of each vector type described.

Virus	Advantages	Disadvantages
Adeno-associated viruses (AAV)	Non-pathogenic, Long term expression	Low level 'production' capabilities
Retroviruses	Long term expression	Infects dividing cells only, potential oncogenesis
Adenoviruses	Good safety profile	Innate immunity

Table 1-1 – Pro/Con list of most popular viral vectors

1.3.1.2.1 Adeno-associated viral (AAV) vectors

AAV's belong to a class of virus called *Parvoviridae dependovirus*, which means they are a single stranded parvovirus and require 'helper' viruses to allow them infect and replicate. The most common helper viruses are adenovirus (AD) or herpes simplex virus (HSV) (Choi *et al.*, 2005; Wu *et al.*, 2006). There are currently 11 classified serotypes of naturally occurring AAV (Choi *et al.*, 2005), all of which share similar advantageous characteristics for use as a vector, depending on the nature of the therapy and the target cell or tissue. Parvoviruses are non-pathogenic to humans and therefore do not generate an innate immune response (Choi *et al.*, 2005; Wu *et al.*, 2006; Hirsch *et al.*, 2010), they are capable of transducing both dividing and non-dividing cells (Hirsch *et al.*, 2010; Tang *et al.*, 2010) and their DNA exists as epsiomes therefore not requiring integration into the host chromosome for expression(Hirsch *et al.*, 2010).
Although parvoviruses do not generate an innate immune response, some recent studies have shown that most people have been exposed to wild type AAV2 and have therefore generated neutralising antibodies (NAbs) which may impede the viral vector transduction and prevent any expression (Zaiss and Muruve, 2008; Li *et al.*, 2012). As the

name suggests, NAbs neutralize the biological effect by binding with the target antigen located on the surface viral particle.

Of the 11 serotypes, AAV 2 was the most commonly used for several reasons, the primary one being that it was the first AAV to be cloned into a bacterial plasmid in 1982 by Samulski and is therefore the serotype that has been most studied (Wu *et al.*, 2006). A basic map and schematic of AAV 2 (encoding eGFP) can be seen in Figure 1-6. However, as AAV use has been increasingly used in research, each of the serotypes has gained prominence in several specific fields.

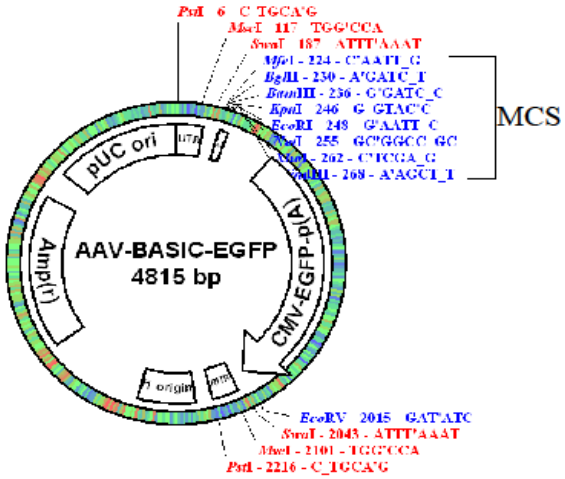
Several AAV serotypes have been used as the gene therapy vector for clinical trials for various diseases and disorders, including haemophilia, muscular dystrophy and cystic fibrosis, which was the first AAV human gene therapy trial which began in 1995 (Grieger and Samulski, 2005; Wu *et al.*, 2006). Studies of particular interest relevant to this project are those using skeletal muscle as a target tissue, such as Murakami *et al (2011)* using a recombinant AAV2 vector demonstrating continued expression of luciferase in mice, using the MCK promoter, post intramuscular (IM) injection (Murakami *et al.*, 2011) and Bosch *et al (2013)* who have demonstrated long-term (4+ years) glucose homeostasis in diabetic beagle dogs using AAV1 (Callejas *et al.*, 2013).

Recombinant AAV (rAAV) vectors are engineered by removing two viral portions of the genome, rep and cap. In their place, a transgene expression cassette is added(Choi *et al.*, 2005).

One major limitation of AAV's is the size of the DNA fragment inserted must be less than 4.8Kb, this causes problems when AAV would be the vector of choice for diseases such as muscular dystrophies, where the entire dystrophin gene is 2.3Mb (Bushby *et al.*, 1992; Wang *et al.*, 2012).

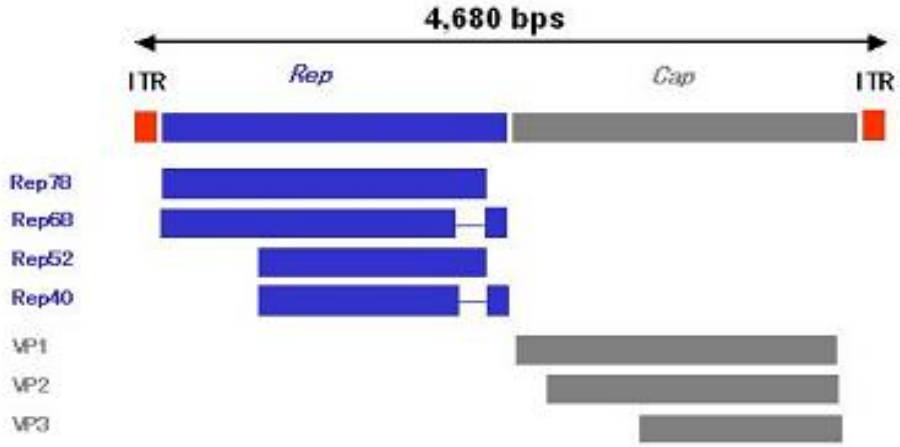
Map of AAV-BASIC-EGFP

MCS INFO:
MfeI(224),
BglII(230),
BamHI(236),
KpnI(246),
EcoRI(248),
NotI(255),
XhoI(262),
HindIII(268)



Integrity of recombinant vector should be double checked with PstI and MscI

AAV2 Genome Map (Schematic)



Rep78/68: involved in genome replication
Rep52/40: involved in genome packaging.
VP 1/2/3: capsid proteins.

Figure 1-6 – Example of an AAV construct which could be used for viral gene therapy. *This particular example shows the map of a basic AAV serotype 2 from VectorBiolabs (Philadelphia, PA) (Vector Biolabs) (Vector Biolabs)*

1.3.1.2.2 Retrovirus vectors

Retroviruses are enveloped, single stranded RNA viruses which contain four main gene regions; *gag* encodes for the structural capsid proteins, *pro* the viral protease enzymes, *pol* encodes for integrase and viral reverse transcriptase and *env* refers to the envelope glycoproteins. Retrovirus vectors are only able to infect dividing cells.

The most commonly used retrovirus for vector usage is the Moloney leukaemia virus (Mo-MLV) although other viruses have been used such as HIV. As with AAVs, retroviruses do not induce any immune response from the host and have been shown to provide sustained expression for over 2 years in humans, due to the virus integrating into the host genome. However, said integration is random and depending on where the insertion occurs, this could alter the host gene which may induce cancer (Stone *et al.*, 2000).

Clinical success with a retroviral vector has been seen in SCID patients, however, four out of nine patients went on to develop a leukaemia-like state (Hacein-Bey-Abina, 2008).

1.3.1.2.3 Adenoviral vectors

Adenoviral Vectors (AV) are suitable for gene transfer into both dividing and non-dividing cells. The AV genome remains extrachromosomal within the nucleus therefore sustained long-term expression would require repeated exposure and is therefore one of the major drawbacks of using this particular vector. The extrachromosomal nature does however remove the safety concerns around random insertion into the host genome, and the resulting cancer fear, as seen with the retroviral vectors. AV does still induce an immune inflammatory response, however, it has been suggested that this may be favourable when using gene therapy for cancer, as this may aid in tumour destruction (Choi *et al.*, 2012).

1.3.1.3 Other methods

Several other methods have been developed in an attempt to increase the efficiency and efficacy of transgene expression. Most of these methods are based on the physical

process of permeabilising the membrane wall to allow the transgene DNA directly into the cell. Some of these are outlined as follows.

Electroporation is a technique which is often also used in conjunction with skeletal muscle injection. This method uses an external electrical field across the site of injection in order to generate small (nano) sized pores in the cell membrane which allow water-soluble molecules to cross through the hydrophobic lipid layer(Lee and Deng, 2012). Electroporation aided delivery has shown very good transfection efficiencies in both *in vitro* and *in vivo* studies. Although some areas of damage can sometimes be seen in muscle fibres post electroporation, this is often minimal and does not have enough effect to deter the high efficiencies seen with this technique.

The electrical field can be induced using several types of electrode, depending on the transfection system. Small calliper electrodes are commonly used *in vivo*, these are non-invasive plates which can be placed either side of a limb to generate the electrical field required across the target site. Other electrodes include, needle electrodes, which is an invasive *in vivo* technique in which the needles are inserted directly into the muscle tissue, and various *in vitro* electrodes.

The electrical field is generated using a range of pulsed voltage 'waves', which have been studied to obtain the most optimum settings for a range of systems. Mir *et al* (1999) determined that the optimal electrotansfer efficiency into skeletal muscle was found with 8 pulses (20ms in length) with a low intensity setting of 200V/cm and at a frequency of 1Hz, when determined experimentally *in vivo* (Mir *et al.*, 1999).

In addition to the enhancement provided by electroporation, Mennuni *et al* (2002) showed that pre-treatment with hyaluronidase further increased gene expression approximately 5 fold with no permanent damage or injury seen to the tissue post procedure (Mennuni *et al.*, 2002). Hyaluronidase hydrolyses hyaluronic acid, a major component of the extra cellular matrix found in connective tissue. Connective tissue protects skeletal muscle fibres against potentially harmful organisms such as bacteria and viruses, and therefore would also hamper any other exogenously introduced compounds, such as DNA/DNA carrying vectors. The hydrolysis of this component of connective tissue provides further permeability to allow DNA access to the target tissue.

Ultrasound or sonoporation utilises the 'microbubble collapse during acoustic cavitation' (Yoon and Park, 2010). There have been a number of different studies investigating the use of sonoporation on various different tissues types. The overall results suggest that whilst sonoporation yields a lower transfection rate than electroporation, the damage caused in doing so is much less. This gives sonoporation some grounds for continued investigation (Wells, 2010).

Nano-particle encapsulation, as described by Zhou et al, the Nano-particles are contained in a calcium-phosphate and lipid shell which encases the transgene DNA, to protect it during delivery. Once inside the target cell, the case 'self-destructs' but does not generate a strong immune response due to it being formed of components found in the body already. This vector has been reported to be 2.5-10 times more effective than other methods (Zhou *et al.*, 2010)

Carbon nano-tube spearing is where nickel-embedded nanotubes are driven into cell membranes by a magnetic field. It has been shown that plasmids containing eGFP were immobilised onto the tubes and 'speared' into target cells. The transfection rates were reported to be 'high'(Cai *et al.*, 2005).

Chitosan nanoparticles are glucosamine-based polymers. They have been shown to have high *in vitro* transfection rates when delivering both GLP-1 receptor agonists and siRNA aimed at silencing DPP4 activity(Jean *et al.*, 2012)

pFAR plasmids, which have been developed at INSERM, France, are novel plasmid vectors that are free from antibiotic resistance (Marie *et al.*, 2010). This group have shown these plasmids remain excellent at providing sustained expression and secretion, as seen with non-viral vectors, without the dangers surrounding any antibiotic resistance that may occur using traditional plasmid vectors whose selectively is based solely on antibiotic resistance.

1.3.2 Muscle targeted gene therapy

Muscle targeted gene therapy is currently under investigation for several diseases and disorders, including, but not limited to; several muscular dystrophies (Mendell *et al.*, 2012; Wang *et al.*, 2012), factor IX expression for haemophilia B (Buchlis *et al.*, 2012) and for the systemic secretion of various therapeutic proteins, including inflammatory cytokines and erythropoietin(Mir *et al.*, 1999) as well as insulin therapy for diabetes (Niessen *et al.*, 2012).

This is mainly due to the ease at which transgene expression is obtained. Skeletal muscle has the ability to uptake naked DNA by a simple injection, the muscle fibres are stable, end-differentiated, multi-nucleated fibres and are thought to persist for the lifetime of the individual. Even if the muscle fibre is damaged, only part of the fibre is affected, the remaining fibre structure, and nuclei contained within, survive. This provides an exceptionally stable substrate for prolonged gene expression (Lu *et al.*, 2003). Furthermore, it is easily accessible and with it being a very well vascularised system, any protein secretions are circulated in a short period of time. (Aihara and Miyazaki, 1998; McMahon *et al.*, 1998).

The safety profile of skeletal muscle targeted gene therapy has been proven in humans previously, through several clinical trials. Bowles *et al* (2012) showed, during a phase 1 trial, that the novel AAV2.5 viral vector was effective in delivering a mini-dystrophin gene into the bicep muscle in Duchenne muscular dystrophy suffers (Bowles *et al.*, 2012).

Prior to *in vivo* studies taking place, *in vitro* investigations can be undertaken using primary muscle cell culture or immortalised cell lines. C2 cells are an immortal mouse myoblast cell line originally obtained by serial passage of myoblasts of the thigh of C3H mice after crush injury in 1977 (Yaffe and Saxel, 1977) and then sub-selected to C2C12 cells by Blau *et al* in 1985 (Blau *et al.*, 1985) . C2C12 cells were employed in this thesis due to their ease of use with regards to growth and differentiation. There are little to no sources which detail any age (passage) limitations.

Other skeletal muscle lines are available, such as L6 – Rat skeletal muscle, and of course human primary lines, however, as the next stage was murine *in vivo*, it was thought logical to utilise the murine cell line.

1.3.3 Current diabetic gene therapy

Several studies have been conducted which demonstrate the feasibility of using gene therapy methods as a promising new route for diabetes treatment.
Shaw *et al* have previously demonstrated that long term secretion of human insulin has resulted in a reduction of blood glucose levels in both disease and non-disease models, when administered via skeletal muscle injection in plasmid format (Shaw *et al.*, 2002) Other groups have had more success utilising viral based methods in conjunction with GLP-1, such as Riedel *et al* (Riedel *et al.*, 2010) who have demonstrated successful expression and secretion of GLP-1 from pancreatic β-cells *in vivo* via a double stranded AAV vector of serotype 8 (DsAAV8) or Choi and Lee (Choi and Lee, 2011) who also demonstrated long term expression of GLP-1 with dsAAV, amongst others (Voutetakis *et al.*, 2010; Gaddy *et al.*, 2011; Gaddy *et al.*, 2012),which suggests that GLP-1 expression may be more compatible with viral based vectors. Samson *et al* (2008) demonstrated Exendin-4 levels were found 15 weeks post injection with an AD vector, and that this administration successfully improved glucose homeostasis, however, the *in vivo* model chosen was diet-induced obesity in standard black 6 mice rather than a model of spontaneous diabetes, which confers a greater affinity to the phenotypes seen in human T2DM.

These studies suggest that whilst great inroads are being made in developing a realistic therapy for T2DM, work is still needed to produce a vector which is consistent in its expression of the transgene of choice and has little chance of inducing any sort of toxicity or immune response.

1.4 Models for studying muscle-targeted gene therapy for diabetes mellitus

Disease models can be studied both *in vitro* and *in vivo*. In this thesis, I used an in vitro model of C2C12 cells to assess the capabilities of the vectors used, and two *in vivo* models: CD1 and db/db mice to assess the efficiency of expression and efficacy.

The C2C12 cell line is a common *in vitro* muscle cell line which is well established and studied and provides a very stable phenotype for repeat studies. Myoblasts can be induced to form multinucleated myotubes (often characterised by having 3 or more nuclei) and are used for various circumstances which require mature skeletal muscle fibre as a model.
It was first established in the Blau lab from primary murine skeletal muscle.
This study aims to confirm the efficacy of muscle targeted gene therapy, with skeletal muscle being the route of ingress for the treatment. As the *in vivo* disease model is murine, it is the most sensible option to use a murine *in vitro* model also (Yaffe and Saxel, 1977; Burattini *et al.*, 2004).

Type 2 diabetes in rodents can be split into three groups; spontaneous, chemically induced and transgenic (Rees and Alcolado, 2005).
The 'db/db' model is a spontaneous homozygous point mutation on the leptin receptor gene (*Lepr* ^{db}) which leads to polyphagia, polydipsia and polyuria. Mice become obese at approximately 3 weeks old and plasma insulin levels being to rise at 10-14 days old. At anywhere between 4 and 8 weeks old blood glucose levels being to rise. Exogenous insulin does not control the blood glucose levels. These elevated levels are uncontrollable and lead to severe β-cell death, which ultimately causes death of the animal at approximately 10 months old.
The back strain is C57BLKS/J, which confers 84% homology with the standard black 6 (C57BL/6) and 16% homology with DBA/2J and was initially maintained by Dr N Kaliss (hence 'KS'). The updated strain name (The Jackson Laboratory) is BKS.Cg-*Dock7*^{m}+/+*Lepr*^{db}/J, homozygous *Lepr*^{db}. The abbreviated form of this is 'db/db'. The homozygous strains are maintained with *Dock7*^{m} and *Lepr*^{db} in repulsion. This is commonly accepted to be a severe model for type 2 diabetes.

1.5 Overall Objectives

The principal aim of this thesis was to investigate the hypothesis that over-expression of an externally introduced gene, whether in an unmodified form such as GLP-1 or a GLP-1 homologue, such as Exendin-4, will alleviate the symptoms associated with T2DM without the need for daily or even weekly medical intervention. Efficacy, longevity and the most appropriate vector were to be determined experimentally.

To explore this hypothesis, this thesis had the following specific objectives:

- To establish *in vitro* and *in vivo models* for muscle targeted gene therapy.
- Compare expression obtained from plasmid constructs and AAV viral vectors for reporter gene studies.
- Compare GLP-1 and Exendin-4 plasmids and AAV vectors *in vitro.*
- Evaluate GLP-1 and Exendin-4 plasmids and AAV vectors in long term *in vivo* studies, in addition to the potential impact in diabetic models.

2 Chapter 2 - Material and Methods

2.1 Materials

Materials were purchased from several different sources however:

- All general reagents and chemicals were purchased from Sigma-Aldrich, Dorset, UK, unless otherwise stated;
- Tissue culture plastic ware was purchased from Greiner Bio-One Ltd, Gloucestershire, UK;
- Tissue culture media were purchased from Life Technologies, Paisley, UK;
- Primary antibodies were purchased from Abcam, Cambridge, UK and Phoenix Pharmaceuticals Inc., CA, USA;
- Secondary antibodies were purchased from Life Technologies, Paisley, UK;
- Restriction digest reagents were purchased from Promega, WI, USA.

Some assays and reagents needed for certain protocols were readily available as pre-made 'kits'. Table 1 indicates the kits used and the suppliers.

Analytical Kit	Supplier
GLP1 (active) ELISA	Millipore, MA, USA
Exendin 4 EIA	Phoenix Pharmaceuticals, Karlsruhe, Germany
Mouse Insulin ELISA	Mercodia, Uppsala, Sweden
Plasmid Purification and extraction kit	Qiagen Ltd, West Sussex, UK

Table 2-1 - List indicating pre-designed kit-based assays and suppliers

The vectors used are outlined in Chapter 1. (1.3.1.1 and 1.3.1.2).

The plasmid vector was a pVR1012 with a CMV promoter and Kanamycin resistance, and were already available within the group.

The AAV vectors were purchased from the Viral Vector Production Unit (UPV), University of Barcelona. Standard serotype 1 was used for all studies involving GLP-1 and Exendin-4. A series of serotypes were used for reporter analysis, with standard eGFP.

2.2 Methods

2.2.1 Cell culture and characterisation

C2C12 mouse muscle cell line (originally established in the Helen Blau laboratory (Blau *et al.*, 1985)), which was previously obtained and already available within the group, was seeded into a T75 flask from liquid nitrogen frozen stock, in Dulbecco's Modified Eagle Medium (DMEM) media containing 10% foetal bovine serum (FBS) and 1% pen/strep (100 U/ml each for penicillin and streptomycin) . This combination will be referred to as 'growth medium'. The growth medium was changed at least every other day, whilst cells were incubated at 37^{o}C in a humidified environment containing 5% carbon dioxide.
Cells were 'passaged' when they reached 70-80% confluence, which constituted of the following procedures: medium was aspirated, PBS was used to wash off any remaining serum and approximately 2-3 ml of a 0.05% solution of Trypsin/EDTA (containing 0.21 mmol Trypsin and 4.81 mmol EDTA) was added. The flask was then re-incubated for up to 15 minutes to achieve single cell suspension. Cells were regularly monitored during this re-suspension process to ensure no adverse effects occurred. After suspension, an equal amount of serum-containing DMEM was added to the flask to neutralise the action of Trypsin. The total volume of solution was transferred to a Falcon tube and centrifuged at 150 relative centrifugal force (RCF) for 3minutes. The supernatant was aspirated and the pellet re-suspended in an appropriate volume of growth medium. The cells were then prepared for counting and re-seeding as necessary.
If subsequent use of the cells included any staining, seeding would occur in standard size 6 well plates containing 22x22 mm coverslips layered with a fibronectin (Sigma-Aldrich, Dorset, UK) coating (10 µl/ml).
Fibronectin coating was achieved by steeping coverslips in 70% ethanol for 5 minutes and allowing to air dry. Coverslips were dropped into each well of a 6 well plate and 400 µl of fibronectin (10 µl/ml) was used to cover the surface of the coverslip. This was allowed to 'set' for 1 hour at room temperature (RT) or overnight at 4 ^{0}C.

2.2.1.1 Cell Counting

To calculate number of cells in suspension, 10 µl of the cell suspension was combined with 10 µl of 0.4% Trypan Blue (1:1 ratio). 10 µl of the total solution was added to a Neubauer chamber and observed under x20 magnification. The cells in the four corner squares of the counting chamber were counted and averaged. This number was then multiplied by 2 to take into account the 1:1 dilution factor and then multiplied again by 1 x 10^4 to obtain number of cells per ml.

2.2.1.2 Freezing and preservation of cells

To protect and store stocks of C2C12 cells, flasks at 70-80% confluence were trypsinised, separated and re-suspended in 1 ml cell freezing buffer (90% FBS, 10% DMSO) in a 1.5ml cryotube. The cryotubes were then stored at -80 ^{0}C overnight in a slow freeze 'Mr Frosty' Nalgene container (Thermo Scientific, Hemel Hempstead, UK) before being transferred to liquid nitrogen for long term storage.

2.2.1.3 Cell differentiation

Cells were grown in 6-well plates containing 22x22 mm coverslips until approximately 80-90% confluent. Medium was then changed to a reduced-serum medium (Differentiation medium), which consisted of DMEM containing 2% horse serum and 1% pen/strep. Differentiation medium was changed every 3-5 days until myotube formation was seen.

2.2.2 In vitro transfections using Lipofectamine2000

The transfection protocol followed the recommended steps included in the manufacturer-provided literature.

All transfections were carried out in cells growing on coverslips coated with fibronectin in a 6-well plate. The concentration of cells seeded per well was ~$2x10^5$ and transfections were performed at 70-80% confluence.

The following components and volumes are for 1 well of a 6-well plate:

10 µl of Lipofectamine 2000 was added to 150 µl Opti-MEM serum free medium and 2 µl of 1.0 µg/µl of DNA was added to 250 µl Opti-MEM (serum-free media). Both were incubated separately at room temperature (RT) for 5 minutes. After the incubation, both suspensions were mixed together gently by pipetting. Once mixed, 250 µl of combined suspension was added to each well. The plate(s) were returned to incubate at 37 ^{0}C, 5% CO_2 for 1-3 days, or as the study required.

2.2.3 Gene expression analysis of C2C12 cells using either reporter gene GFP or genes of interest Exendin-4 and GLP-1 by immunofluorescence staining

Cells grown on coverslips in 6-well plates were fixed using fresh 4% paraformaldehyde (PFA) for 15-20 minutes on a shaker. After fixing, the cells were washed twice in phosphate buffered saline (PBS) (at this point, cells could be stored at 2-8 ^{0}C in PBS until needed). PBS was removed and cells were permeabilised in 0.5% Triton X-100 in PBS by shaking at RT for 10 minutes. The Triton X-100 solution was removed and cells were washed twice in PBS. PBS was removed and the non-specific binding blocking buffer 20% FBS in PBS was added to the wells. Subsequent incubation was at RT for approximately 1 hour on a shaker. The blocking buffer was removed and the cells washed twice in PBS. PBS was removed and the required primary antibody was added, at the required concentration (see Table 2-2) in 0.05% FBS in PBS. For all fluorescent staining, primary antibodies were used in conjunction with Invitrogen Alexa Fluor 488 or 568 secondary antibodies. The cells were incubated at RT for 1 hour on a shaker. After incubation, the cells were washed twice in PBS. The PBS was removed and the appropriate secondary antibody was added, at a 1:500 dilution in 0.05% FBS in PBS. The plate was incubated in the dark at room temperature for 1 hour on a shaker. After incubation, the solution was removed and the cells were washed twice in PBS. PBS was removed and a 10 µg/ml working solution of 4',6-diamidino-2-phenylindole (DAPI) was added. This was incubated

at RT in the dark on a shaker for 5 minutes. The DAPI solution was removed and cells were washed twice with PBS. The second wash was left for 3-5 minutes to reduce the intensity of the DAPI stain. After 5 minutes, the coverslips were removed from the wells and mounted onto microscope slides using Vectashield and sealed with clear nail varnish. The slides were labelled appropriately and stored in the dark at 2-8^{0}C until needed.

Alternatively, Vectashield containing DAPI was used; in which case, coverslips were washed with PBS and mounted directly onto slides without the need for a DAPI staining step.

Primary Antibody	Supplier	Dilution (In 0.05% FBS in PBS)
Desmin	Abcam, Cambridge, UK	1/100
Exendin-4	Phoenix Pharmaceuticals, Karlsruhe, Germany	1/50
GLP-1	Abcam, Cambridge, UK	1/1000

Table 2-2 – primary antibody dilutions used for immunohistochemical analyses

2.2.4 Visualising stained cells

To analyse cells from *in vitro* samples (e.g. C2C12 transfection studies), the cells were seeded on fibronectin coated coverslips (10 µl/ml) which were then used in various capacities, depending on requirements.

In all cases, either a Nikon Eclipse E400 fluorescent microscope with a DXM1200 camera (Nikon, Sendai, JP) or a Zeiss AxioImager Z1 with CoolSnap HQ2 camera (Zeiss, Oberkochen, DE), in conjunction with either Qcapture Pro image capture program (QImaging, British Columbia, CA) and Lucia GF counting software (version 3.81)) or Axiovision (version 4.8.2) (Zeiss, Oberkochen, DE), respectively, were used for analysis. Images were subsequently further analysed using ImageJ (version 1.48u) (Wayne Rasband, NIH, US.

2.2.4.1 Percentage transfection efficiency

Using the pVR1012-CMV-GFP plasmid, transfections were performed (see 2-2-2) and DAPI stained (see 2-2-3). Ten fields of view per coverslip were counted in a randomised yet consistent procedure in an attempt to eliminate any bias, with percentage transfection efficicncy established by calculating the number of cells showing positive GFP expression as a proportion of the number of nuclei in a particular field of view.

2.2.5 Growth medium

Luria Bertani (LB) agar containing the antibiotic kanamycin (50 mg/ml) was made using the following recipe:

Component	Amount
Tryptone	10.0 g
Sodium chloride	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
Antibiotic	50 µg/ml final concentration (added once media cool to touch)

Table 2-3 *Kanamycin containing LB agar recipe*

LB broth was made to the same ratios as in Table 2-3, minus the agar.

2.2.6 Transformation of competent bacteria

Bacterial competent cells (*E.coli* DH5α) were used for transformations.
The transformation method was provided with the competent DH5α cells.
In short, sterile, polypropylene culture tubes, the frozen (-80 ^{0}C) competent cells, cold super optimal broth (SOC) medium and pipette tips were placed on ice to cool, or, in the case of the cells, thaw. Once thawed (approx. 5 minutes), the cells were mixed gently and 100 µl was transferred to a culture tube (100 µl per transformation). Ten ng (in 10

µl) of DNA was added to the 100 µl of competent cells. The suspension was mixed by flicking. The tubes were returned to ice for 10 minutes before being heat-shocked for 45-50 seconds at 42 ^{0}C. After heat shock, the tubes were returned to ice once more for a further 2 minutes. After the 2 minute ice incubation, 900 µl of SOC medium was added to each transformation reaction. The tubes were then incubated at 37 ^{0}C on a shaker at approximately 225 rpm for 60 minutes.

After incubation, the transformation reaction was plated onto LB/kanamycin plates and incubated at 37 ^{0}C for 12-14 hours.

The antibiotic Kanamycin was added once the LB medium was cooled to touch (The Duran bottle containing the sterilised media should be warm to the touch but not so hot that it can't be held with bare hands) after steam sterilisation – 121^{0}C for a minimum of 15minutes, before being poured aseptically into a 90 mm Petri dish.. The plates were left to solidify in an aseptic atmosphere before being stored in a cold room until needed.

Fifty ng (in 1 µl) of DNA was added to 50 µl of competent DH5α cells and were mixed gently (not pipetting). The tubes were incubated on ice for 30 minutes, then transferred to a heat block for heatshock treatment at 45 ^{0}C for 30-45 seconds, then transferred to ice again for 2 minutes. Subsequently, 950 µl of SOC media was aseptically transferred to each tube and incubated for a further 60 minutes at 37 ^{o}C in a shaking incubator at 225 rpm. After incubation, 250 µl of the mixture was spread onto an LB plate, containing the antibiotic. The plates were re-incubated at 37 ^{0}C for 24-48 hours, or until bacterial growth was observed. Once growth was observed, the plates were stored at 4-8 ^{0}C until needed.

2.2.7 Obtaining plasmid

One colony-forming unit (cfu) of transformed *E.coli* was inoculated into 5 ml sterile LB broth containing kanamycin (see Table 3 – without agar) in a 50ml falcon tube and incubated at 37 ^{0}C and 300 rpm for 8 hours. This 5 ml so-called 'starter culture' was then inoculated into 500 ml sterile LB in a 2 L flask and again incubated under the same conditions for 12-16 hours. Post-incubation, the Qiagen Mega Prep protocol was

followed, with no deviations, as per literature provided within the kit. This resulted in a 1 ml stock solution of required plasmid DNA.

2.2.8 Spectrophotometry

The NanoDrop 2000 (Thermo Scientific, Hemel Hempstead, UK) Spectrophotometer was used to determine the concentration of DNA obtained from the Qiagen Mega prep kit (2.2.4.3). One µl of DNA was placed onto the loading pad. The two pads were then made to touch creating a column of fluid through which the light beam could be passed through. An absorbance curve was generated, measuring DNA peak at 260 nm and contaminating protein peak at 280 nm. The amount of DNA, calculated in ng/µl, was then used to standardise all DNA samples to 1 µg/µl.

2.2.9 Restriction Enzyme Digest

Restriction enzyme digests (double digests) were performed on GLP-1 and Exendin-4 plasmids, as per the Promega protocol (which was included as part of the literature provided with the enzymes), using enzymes BamHI and EcoRV.

The digest mix consisted of:

Component	Volume
Sterile, deionised water	16.3 µl
Restriction enzyme 10x Buffer	2.0 µl
Acetylated BSA (10µg/µl)	0.2 µl
DNA (1.0 µg/µl)	1.0 µl
Mix the above by pipetting, then add:	
Restriction Enzyme (10 u/µl)	0.5 µl
FINAL VOLUME	**20.0 µl**

Table 2-4- Restriction digest master mix

Tubes were then centrifuged briefly and gently mixed by pipetting before being incubated at ~37 ^{0}C for 1-4 hours.

2.2.10 Agarose gel electrophoresis

Plasmid fragments were separated and visualised on a 1% agarose gel, which was prepared by dissolving 1 g agarose powder (Melford, Suffolk, UK) in 100 ml of 1x TBE buffer (89 mM Tris-base, 89 mM Boric acid, 2 mM EDTA). Then, 4 µl of 10 mg/ml ethidium bromide (Sigma, Dorset, UK) was added to the molten gel. The gel was run with a potential difference of 70-90V across the gel using 1X Tris/Borate/EDTA (TBE) buffer.

2.2.11 In Vivo Studies

2.2.11.1 Ethics Statement and Animal details

All experiments were conducted under the Animals (Scientific Procedures) Act 1986 in accordance with the British Home Office animal licence guidelines and under a Project Licence (Project Licence holder: Professor James Shaw, Personal Licence holder: Gillian Patterson).
All animals were housed within the Comparative Biology Centre located within the University of Newcastle and kept on 12 hour light / dark cycles. All animals were allowed free access to stock chow and water, unless they were subject to fasting for glucose tolerance tests (see individual *in vivo* protocols in Appendix), at which stage they were allowed free access to water only.

All mice were sourced from Charles River, via the CBC facility within the university.
All 'normal' model mice used were CD1 females, with the exception of the initial eGFP reporter expression *in vivo* study, which used males (see 5.4.3).
All 'diseased' model mice were dbdb females.
The CD1 mouse is general-purpose model often used for wide range of studies with reliable, consistent baseline parameters.
Dbdb mice, originally developed from The Jackson Laboratory (JAX), form from a homozygous point mutation on the leptin receptor gene (*Lepr* ^{db}) which leads to

polyphagia, polydipsia and polyuria. Mice become obese at approximately 3-4 weeks old and plasma insulin levels being to rise at 10-14 days old. At anywhere between 4 and 8 weeks old blood glucose levels being to rise. Exogenous insulin does not control the blood glucose levels. These elevated levels are uncontrollable and lead to severe β-cell death, which ultimately causes death of the animal at approximately 10 months old.
The back strain is C57BLKS/J, which confers 84% homology with the standard black 6 (C57BL/6) and 16% homology with DBA/2J and was initially maintained by Dr N Kaliss (hence 'KS'). The updated strain name (The Jackson Laboratory) is BKS.Cg-*Dock7*^{m}+/+*Lepr*^{db}/J, homozygous *Lepr*^{db}. The abbreviated form of this is 'db/db'. The homozygous strains are maintained with *Dock7*^{m} and *Lepr*^{db} in repulsion. This is commonly accepted to be a severe model for type 2 diabetes.

All mice were brought into the university facility at approx. 3-4 weeks old, which is the earliest possible age mice can be sourced. After adhering to the 1 week acclimatisation period in place at the CBC, mice were approx. 4-5 weeks old at the onset of any study.

2.2.11.2 Non-invasive imaging using the In Vivo Imaging System (IVIS)

A non-invasive imaging technique was employed to monitor eGFP gene expression in the mice during the study. The mice were lightly anaesthetised using the inhalant isoflurane so as to render them immobile during the imaging process.
Mice were imaged under epi-illumination conditions from both dorsal and ventral views. The spectral un-mixing function in the software was utilised to subtract any possible auto fluorescence from the fluorescence seen from GFP expression. Regions of interest (ROI) were established and GFP intensity measured under the same conditions for each subject.
Living Image In Vivo Imaging software (Version 4.1) was used (PerkinElmer, MA, US).

2.2.11.3 Vector Administration

Plasmid administration was coupled with hyaluronidase and electroporation as a method to increase the efficiency of vector uptake and subsequent gene expression.

For plasmid administration, the mouse was anaesthetised lightly to prevent movement and reduce pain and stress via inhalant anaesthetic isoflurane. The analgesic carprofen was administered subcutaneously into the scruff of the mouse (dose 50 mg/kg, total volume 0.1 ml). Hyaluronidase was administered into the muscle in the location of the subsequent plasmid administration, at a concentration of 0.4 U/µl in a total volume of 25µl prior to plasmid injection, which was at a concentration of 1 µg/µl in a total volume of 50 µl. Post plasmid injection, the leg was subjected to electroporation (settings used: 200 V/cm, eight 20 ms pulses). The calliper electrodes were stainless steel plates and ECG gel was applied to the plates prior to electroporation taking place. All injections were made percutaneously to both tibialis anterior and gastrocnemius muscles, perpendicular to the central portion of the muscle.

AAV vectors were administered under the same conditions, without the use of the hyaluronidase or electroporation. The AAV vectors were administered in a total volume of 50 µl in the following titre of vector genome (vg) (as determined appropriate from published literature (Zincarelli *et al.*, 2008):

- Exendin 4 - $1.776x10^{11}$ vg/mouse
- GLP1 - $1.784x10^{11}$ vg/mouse
- eGFP - $1.745x10^{11}$ vg/mouse

Whole blood samples were obtained using standard tail venepuncture, as larger volumes (approximately 100 µl, depending on the weight of the animal) were required. The exact volume obtained was kept within the NC3R guidelines for blood sampling (which was also reflected within the PPL - https://www.nc3rs.org.uk/blood-sample-volumes

2.2.12 Tissue harvesting

2.2.12.1 Muscle samples

When indicated and after humane Schedule 1 euthanasia, muscle samples were harvested from mice and subjected to further processing (snap freezing or buffer immersion).

Samples for freezing were snap-frozen by placing them directly into isopentane, pre-cooled in a 70% ethanol/dry ice bath, until uniformly frozen. Samples were then placed into a pre-labelled cryotube and maintained on dry ice until longer term storage at -80 ^{0}C was possible.

Other samples were placed directly into 1 ml of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1.0 mM dithiothreitol and 10 µl/ml protease inhibitor (Roche, UK).

Frozen samples were sectioned using a Leica cryostat (Leica, Nussloch, DE), creating sections of 4-5 µm thickness after which they were mounted onto superfrost glass slides for subsequent staining.

Samples in buffer A were then homogenised by depositing them into Lysing Matrix D green-cap tubes containing 1.4mm ceramic beads (mpBio, CA, USA), and shaking them in a FastPrep instrument (mpBio, CA, US). The instrument was run for 20 seconds at a speed setting of 6.5 m/s to fully homogenise the tissue. If any further homogenisation was required, the sample was held on ice for ~5minutes before another run was performed. The resulting homogenate was centrifuged for 5 minutes at 12,000 RCF. The supernatant was collected in fresh eppendorfs and frozen at -80 ^{0}C until needed.

2.2.12.2 Pancreas samples

The pancreas was removed from each animal, weighed and placed directly into small universals containing 5 ml 10% formalin.

Samples were sent for processing into paraffin blocks and sectioned, using a Microm HM330 microtome (Microm, Heileberg, DE), at 4-5µm and mounted onto superfrost glass slides for staining.

2.2.13 Blood sampling

Blood samples were taken as directed by the protocol and in accordance with the Home Office Project Licence (PPL). Peripheral blood samples were taken using tail venepuncture method and collected in a blood capillary microvette (Sarstedt, Nümbrecht, DE).
Terminal samples were taken under terminal anaesthesia through cardiac puncture.

2.2.14 Analytical Methods

The following methods were employed for analysing the blood samples and the homogenised tissue samples.

2.2.14.1 Exendin-4 EIA

Exendin-4 concentrations in serum derived from blood samples from mice and the supernatant derived from homogenised muscle samples were analysed using a commercial exendin-4 EIA kit (Phoenix Pharmaceuticals, Karlsruhe, DE). The manufacturer-recommended protocol was followed without deviation.

2.2.14.2 GLP-1 ELISA

Equally, serum and muscle GLP-1 concentrations were evaluated using an active GLP-1 ELISA kit (Millipore, MA, US). Prior to serum separation, 10 µl/ml of DPP4 inhibitor

(Millipore) was added to the blood sample. The manufacturer's protocol was followed without deviation.

2.2.14.3 Mouse Insulin ELISA

Serum separated from blood sampled through cardiac puncture (under terminal anaesthesia) was analysed for insulin content using a mouse insulin ELISA kit (Mercodia, Uppsala, SWE). The blood was allowed to clot at room temperature before being quick spun to separate the serum from the clot. The manufacturer's protocol was followed without deviation.

2.2.15 Analytical method validation

Where appropriate, each assay was further validated to demonstrate accuracy and precision within the laboratory and across the length of the studies. Repeatability and intermediate precision was considered when performing these tests and additional samples were included where needed.

2.2.15.1 Inter assay Coefficients of Variability (CV) determination

Each ELISA/EIA type used was subject to both inter and intra assay validation. As this study relies on comparison of studies conducted at different times, it is important to have confidence in the robustness of the assays generating the data.
The generally accepted rule (as described by the Statistics department, Newcastle University) is inter assay % CV values should be less than 15 and intra assay % CV values should be less than 10. Inter assay % CV was determined by assessing the high, medium and low values, which are used to form the standard curve. The % CV for each assay type is determined by averaging each % CV gained.

Table 2-5 outlines the data obtained the data obtained from each Exendin-4 EIA used throughout the study. The instructions included in the kit protocol described manually obtained figures in ng/ml from the graph obtained from the standard curve. The results obtained in nm from the absorbance readings were more accurate and this raw data was used in inter assay precision determination.

	100ng/ml	1ng/ml	0.01ng/ml
Mean	1.892	0.574	0.111
SD	0.271	0.067	0.017
CV	14.339	11.642	15.703
Total % CV	13.9		

Table 2-5 – Inter assay % CV for Exendin-4 EIA. *Precision data from 3 different EIA plates, performed on 3 separate occasions using a high (100 ng/ml), medium (1 ng/ml) and low (0.01 ng/ml) concentration of protein standard as the sample.*

Table 1-6 outlines the data obtained from each GLP-1 ELISA used in the study. As with the Exendin-EIA, the determination of concentration was obtained manually from the subsequent standard curve. The raw data spectrophotometry absorbance was again used as this was more precise.

	2pM	10pM	100pM
Mean	432.458	1223.501	19551.776
SD	20.344	201.785	1678.065
CV	4.704	16.492	8.583
Total % CV	9.9		

Table 2-6– Inter assay % CV for GLP-1 ELISA. *Precision data from 3 different ELISA plates, performed on 3 separate occasions using a high (100 pM), medium (10 pM) and low (2 pM) concentration of protein standard as the sample.*

2.2.16 Solid tissue analysis

2.2.16.1 Haematoxylin and eosin staining

If stored at -80 ^{0}C, the frozen sections were left to warm up to RT prior to the staining procedure. The paraffin-embedded pancreas sections were de-paraffinised by submerging the slides in histoclear for 10 minutes and then re-hydrated by submerging in 100% ethanol for 3 minutes, 95% ethanol for 1 minutes and then rinsed in distilled water (dH_2O). The slides were then submerged in haematoxylin solution for 5 minutes before being rinsed with dH_2O. The next step consisted of washing the sections with Scott's tap water for 30-60 seconds followed by washing with dH_2O. After the wash, the slides were submerged in eosin solution for 1 minute before being washed again with dH_2O. After this final wash, slides were dehydrated by serial submersions in 50%, 70%, 95% and twice in 100% ethanol solutions, all for 1 minute each. The slides were then placed in 2 x histoclear solutions before being mounted with DPX using a coverslip. The sections were visualised using a Zeiss AxioImager Z1 with CoolSnap HQ2 camera in conjunction with Axiovision 4.8.2 software.

2.2.16.2 Immunoperoxidase staining – double labelling

Slides were de-paraffinised and re-hydrated as per standard protocol (10 minute de-wax in histoclear, 3 minute re-hydrate in 100% ethanol, 1 minute rehydrate in 95% ethanol, and rinse in PBS). The de-paraffinised slides were then submerged in methanol and hydrogen peroxide (200 ml and 3 ml respectively) for 10 minutes to block peroxidise activity. To retrieve the antigen (epitope exposure) the slides were placed in a pressure pan containing 10 mM sodium citrate (pH 6.0) and boiled at full pressure for 1 minute. The slides were immediately rinsed with cold running tap water and held in PBS until needed. To perform non-specific blocking, the sections were incubated in 20% FBS for 1 hour at RT, after the sections had been demarcated with DAKO marker (DAKO, Cambridgeshire, UK). Following blocking, the slides were washed twice with PBS before being incubated overnight (refrigerated) with primary antibody in 0.05% FBS in PBS. After this overnight incubation, the slides were washed twice with PBS before being incubated at RT for 1 hour with the secondary antibody. After incubation, the slides were again washed twice with PBS before being incubated for approximately 10 minutes with primary substrate, which varied depending on what was being examined (see Table 2-2). The slides were then washed twice with PBS and peroxidase action was again blocked by incubating in a solution of 1 ml hydrogen peroxide and 9 ml PBS for 10 minutes. The slides were washed twice with PBS and incubated in the blocking solutions avidin and biotin for 15 minutes each at RT. The slides were washed twice with PBS before being incubated in a non-specific block, 20% FBS in PBS, for 1 hour at RT. The slides were rinsed with PBS and cold incubated at 4 ^{o}C with the second primary antibody overnight. After the overnight incubation, the slides were washed twice with PBS and the second secondary antibody was added, slides were incubated for 1 hour at RT. The 'ABC' solution (Vectastain ABC kit, Vector Labs, CA, US) was made (30 minutes before use). The slides were washed twice with PBS before being incubated in the ABC solution for 30 minutes. The slides were given a final wash with PBS before being incubated for 5 minutes with the secondary substrate, rinsed in PBS, counterstained in haematoxylin for 5 minutes, washed with tap water and mounted using DPX (Sigma-Aldrich, Dorset, UK) and a coverslip.

2.2.16.3 Whole section imaging for transfection evaluation

Whole sections of frozen muscle were visualised using the Zeiss AxioImager software mosaic function. This allows each field of view to be stitched together to form an image the entire section at the desired magnification. Standard exposure limits were determined to ensure no variability from the software interfered with the intensity of the staining observed.

The image of the whole section was then imported into Image J software programme (http://imagej.nih.gov/ij/) to analyse the entire area in question. Image J is a free downloadable open access tool used globally by many researchers, and as such is classed as an approved peer reviewed system for analysing images.

For cell based analysis (myoblasts/myotubes), the field of view was uploaded to the software and a RGB colour deconvolution was performed. This separated the red, green and blue stained images from one another, ensuring no cross-over in expression was observed. The 'green' (eGFP) was converted to a binary image and the resulting area calculated as a percentage.

The same principle was applied to section images, however, as the image was the entire tiled area of the section, the section was drawn around to obtain a total area. The image was then RGB deconvoluted, turned to binary and the area obtained. The percentage area transfected was then manually calculated using the two figures.

2.2.17 Statistical analysis

Intra-peritoneal glucose tolerance test (IPGTT) analyses were performed using repeated measures analysis of variance (rANOVA) with the Bonferroni correction applied. The rANOVA is used in longitudinal studies when the same subjects are repeatedly measured under different conditions but the results are linked (a standard ANOVA assumes independence). The Bonferroni correction is applied as this controls the 'family wise

error rate' (the probability of making false discoveries) and is a standard correction filter when analysing multiple comparisons.

All other statistical measurements were performed using either t-test (paired or un-paired) or two-way ANOVA, depending on the nature of the data.

All data are reported as mean ± SD, unless otherwise stated.

3 Chapter 3 – Establishment of *in vitro* and *in vivo* muscle targeted gene transfer protocols and evaluation of the resulting expression, driven by viral and muscle specific promoters in plasmid constructs and AAV vectors in eGFP reporter gene studies

3.1 Introduction

For the purposes of this thesis, all 'reporter gene' studies refer to the expression and visualisation of the fluorescence gene GFP.

Initially, the wtGFP compound was isolated in the 1960's from the jellyfish Aequorea Victoria (Shimomura *et al.*, 1962). However, several engineered mutations have since allowed more widespread use of GFP derivatives and have provided several other colour formats to be used in multiple applications. The most commonly associated green colour was first reported by Tsien, 1995 which allowed the fluorescence to be observed under the FITC filter, remaining the most frequently used reporter gene format used today (Heim *et al.*, 1995).

In order to predict the outcome for skeletal muscle expression studies *in vivo*, *in vitro* models can be used to establish proof of concept. As discussed earlier, skeletal muscle is an attractive target for gene therapy as it provides a stable end-differentiated tissue platform for long term expression with good vascularisation for easy delivery into the circulating bloodstream.

C2C12 cells are a murine muscle cell line, initially derived from the C2 cell line, which in turn was obtained from the skeletal muscle of normal C3H mice in the Blau lab in the 1980's (Blau *et al.*, 1985). The C2C12 line is a very good model for *in vitro* muscular based studies as the cells readily proliferate but myogenic phenotype remains stable, generally up to approximately Passage 50 when cells are maintained appropriately. The cells can also be differentiated from myoblasts into myotubes which mimic somewhat the events seen *in vivo* with reference to skeletal muscle myogenesis from proliferative satellite precursor cells. Several studies have shown that C2C12 is a good cell line for evaluation of muscle-targeted gene therapy (Shaw *et al.*, 2002; Ratanamart *et al.*, 2007).

Three types of vector were used in GFP reporter gene studies to undertake initial evaluation of constitutive viral promoter driven plasmids (pVR1012.CMV.GFP);

eukaryotic muscle-specific promoter driven plasmids (pRRL.sin.PPT.DES.eGFP) and adeno-associated viral vectors:

- pVR1012.CMV.GFP is a plasmid construct which was originally obtained from Vical (CA, USA) with the genes of interest previously cloned between the EcoRV and BamHI regions within the group and therefore ready to use.
- pRRL.sin.PPT.DES.eGFP was donated by Prof. H. Lochmuller of the Musculoskeletal Research Group located in the Institute of Genetics at Newcastle University. This vector was included to evaluate a muscle specific promoter, in this case DES (desmin), in comparison to the promoter used in all other vectors CMV (cytomegalovirus).
- AAV.CMV.GFP is an AAV vector obtained from the Viral Vector Production Unit (UPV) of the University of Barcelona. Several serotypes were trialled *in vitro* methods to determine the most appropriate vector for *in vivo* study, including; AAV2/1, AAV2/5, AAV2/6, AAV2/8, AAV2/9 and AAV2/10.

3.2 Objectives

The potential for various muscle-based cell lines to successfully take up both plasmid and viral DNA has been shown previously. However, using the C2C12 cell line specifically and comparing directly with normal murine skeletal muscle *in vivo*, using a variety of methods was important to establish prior to starting investigations using Exendin 4 and GLP1 vectors.

Therefore, the main aims of this chapter were;

- To establish an *in vitro* muscle cell model and standard conditions for plasmid mediated transfection.

- To determine conditions for achieving expression in multinucleated myotubes.

- To establish an effective MOI for AAV expression in HEK293 cells and determine the most appropriate AAV serotype in C2C12 cells.

- To establish an *in vivo* live imaging protocol to evaluate reporter expression and delivery.

3.3 *In vitro* studies using the C2C12 cell line, employing Lipofectamine2000 as a transfection agent for plasmid DNA, and standard infection protocols for AAV vectors.

3.3.1 C2C12 Cell characterisation

C2C12 cells were seeded in 6 well plates with DMEM media containing FBS and pen/strep (2.2.1) and incubated in a humidified environment containing 5% CO_2 until 80-90% confluent myoblasts were observed. Once confluent, the media was changed to a reduced serum media (2.2.1.3) for differentiation into myotubes.

Skeletal muscle requires the mononucleated myoblast precursor satellite cells to fuse together to form multinucleated myotubes (confirmed *in vitro* by presence of 3 or more nuclei within a single cell).
Once the cells had been differentiated into myotubes, staining with the muscle specific marker Desmin confirmed myogenic origin. Desmin is a protein which is only expressed in certain cytoskeletal components found in vertebrates.

Figure 3-1 shows characteristic bipolar morphology of C2C12 myoblasts visualised under phase contrast microscopy.

Figure 3-2 shows standard myotube morphology, as seen under fluorescence microscopy, after the media serum concentration was stepped down to induce the myotube formation.
Desmin, a muscle specific marker, was used to confirm myogenesis. 100% of fused multinucleated myotubes showed desmin expression.

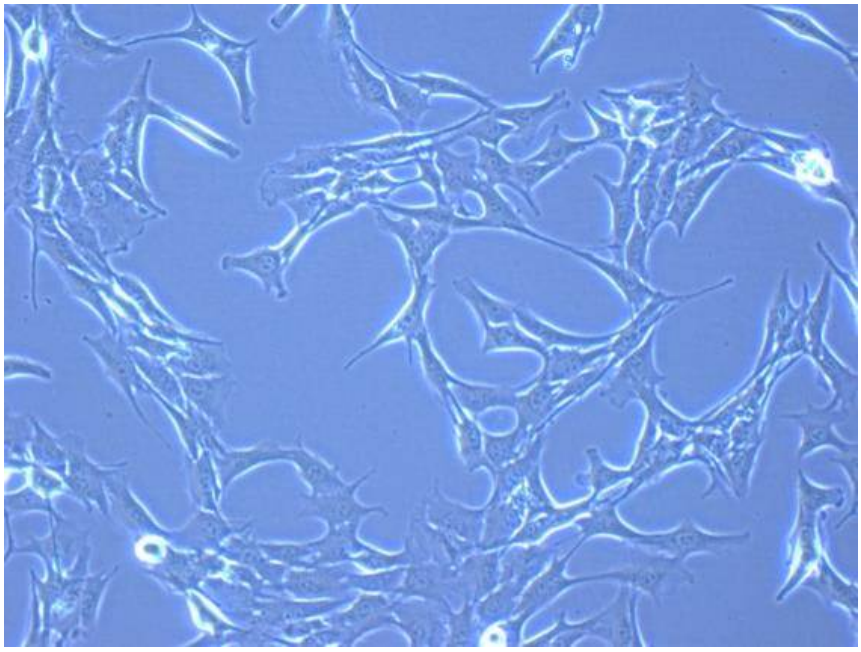


Figure 3-1 C2C12 myoblasts under bright field at x20 magnification. *All cells are mononucleated indicating that no end-differentiation has occurred and the cells are still in the precursor format. Standard growth media was used. Image was taken 48 hours after seeding.*

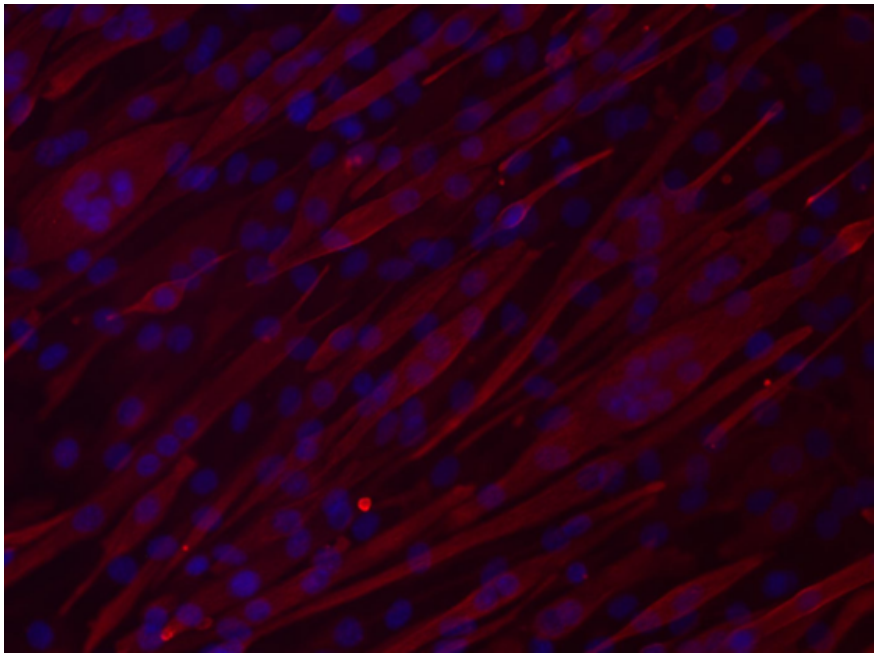


Figure 3-2 C2C12 myotubes stained for Desmin and highlighted with DAPI at x20 magnification. *Whilst not all of the myoblasts shown in the image have formed into tubes, 100% of tubes are showing positive desmin staining, shown in red, with the characteristic multi-nucleated structure. Cells were subject to stepped down media (reduced serum content) at 70-80% confluence. Image was taken 6 days after seeding, 2 days post reduced serum media change.*

3.3.2 *Determination of optimal in vitro transfection conditions for C2C12 myoblast cells with pVR1012.CMV.eGFP plasmid using Lipofectamine2000*

C2C12 cells were transfected using the standard Lipofectamine2000 protocol (2.2.2).

$1x10^6$ C2C12 cells were seeded into each well of a 6 well plate ($6x10^6$ C2C12 cells in total) containing 22x22mm coverslips coated with fibronectin (10 µl/ml) and were incubated at 37 ^{0}C until 70-80% confluent.

The Lipofectamine2000 transfections were performed using the pVR1012.CMV.eGFP plasmid at various concentrations. After 48 hours incubation, the coverslips were removed, the cells were fixed and a DAPI nuclear stain applied (2.2.3, 2.2.3.1).
The total number of cells adhered to the coverslip (counted as number of nuclei stained positive with DAPI) was compared to the number of cells showing positive GFP expression, (2.2.3.2), enabling determination of percentage transfection efficiency (%) for different Lipofectamine2000 concentrations (Figure 3-3).

This procedure was then repeated using the amount of DNA as a variable and the Lipofectamine2000 concentration as the constant (Figure 3.4).

Both of these studies conferred successful gene transfer, as confirmed by eGFP fluorescence (see Figure 3-3).

As there were no significant differences between any of the Lipofectamine2000 and DNA concentrations evaluated, the amounts of lipofectamine2000 reagent and DNA used in all subsequent protocols were 10 µl and 2 µg respectively. These concentrations have previously been demonstrated to be very successful in similar protocols used previously within the group.

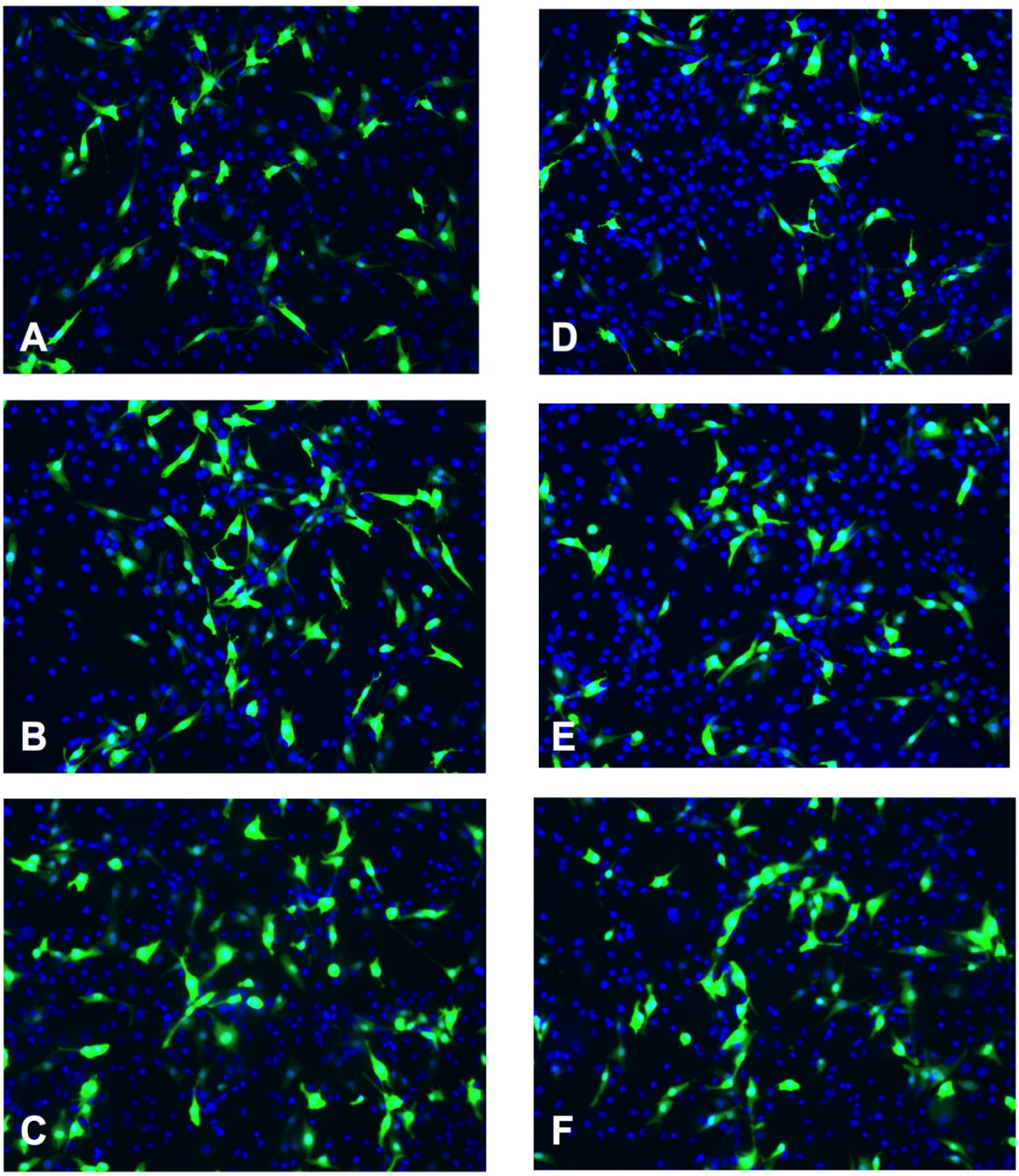


Figure 3-3 Examples of C2C12 myoblasts transfected with Lipofectamine2000 using the standard protocol. *Using 10µl Lipofectamine2000 and 2µg of 1µg/µl pVR1012.GFP.CMV as observed at 48 hours post transfection time using the variant described in figures 3-4 and 3-5. 2µg DNA/5µl lipofectamine2000 (A), 2µg DNA/10µl lipofectamine2000 (B), 2µg DNA/15µl lipofectamine2000 (C), 4µg DNA/10µl lipofectamine2000 (D), 3µg DNA/10µl lipofectamine2000 (E), 2µg DNA/10µl lipofectamine2000 (F). 10 fields of view were counted per condition. Each condition was assessed x6.*

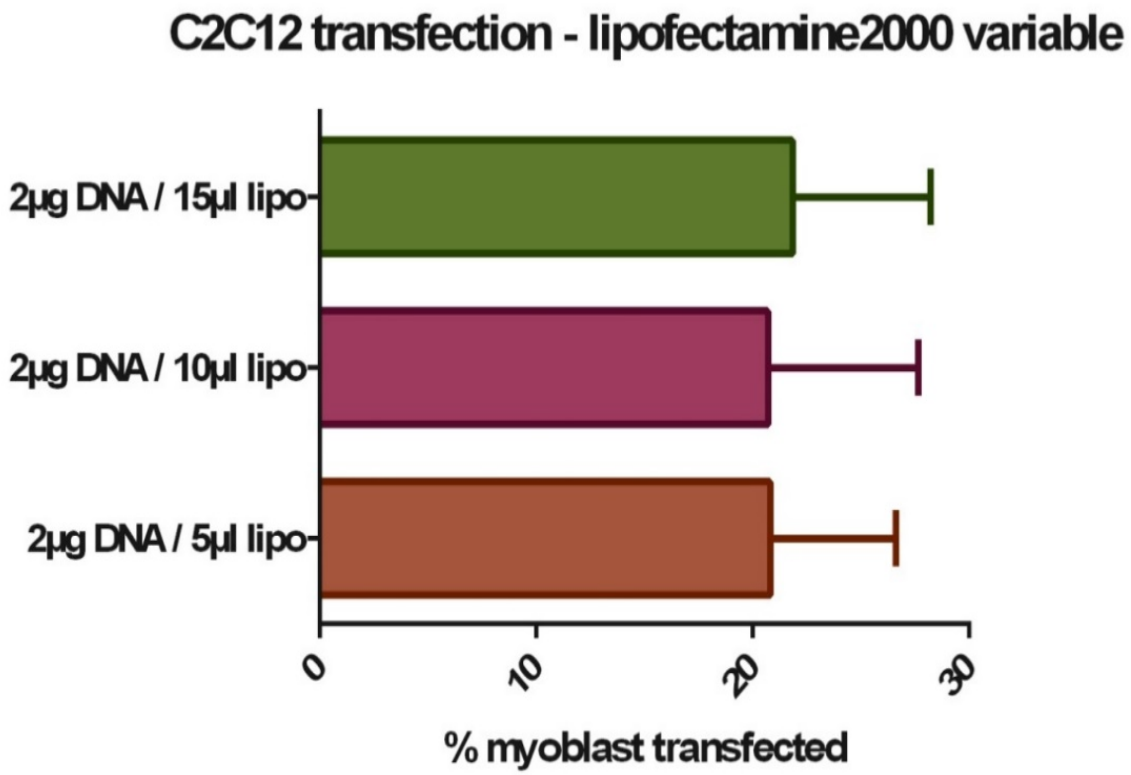


Figure 3-4 - % Transfection rates for variable amounts of Lipofectamine2000 reagent used in the transfection procedure. *Cells were seeded onto coverslips in each well of a 6 well plate. The coverslips were removed, cells fixed and mounted and visualised under a fluorescence microscope.10 fields of view were taken from each coverslip, the nucleus (DAPI) and transfected cell (GFP) were counted and the % transfection rate calculated (n=6).*
There was no significant difference in the percentage of myoblasts transfected when using 5-15 µl of Lipofectamine2000 and 2 µg of plasmid DNA ($p>0.05$). 10 µl was deemed to be the appropriate amount and was the amount used in similar protocols previously used within the group.

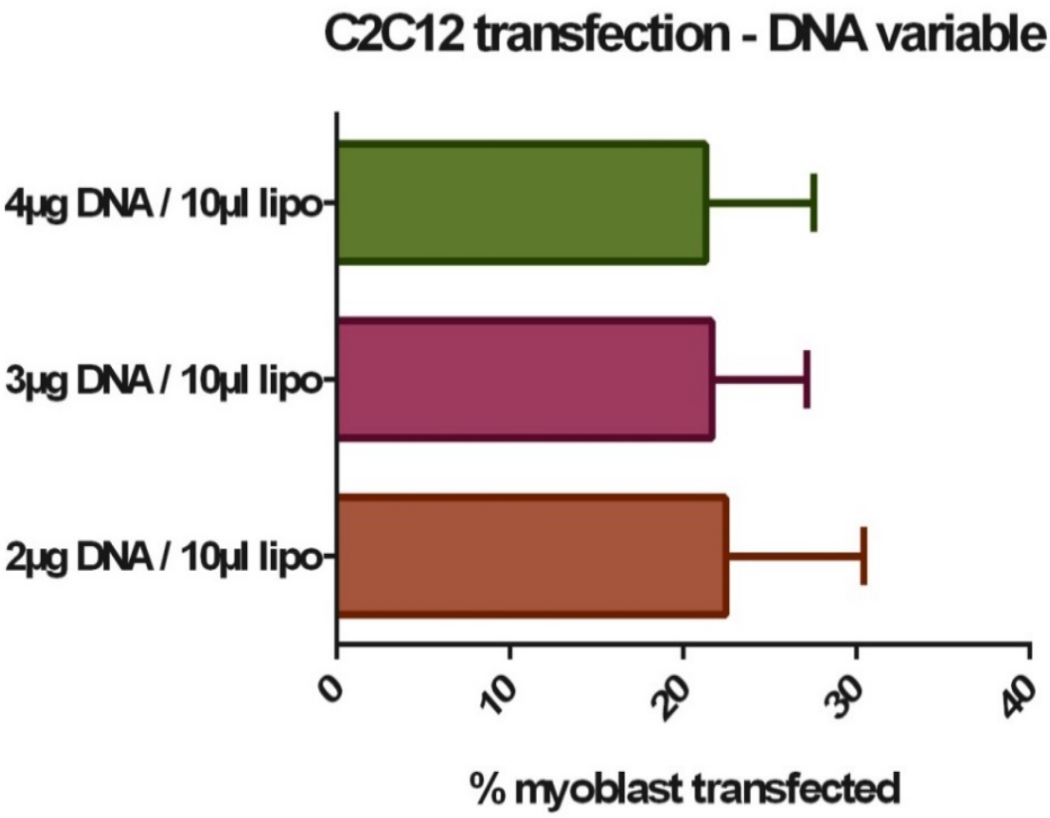


Figure 3-5 Transfection rates for variable amounts of plasmid DNA used in the transfection procedure*. Cells were seeded onto coverslips in each well of a 6 well plate. The coverslips were removed, cells fixed and mounted and visualised under a fluorescence microscope.10 fields of view were taken from each coverslip, the nucleus (DAPI) and transfected cell (GFP) were counted and the % transfection rate calculated (n=6).*

There was no significant difference in the percentage of myoblasts transfected when using 2-4 µg of plasmids DNA with 10 µl of lipofectamine2000 reagent when analysed (p>0.05). 2 µg was deemed to be the appropriate amount of plasmid DNA to be used in all future protocols.

3.3.3 *Determination of optimal in vitro transfection conditions for C2C12 myotubes with pVR1012.CMV.eGFP plasmid using Lipofectamine2000 and hyaluronidase*

As myotubes are more akin to the differentiated skeletal muscle targeted in *in vivo* transfection, C2C12 myotubes were transfected using the same protocol outlined in 2.2.2 for C2C12 myoblasts.

Myotubes did not uptake the pDNA as readily as the myoblasts, in fact, there was less than 2% percentage transfection efficiency observed using the standard Lipofectamine2000 protocol (Figure 3-6). In an attempt to increase this efficiency, hyaluronidase was added to the lipofectamine2000 protocol.

In order to determine the appropriate amount of hyaluronidase, 4 conditions were examined. Transfection of myoblasts that were allowed to differentiate into myotubes, 0U hyaluronidase, 32U hyaluronidase and 96U hyaluronidase.

In some instances, myoblasts were allowed to differentiate into myotubes before transfection took place. Hyaluronidase was added to standard DMEM growth media at 32U/ml or 96U/ml, 1 hour prior to transfection.

In other instances, myoblasts were transfected as per normal lipofectamine2000 protocol.

Control myotubes were not subject to hyaluronidase and underwent the standard lipofectamine2000 protocol, all under the same conditions.

Myotubes were allowed to grow up to 5 days post transfection.

As can be seen in Figure 3-7, the addition of the hyaluronidase substrate greatly increased the transfection efficiency in C2C12 myotubes. The images shown in Figure 3-7 are representative examples of the transfection efficiency shown for each varying amount of hyaluronidase. Based on the images alone, these suggest that the as the amount of hyaluronidase is increased, the transfection efficiency is increased.

Figure 3-8 substantiates the theory from Figure 3-7 that the increased amount of hyaluronidase increases transfection efficiency. The myotubes that have not been

exposed to hyaluronidase show negligible transfection efficiency, whereas 32U and 96U hyaluronidase double the transfection efficiency respectively.

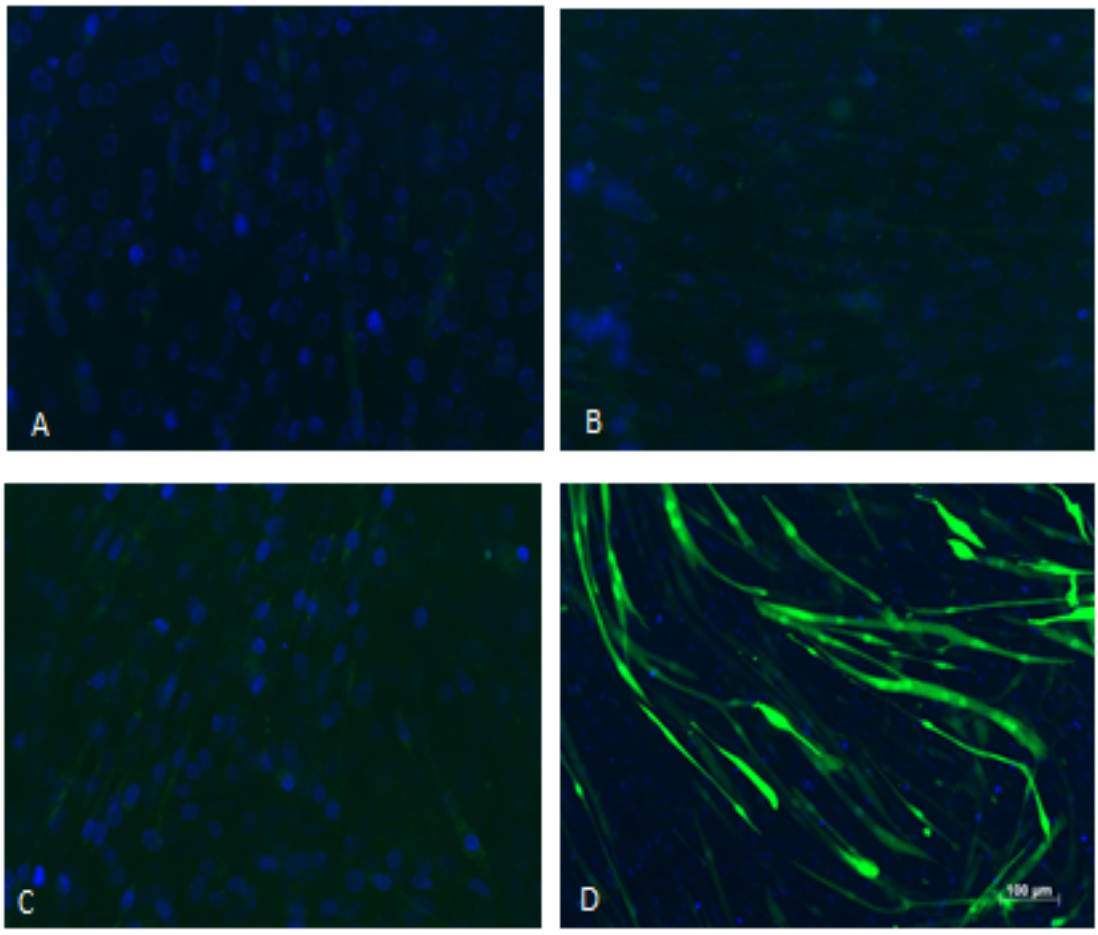


Figure 3-6 – Examples of transfected myoblasts and myotubes*. A, B and C are examples of C2C12 myotubes transfected with pVR1012.CMV.GFP, using the standard lipofectamine2000 protocol. D is an example of transfected C2C12 myoblasts which have been allowed to differentiate into myotubes. Images were taken 5 days post transfection, using Zeiss AxioImager Z1 with CoolSnap HQ2 camera and Axiovision software v4.8.2. Exposure times were set at 400ms for each image. Due to the difficulty in separating individual tubes in order to count the % transfection, in this case, each field of view was turned into a binary image and actual percentage area transfection calculated using ImageJ software.*

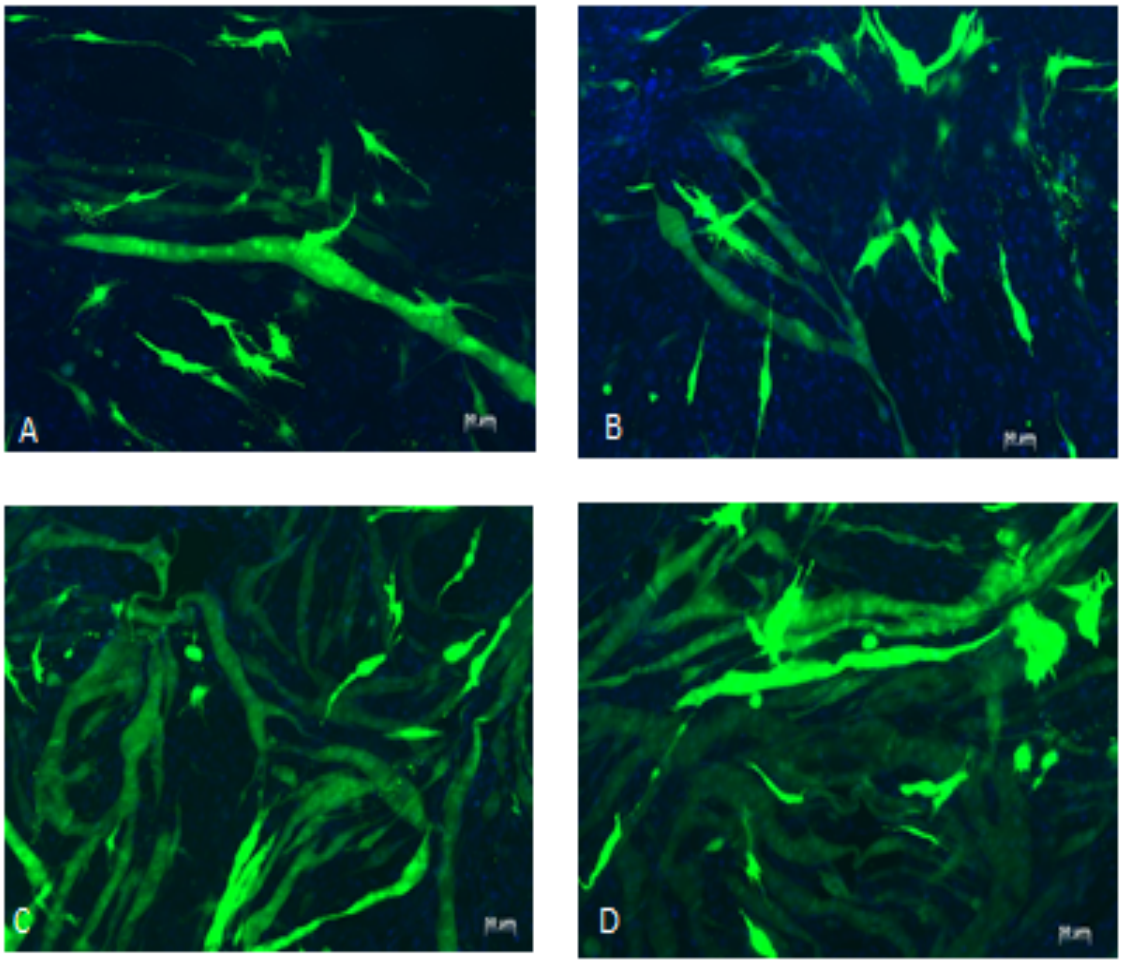


Figure 3-7 – Examples of myotube transfection using hyaluronidase as an adjuvant technique. *A and B show myotube transfection with the addition of 32U of hyaluronidase. C and D show myotube transfection with the addition of 96U of hyaluronidase. Images were taken 5 days post transfection, using Zeiss AxioImager Z1 with CoolSnap HQ2 camera and Axiovision software v4.8.2. Exposure times were set at 400ms for each image. Due to the difficulty in separating individual tubes in order to count the % transfection, in this case, each field of view was turned into a binary image and actual percentage area transfection calculated using ImageJ software.*

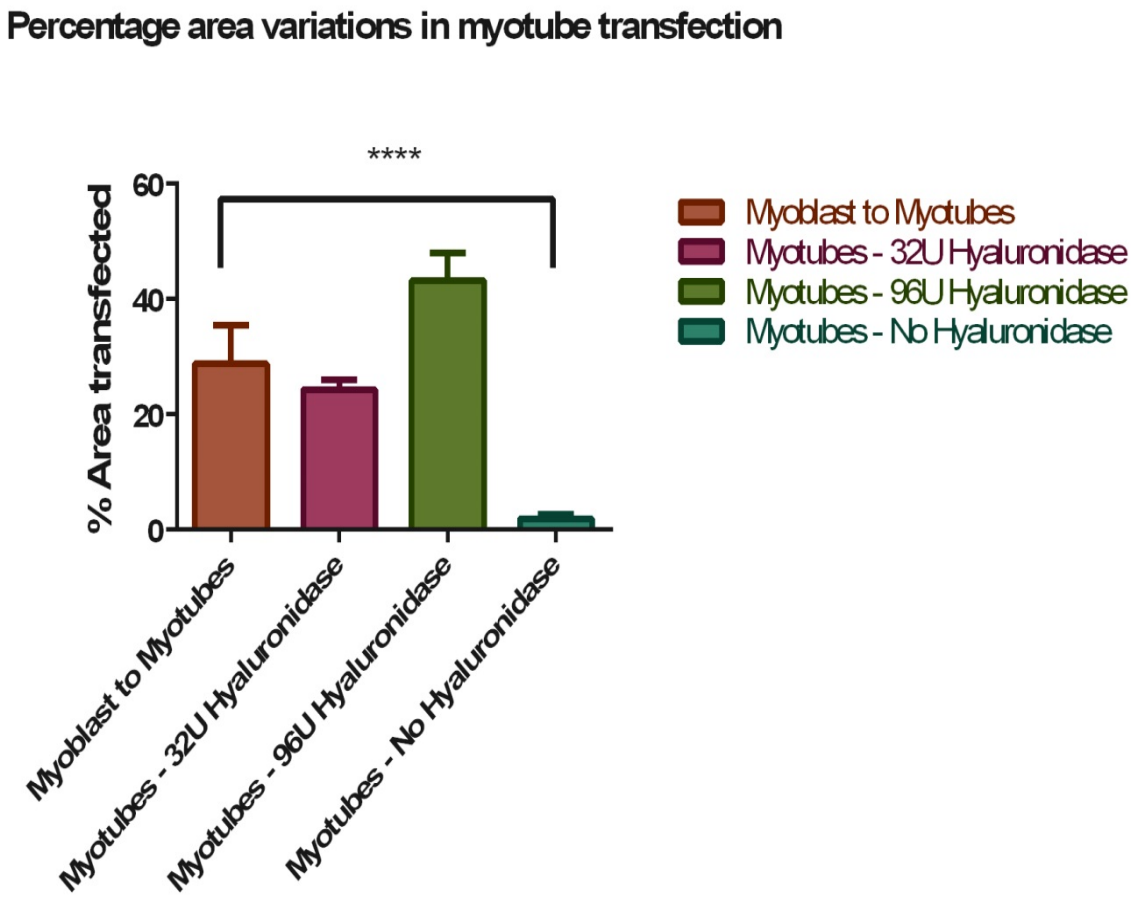


Figure 3-8 – Determination of the most efficient method of transfecting C2C12 myoblasts or myotubes*. The standard Lipofectamine2000 method was employed, with the addition of 32U or 96U hyaluronidase immediately prior to the addition of the Lipofectamine2000/DNA complex. Analysis using Image J software shows the addition of 96U hyaluronidase shows a significant improvement of gene expression (over 20 fold increase) in C2C12 myotubes over no hyaluronidase added (n=6; p<0.0001)*

3.3.4 *In vitro infection of AAV vectors to determine most appropriate serotype for use and the multiplicity of infection (MOI) required.*

AAV vectors are available in several different serotypes. Serotype 2 has been the most extensively studied, however, different serotypes have been shown to exhibit a greater affinity to different tissue types (Wu *et al.*, 2006).

A literature search returned variable results with regards to the most appropriate serotype for use in the remainder of this study. As a results, a suite of 'off the shelf' serotypes were purchased from the Viral Vector Production Unit (UPV) of the University of Barcelona to use in an *in vitro* assessment with C2C12 myoblasts.

Along with the most appropriate serotype, the most appropriate MOI also needs to be determined. As the amount of AAV vectors obtained for the initial serotype trial was limited, it was decided to use a well-known 'easy to transfect' cell line as opposed to the C2C12 cell line in order to reduce the risk associated with failed experiments. The cell line chosen was HEK293. Figure 3-9 demonstrates the difference in transfection efficiency between the C2C12 and HEK293 cell lines, with an almost 2 fold increase in percentage transfection obtained in the HEK293 cells.

A literature search indicated a suitable MOI, with the titre of vector that was available to us, was between 0.5 and 1. Again, due to the limited volume of each vector serotype and the number of studies planned, a basic experiment to assess the difference in infection efficiency between these two MOI's was established (Figure 3-10).

As no significant difference was observed between an MOI of 0.5 or 1, an MOI of 0.5 was used for all future in vitro experiments involving AAV infection in order to preserve as much of the vector as possible in the case of any adverse event.

Once the MOI had been established, an *in vitro* experiment using C2C12 myoblasts to determine most suitable AAV serotype for use in future *in vivo* experiments returned AAV1 as the most appropriate. The switch from HEK293 to C2C12 cells was deemed appropriate in order to verify the use of a muscle cell line before progressing to *in vivo* experiments. The Lipofectamine2000 protocol was mimicked, without the use of Lipofecatmine2000 itself. AAV is able to infect cells without the use of any adjuvant

techniques, however the results gained from this study were disappointing and much lower than expected.

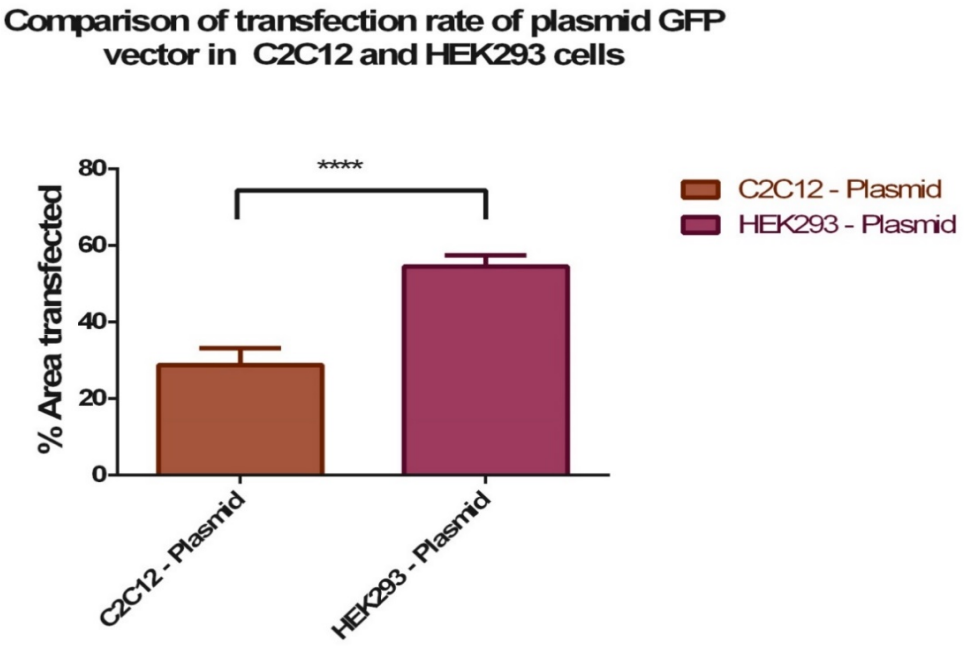


Figure 3-9 Comparison of % transfection rates between C2C12 and HEK293 cells using pVR1012.GFP.CMV*. An almost 2 fold increase in transfection efficiency in HEK293 cells indicates the ease at which HEK293 cells are transfected, which can be extrapolated for use with AAV vectors (n=6; p<0.0001).*

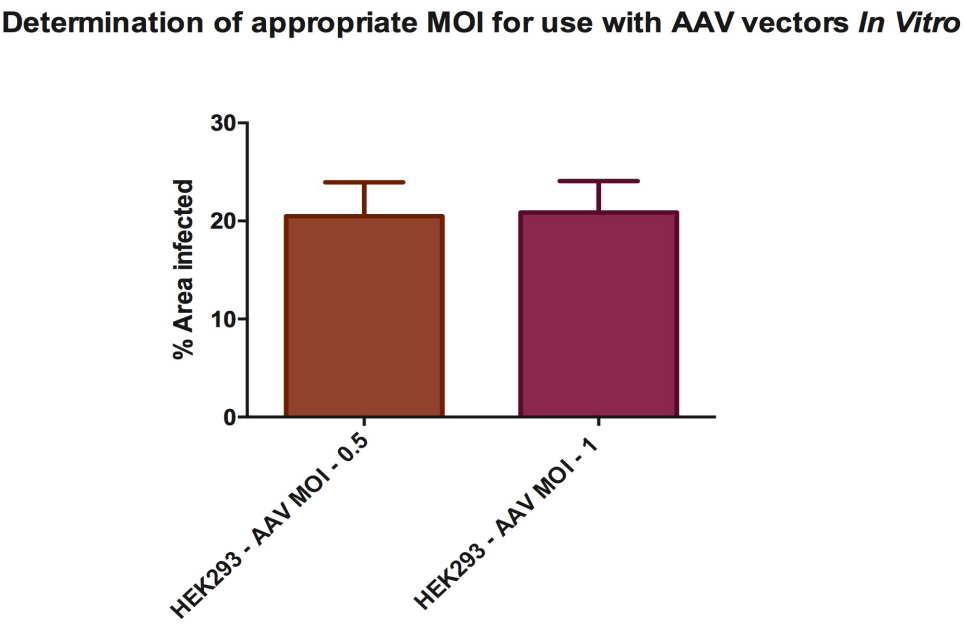


Figure 3-10 - Determination of appropriate MOI for use with AAV vector in HEK293 cells*. No significant difference was observed between each MOI (n=6, p>0.05). As the volume of AAV1 was sparse, the lower MOI was deemed acceptable and appropriate for all future studies. .*

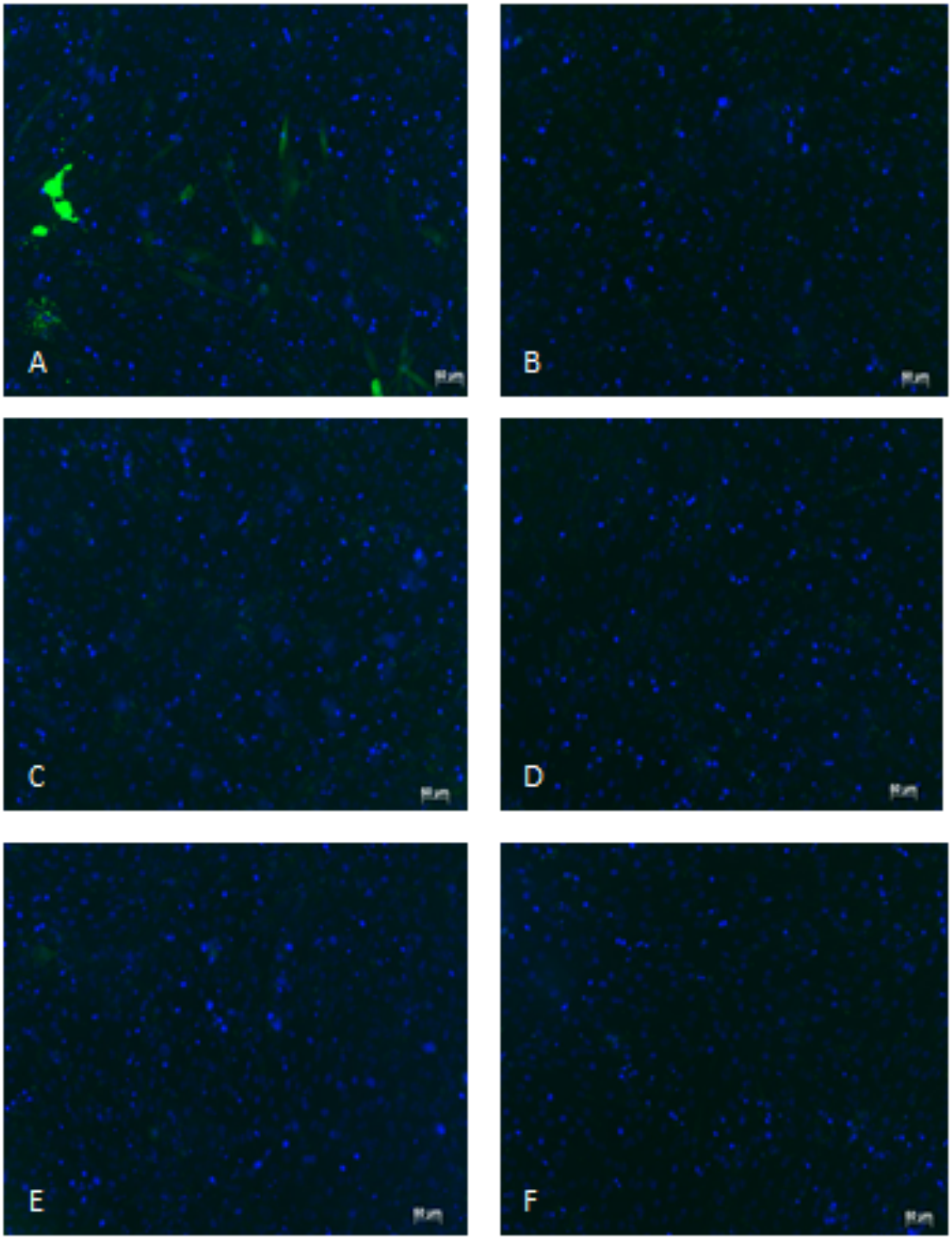


Figure 3-11– In Vitro assessment of AAV serotype to determine most appropriate for use*. C2C12 cells were used in the serotype assessment to ensure the vectors were capable of infecting a muscle line before moving the studies in vivo. C2C12 myoblasts were infected as per standard Lipofectamine2000 protocol, without the Lipofectamine2000. The only serotype to show any confirmed expression, however limited, was serotype 1 (AAV1) (A). The other serotypes assessed were AAV2 (B), AAV5 (C), AAV6 (D), AAV9 (E) and AAV10 (F).*

3.4 *In vivo* studies to determine the efficacy and efficiency of the conditions established during in vitro trials.

Two sets of in vivo transfections were performed on normal (CD1) mice.
The first was to determine length of expression using the current CMV promoted plasmid (pVR1012-CMV-eGFP), and the second to determine if CMV was the most appropriate promoter or if a muscle specific promoter would achieve greater expression (pRRL.sin.PPT.DES.eGFP).

3.4.1 In vivo transfection to assess predicted optimal procedure for gene expression efficiency in CD1 males using plasmid vector

The IVIS spectrum is a non-invasive method of determining the location and intensity of fluorescent (or luminescent) biomarkers without needing to sacrifice the animal.
Each animal is lightly anaesthetised purely to prevent any movement whilst the scans are taking place. Any and all auto-fluorescence is detracted from the expression detected by the transgene by the software associated with the equipment. The specific intensity of each individual region of interest is then calculated, and the corresponding images can be used to assess locale.
All mice were subjected to IVIS scans on a weekly basis. This evaluates the eGFP expression found in a rodent, independent of any auto-fluorescence that may be found in all mammals.
5 mice in total were subjected to transfections using pVR1012-CMV-eGFP. eGFP expression was monitored over the course of 30days using the non-invasive imaging system IVIS Spectrum.

Whilst the standard deviations are relatively high, the GFP expression, as observed using the IVIS spectrum and associated software, shows a significant trend to increasing expression over time in a normal rodent model, with no indication of diminishing intensity even at day 30.

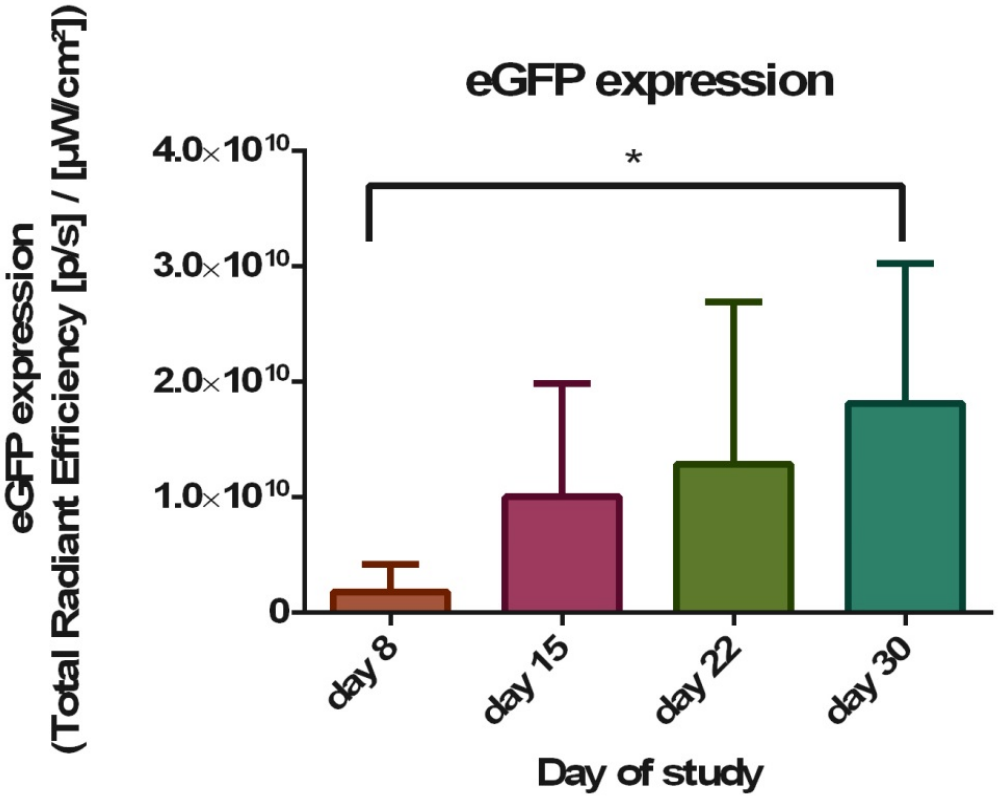


Figure 3-12 - GFP expression, as identified using IVIS spectrum software, during course of the study. *The total radiant efficiency takes into account the level of auto- fluorescence that may be observed from standard tissue samples. The fluorescence obtained was normalised against the auto-fluorescence of each animal, and therefore the pre-treatment level was zero (n=5; p<0.05).*

3.4.2 *In vivo transfections to assess promoter effectiveness in CD1 females*

10 mice in total were transfected with both pVR1012.CMV.eGFP and pRRL.sin.PPT.DES.eGFP, 5 in each batch. GFP expression was monitored over the course of 30 days to establish if a muscle specific promoter is necessary.

The significant difference in expression observed, in favour towards the CMV promoter, indicated that not only is a muscle specific promoter not necessary, but the expression efficiency gained from the use of the CMV promoter is far superior. In fact, some cases of the CMV promoted plasmid yielded such expression that it was visible to the naked eye on harvesting the muscle (see Figure 3-15).

In addition to the IVIS scans to obtain visualisation of region of expression, a technique which incorporated both IVIS and CT was trialled. The resulting image can be seen in Figure 3-16. Unfortunately, shortly after this initial trial, the CT equipment located in the CBC facility was no longer available for use. The single mouse that was used for this trial unfortunately died shortly after this procedure. On consultation with the NVS, the procedure was not deemed to be the cause of this death.

In order to assess the validity of the results obtained from IVIS scanning, a selection of muscle samples were harvested, snap frozen, sectioned and mounted and assessed microscopically for GFP expression.

Each section was imaged using the Zeiss Axioimager Z1 fluroescence microscope and the associated AxioVision software. The entire muscle section was imaged using the mosaic function, which allows multiple fields of view to be 'stitched' together to form the full section. These full stitched images were then analysed in ImageJ, in which the entire area of the section was calculated. The eGFP expression was then transformed into a binary image, and the percentage area of eGFP positive was gained from the original area of the entire section. The full harvested sample was sectioned, and every tenth section analysed in this way. An example of the results is shown in Figure 3-17.

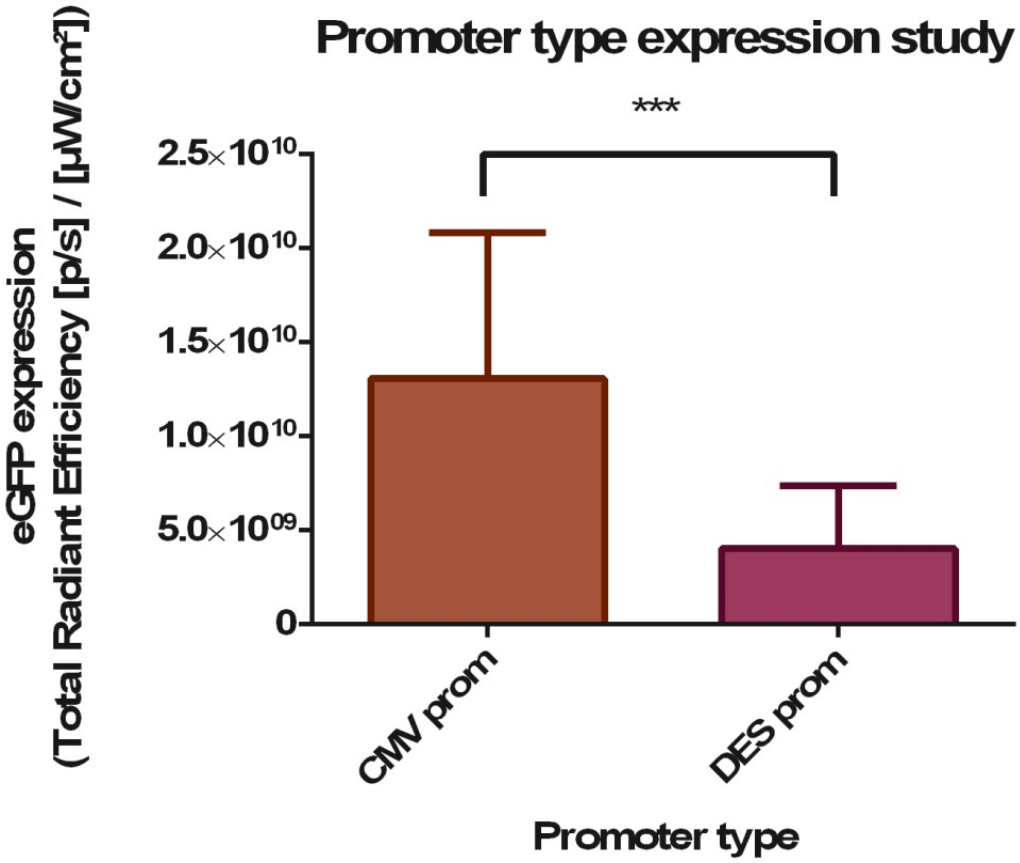


Figure 3-13 Using GFP expression, as identified using the IVIS spectrum software. *Data was analysed using T-test, there was a statistical significance ($p<0.05$) between the CMV and muscle specific DES promoters, in favour of the CMV ($n=5$, $p<0.001$).*

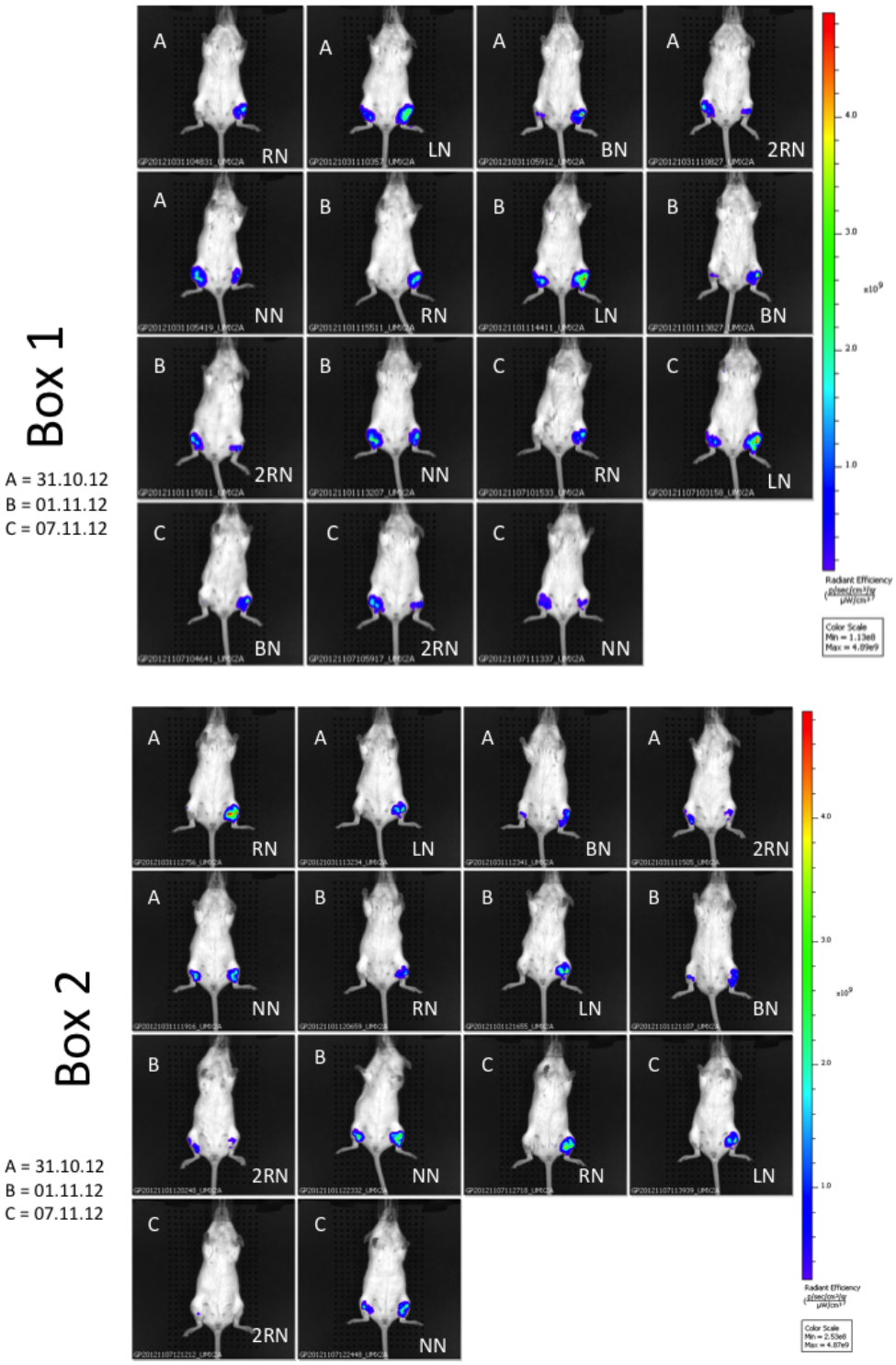


Figure 3-14 – IVIS scan images showing the expression gained from both DES and CMV promoted plasmid vectors*. A mix of plasmid was used in each animal in an attempt to remove mouse-mouse variability. See Table 3-1 for a description of plasmids used.*

Box	Mouse ID	Leg	Plasmid
1	RN	Left	CMV
		Right	DES
	LN	Left	CMV
		Right	DES
	BN	Left	CMV
		Right	DES
	2RN	Left	DES
		Right	CMV
	NN	Left	DES
		Right	CMV
2	RN	Left	CMV
		Right	DES
	LN	Left	CMV
		Right	DES
	BN	Left	CMV
		Right	DES
	2RN	Left	DES
		Right	CMV
	NN	Left	DES
		Right	CMV

Table 3-1– List referencing the plasmid used for the expression as determined by IVIS spectrum scan, shown in Figure 14. *In an attempt to remove variability in using different animals, each promoter was injected into the same mouse, using different hind limbs, and alternating between left and right.*

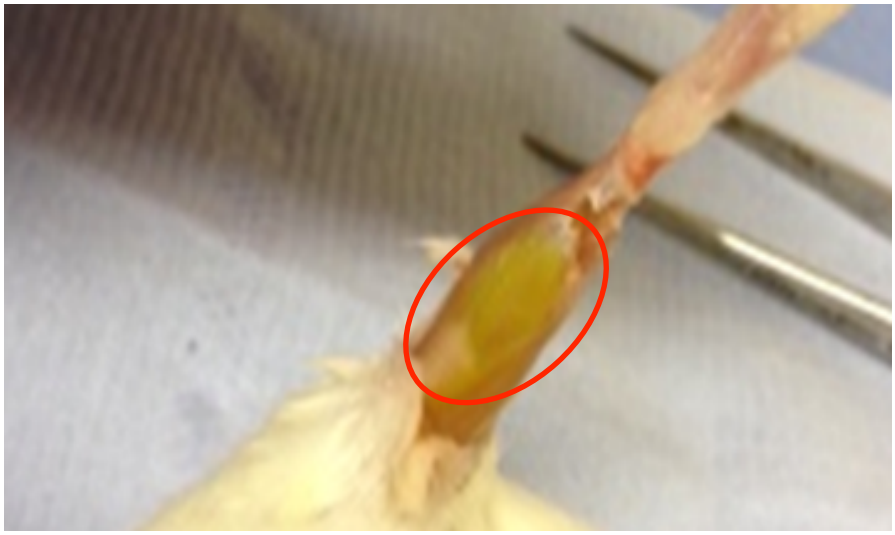


Figure 3-15 – Example of strong eGFP expression in rodent muscle as observed 'by eye'*. In some cases, the GFP expression was strong enough to be seen with the naked eye. This is an example of a gastrocnemius (and surrounding area) transfected muscle in a CD1 mouse using CMV promoted plasmid vector.*

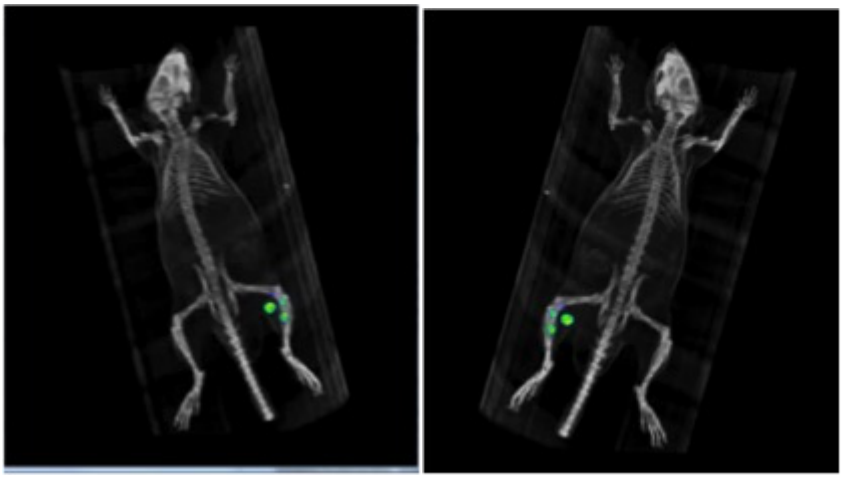


Figure 3-16 – Dorsal and Ventral view images of combined IVIS and CT scans showing the location of expression combined with skeletal structure. *This procedure was trialled with one mouse (BN) from the plasmid promoter trials (3.4.2). The mouse died shortly after the procedure (hence missing from the image shown in Figure 3-14, box 2), however, the NVS did not surmise the death was procedure related.*

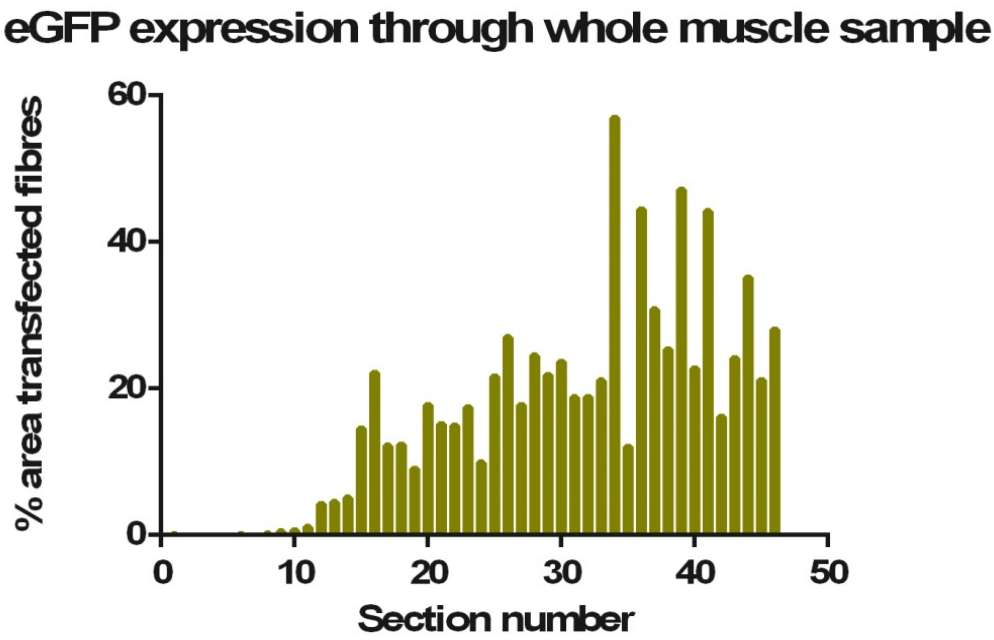


Figure 3-17 GFP expression was determined throughout the entire cross-section of a randomly selected muscle sample*. GFP was visualised using mounting medium containing DAPI only and analysed using the tiling feature on the Zeiss AxioImager Z1 with CoolSnap HQ2 camera and Axiovision software v4.8.2 and Image J software.*

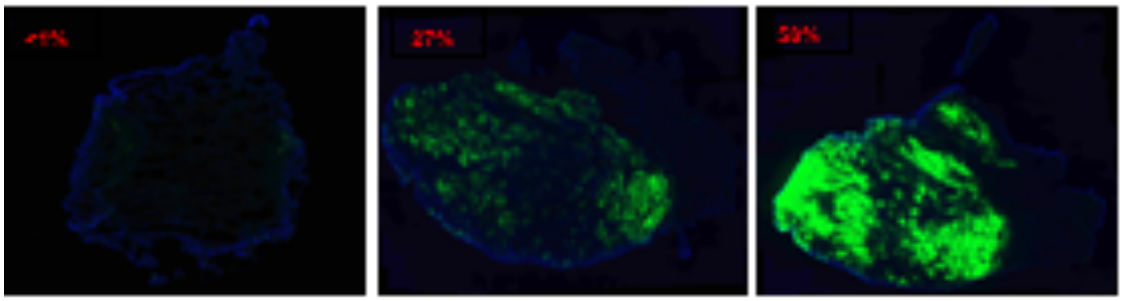


Figure 3-18 a selection of low (<1%), medium (27%) and high (58%) transfected area images used to complete the analysis in Figure 3-17

3.5 Discussion

In these studies, the use of the reporter gene GFP was used to establish the extent at which the proposed protocols would successfully express the gene of interest, which may be more difficult to determine levels.

The plasmid vector, which was previously established within the group, contained the GFP gene sub-cloned between the BamHI and EcoRV sites of the pVR1012 vector, obtained from Vical (CA, USA).

The AAV vector was a standard AAV2/1 vector containing GFP, both vectors use the standard CMV promoter for constitutive expression.

The plasmid transfection protocol followed on the standard Lipofectamine2000 protocol provided with the reagent from the manufacturer (Life Technologies), with the variations in reagent and pDNA used to establish an optimum process. Each variable assessed provided no significant difference in expression efficiency, indicating the reagent, Lipfectamine2000, is very robust method for incorporating transgenes. Good transfection efficiency was obtained in myoblasts, however the adjuvant addition of 96U hyaluronidase greatly increased the expression achieved in myotubes, an increase from <2% to >40% positive transfection area was observed.

Gene transcription is easily identifiable using a fluorescence marker such as GFP. Standard protocols for observing fluorescence in cells and tissues are commonly known and well established for use *In Vitro* or post mortem, however, in order to establish the approximate duration of expression, based on vector, an alternative method was required which would allow the vectors to be tracked *In Vivo.* The system used for this is known as the In Vivo Imaging System – IVIS. The IVIS system used in this instance is the Perkin-Elmer pre-clinical IVIS spectrum system. Whilst the system is capable of a multitude of various detection methods, those employed for this purpose, and for any other reported uses hereafter, epi-illuminated fluorescence was detected whilst the

animals were under light inhalant anaesthesia (isoflurane) in a specialised chamber. Detection was determined using a combination of grade 1 CCD and a 150W extended tungsten lamp. The camera temperature was constant at -90^{0}C and the images captured have a pixel ratio of 2048 x 2048. The analysis of the images was performed using the proprietary 'Living Image' imaging software.

GFP expression was confirmed using a combination of staining methods and IVIS analysis. The staining analysis showed, that by traditional methods, GFP expression can be shown in cells and tissues that have been manipulated, fixed and stained. Although this gives a clear indication that GFP expression has been consistent and persistent, to be of use for In vivo applications, the animal(s) in question must be deceased prior to obtaining the information. Using the IVIS system, the transgene expression (either fluorescent or bioluminescent) can be monitored in situ in real time, without any harm or distress to the animals. The robustness of the IVIS system has been somewhat validated by use of the staining methods of tissue once the study was completed. However, attempts to fully, quantitatively establish a direct lineage between muscle content and IVIS expression failed. eGFP ELISA's were repeated many times, however, precision analysis performed on the eGFP ELISA's failed. Due to the nature of the studies (not being performed simultaneously), the reliability of the data was not sufficient to allow any firm conclusions to be drawn. On a basic scale, the conclusion could be made that if no eGFP expression was found from IVIS scanning, no eGFP expression was found during ICC. Unfortunately, this was the only conclusion that could be formed.

The experiment to determine promoter efficacy and efficiency showed a significant affinity to CMV promotion over the muscle specific promoter. However, in order to make the experiment more robust, the plasmid backbone of both vectors should be the same, with the only difference being the promoters. This was not the case in this instance. Nevertheless, this did demonstrate the superior ability of the CMV based plasmid and does confer confidence that this is a more than appropriate vector for use in this study.

4 Chapter 4 – Assessment of constitutive expression and secretion of Exendin-4 and GLP-1 through plasmid and AAV transfection *in vitro*

4.1 Introduction

Given the tremendous potential of GLP-1 and GLP-1-based therapies, especially when it comes to the limited potential for iatrogenic hypoglycaemia due to its endogenous glucose responsiveness, a wide range of applications have been considered (Kim and Egan, 2008; Salehi *et al.*, 2010; Ahrén, 2011; Kielgast *et al.*, 2011; Butler *et al.*, 2013). Treatment potential of GLP-1 is however limited by its short half-life, due to DPP4 degradation. The constitutive/ continuous over-expression of GLP-1 by muscle-targeted gene therapy is thought to be a possible route to overcome this (Mahmoud, 2010). The fact that by using this means, the GLP-1 amino-acid sequence and structure remains unaltered, it is anticipated that this will also reduce the potential of side-effects which can be seen with modified versions of endogenous hormones. Whilst it is generally accepted that the benefits outweigh any potential side effects, studies examining the potential link between overexpression of GLP-1 R agonists and serious illness, including pancreatitis, pancreatic cancer and thyroid cancer, both for and against the theory, have been published.

A cohort study looking at the link between exenatide use and pancreatitis found exenatide use was no more influential in the development of pancreatitis than any other anti-hyperglycaemic drug (Dore *et al.*, 2011) whereas Gale *et al* believe there is still insufficient evidence to proclaim GLP-1 therapies as safe and further investigation is warranted (Butler *et al.*, 2013)

Notwithstanding this possible advantage, uncertainties remain around this concept; for instance will the increase in GLP-1 trigger a concurrent increase in DPP4 activity? Will a chronic pharmacological increase in GLP-1 cause another adverse physiological or pathophysiological effect? Can inherent inefficiencies in achieving desired circulating protein concentrations associated with plasmid-transfection be overcome or are more effective adenovirus-mediated transfections warranted? Would the use of a longer-acting homologue such as Exendin-4 be more effective? These questions, combined with the obvious potential of constitutive GLP-1 or Exendin-4 secretion through gene therapy,

warranted the further evaluation of both constructs in *in vitro* experiments, mainly to assess efficiency, prior to application in *in vivo* studies.

After the initial proof of concept studies using reporter gene eGFP (see chapter 3), GLP-1 and Exendin-4 gene constructs were included in future studies, with the use of GFP as both a control vector and a reporter gene.

GLP-1 (7-36) amide or Exendin-4 sequences had previously been subcloned into the pVR1012 plasmid (refer to vector map). This particular plasmid has been specifically designed for use with gene therapy applications and is driven by the constitutive CMV promoter.

The GLP-1 and Exendin-4 constructs of the pVR1012 plasmid were initially assessed *in vitro,* to confirm they conferred expression as seen with the reporter gene vectors, before progressing to studies *in vivo*.
The C2C12 murine muscle cell line was chosen as it is an extremely stable line which can be used to form muscle-like myotubes and has been used extensively as a model for in vitro studies with a view to progression in *in vivo* muscle related investigations (Burattini *et al.*, 2004; Veliça and Bunce, 2011) .

4.2 Objectives

This chapter reports attempts to evaluate the constitutive expression of both genes of interest, GLP-1 and Exendin4, using the GFP reporter gene as a control, in an *in vitro* muscle model, before progression onto an *in vivo* model. Specific objectives were:

- To compare percentage transfection efficiency of eGFP reporter with GLP-1 and Exendin-4.

- To compare percentage transfection efficiency of plasmid constructs with AAV viral vectors.

- To evaluate the differences observed in the therapeutic peptide secretion of plasmid and AAV delivery systems.

4.3 In vitro confirmation of the biosynthesis and constitutive secretion of Exendin-4 and GLP-1

As mentioned previously, the pDNA vectors had already been established within the group. The GLP1 (7-36) amide and the Exendin-4 sequences were subcloned between the bamHI and EcoRV restriction sites (see plasmid map, Chapter 1, Figure 1-5).
The eGFP sequence was subcloned into two XbaI sites. All that remained for the purposes if this study was to transform the vectors using competent E.coli cells to ensure the stocks persisted.
The sequences of each of the transgenes are shown in table 4-1.

Gene	Sequence
GLP1	CATGCTGAAGGGACCTTTACCAGTGATGTGAGTTCTTACTTGGAGGGCCAGGCAG CAAAGGAATTCATTGCTTGGCT
Exendin-4	CATGGCGAAGGCACCTTTACCAGCGATCTGAGCAAACAGATGGAAGAAGAAGCG GTGCGTCTGTTTATTGAATGGCTGAAAAACGGTGGTCCAAGCAGCCGTGCACCAC CACCAAGC

Table 4-1 – GLP1 and Exendin-4 gene sequences subcloned into pVR1012 (Mahmoud, 2010)

The *in vitro* transfection procedure was repeated for pVR1012.Ex4.CMV and pVR1012.GLP.CMV, as per the optimised conditions determined in Chapter 3.

C2C12 myoblasts were cultured on standard 6 well plates in standard growth medium until 60-70% confluent. The standard Lipofectamine2000 protocol was employed and 48 hours post-transfection, the cell culture media was retained and analysed via EIA for Exendin-4 and ELISA for GLP-1, to assess secretion of transgenically expressed peptide. In order to prevent GLP-1 degradation, DPP4-inhibitor (Merck Millipore, Germany), at a concentration of 10 µl/ml, was added to the wells being used for GLP-1 analysis, including the negative controls, immediately post transfection.
Immunofluorescence staining confirmation of successful transfection was obtained up to 7 days in culture.

C2C12 myoblasts were cultured in standard 6 well plates in standard growth media, except this time, a fibronectin coated coverslip was placed on the bottom of the well to allow a stable growth platform for the cellular matrix that would form after this time. The standard Lipofectamine2000 protocol was employed and the cells were transfected at approximately 50% confluence. Post transfection, cells were fed as needed, coverslips were removed and stained at day 1, 2, 5 and 7.

Both Exendin-4 and GLP-1 plasmid transfections attained high levels of peptide secretion(2439 pmol/L +/- 0 and 51.75 pmol/L +/- 37.57) at a significantly different level ($n=6$; $p<0.0001$) in comparison to reporter gene control (GFP plasmid) when analysed via ELISA / EIA (Figures 4-1 and 4-2). Both plasmids showed high levels of extracellular hormone was excreted into the media, however the levels of Exendin-4 were found to be almost 50 times higher than those found in the GLP-1 media. This is possibly due to the occurrence of DPP4-degradation, even after adding the DPP4 inhibitors to the wells whilst cells were cultured and post collection.

Both Exendin-4 and GLP1 vectors (plasmid and AAV) showed evidence of expression when analysed via staining. However, the intensity of the stain observed and the day of peak activity varied.
Exendin-4 via plasmid transfection and AAV infection both showed highest levels of expression at day 1, however, the AAV-based expression persisted with a greater intensity into day 2 than plasmid based expression ($p>0.05$; Figures 4-3, 4-4 and 4-7). GLP-1 showed all round less expression than Exendin-4 in both systems. Although plasmid based expression peaked at day 2, the AAV based infection did not show any expression of note throughout the study ($p>0.05$; Figures 4-5, 4-6 and 4-8).

Whilst the staining evidence captured the intracellular expression of cells at a fixed point in time, indicating the peak level of activity is day 2, when the media was analysed at the same time points, the peak level of activity for the Exendin-4 plasmid system was day 5. The AAV system showed variable levels with days 2 and 7 showing similar concentrations, with a dip at day 5 (Figures 4-9 and 4-10).

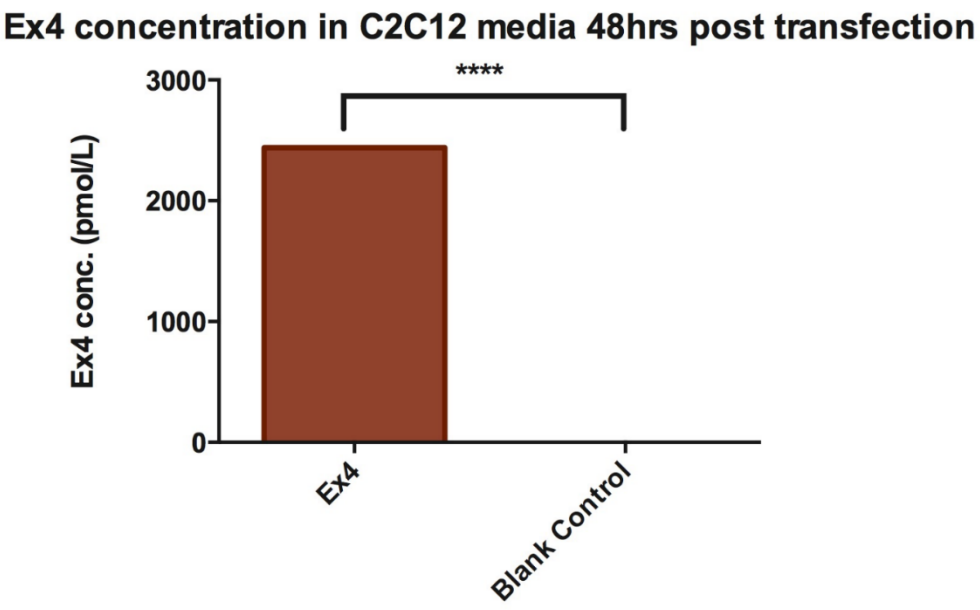


Figure 4-1 Exendin-4 concentration in C2C12 standard growth media. *Media was removed 48hours post transfection, using 2µg plasmid DNA and 10µl Lipofectamine2000. Media was not refreshed within the 48 hours (n=6, ****p<0.0001).*

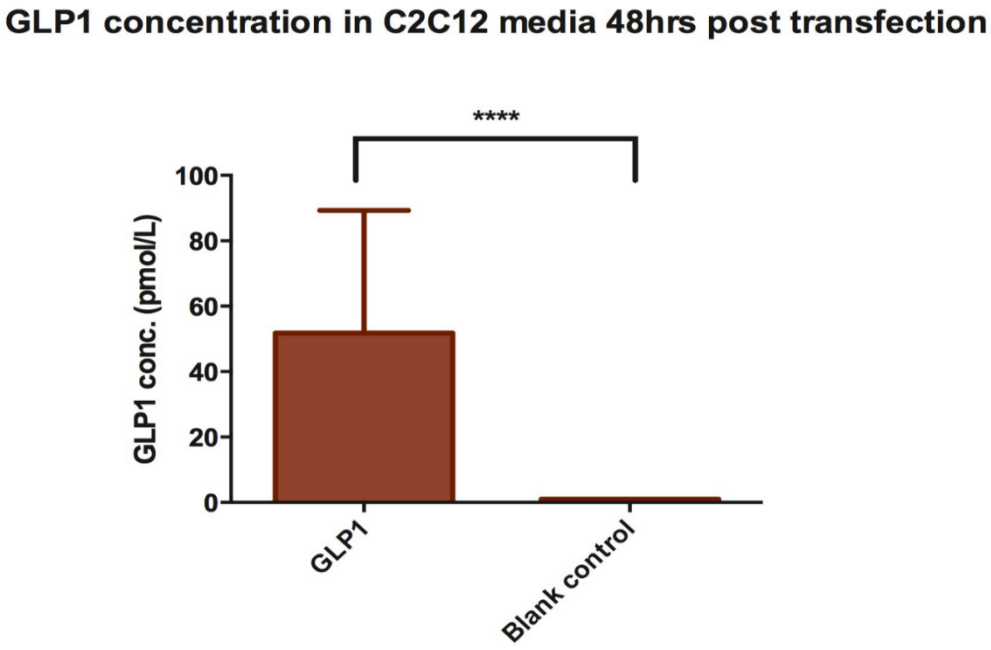


Figure 4-2 GLP-1 concentration in C2C12 standard growth media*. Media was removed 48 hours post transfection, using 2µg plasmid DNA, 10µl Lipofectamine2000 and 10µl/ml DPP4-inhibitor. Media was not refreshed within the 48 hours (n=6, ****p<0.0001).*

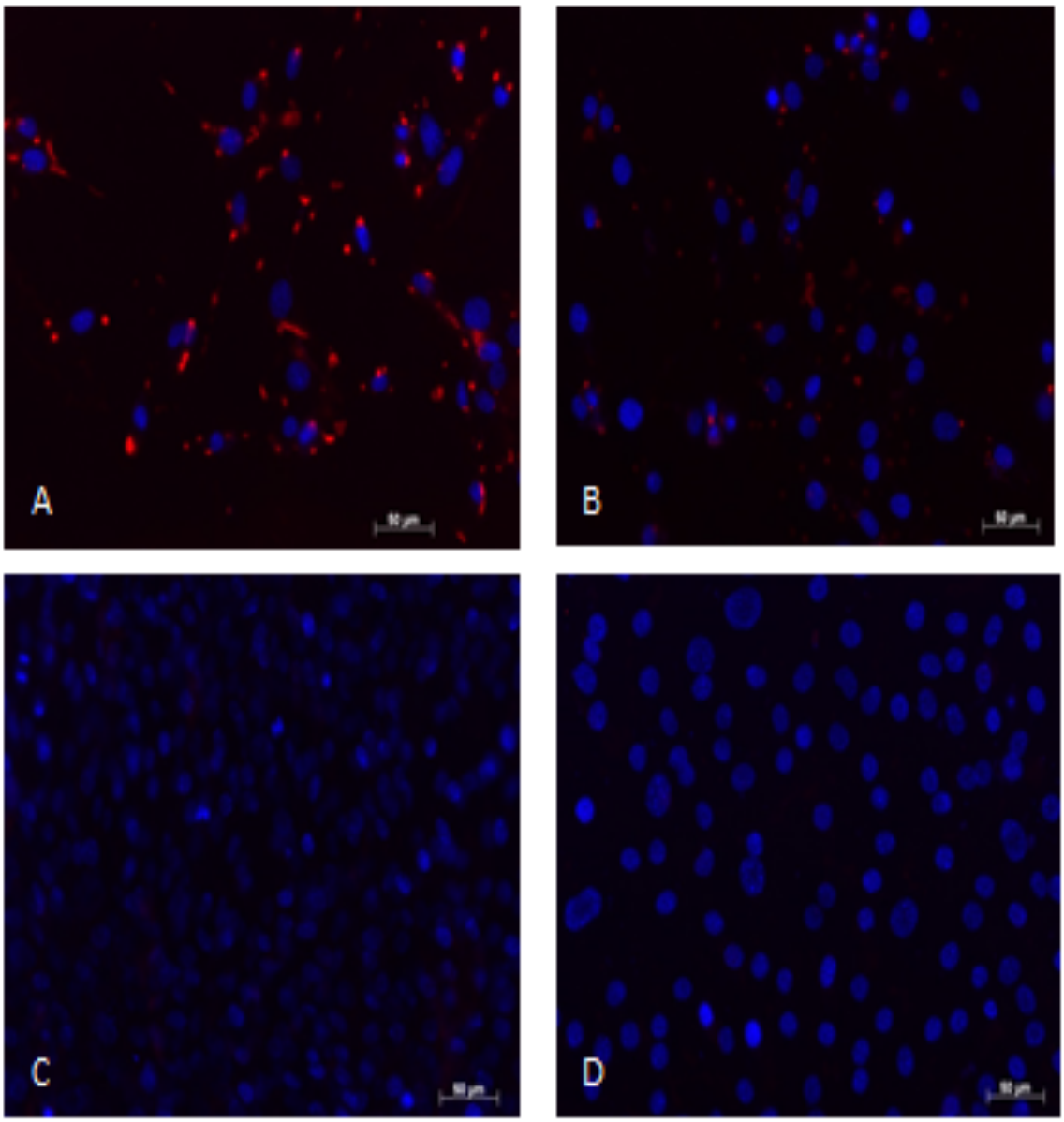


Figure 4-3 – Time course of plasmid transfection using Exendin-4 in C2C12 myoblasts*. x20 mag, 100ms exp. Cells were grown on fibronectin coated coverslips (to support the cell matrix) and removed at day 1 (A), day 2 (B), day 5 (C) and day 7 (D), post transfection. Cells were fixed in fresh 10% formalin and stained with Exendin-4 primary antibody. Red fluorescence areas indicate Exenedin-4 positive cells; blue areas are DAPI positive nuclei.*

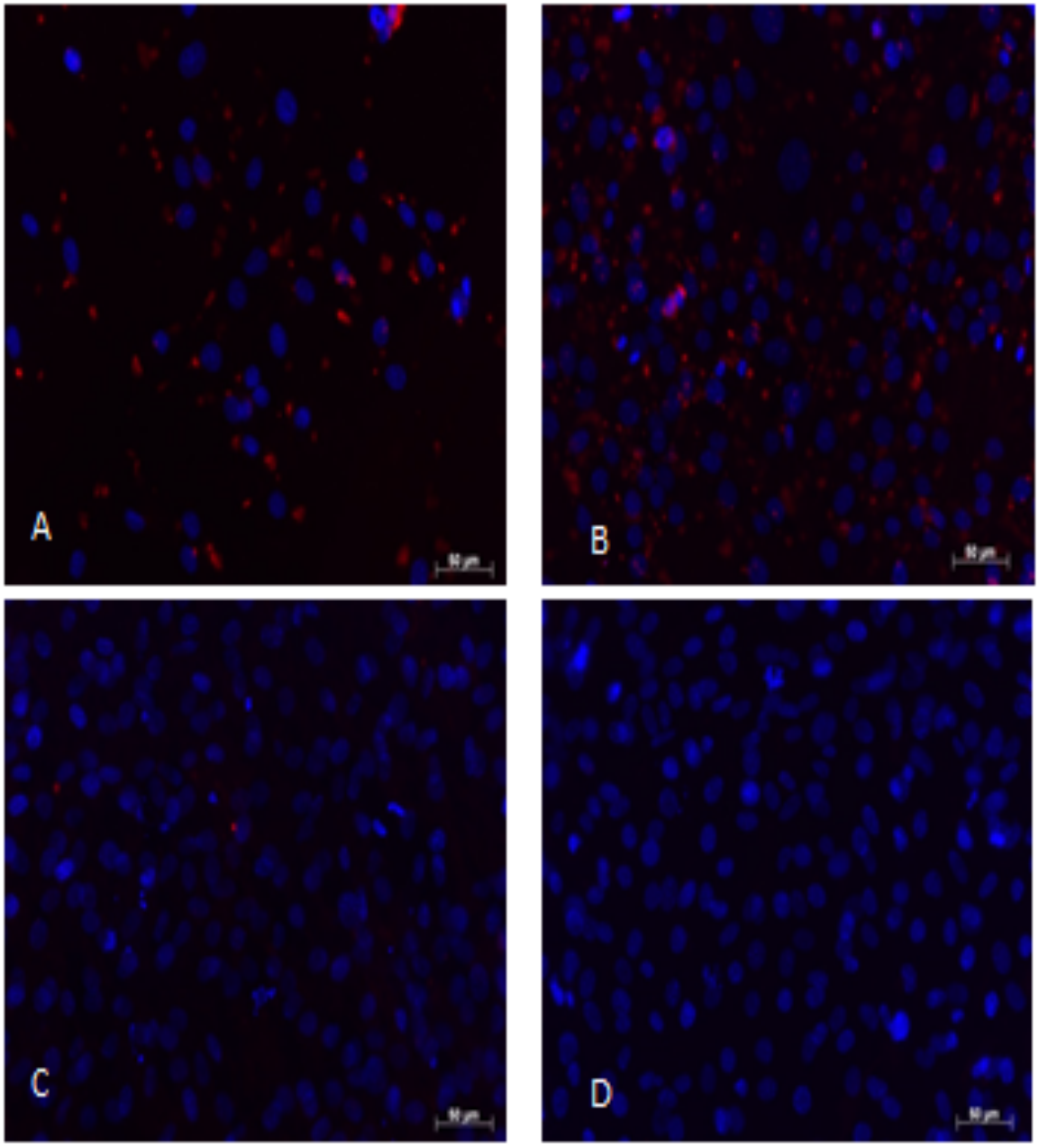


Figure 4-4 - Time course of AAV infection using Exendin-4 in C2C12 myoblasts*. x20mag, 100ms exp. Cells were grown on fibronectin coated coverslips (to support the cell matrix) and removed at day 1 (A), day 2 (B), day 5 (C) and day 7 (D, post transfection. Cells were fixed in fresh 10% formalin and stained with Exendin-4 primary antibody. Although the pattern of staining obtained using AAV is similar to that of plasmid, the intensity appears to be less with the AAV vectors. Red fluorescence areas indicate Exenedin-4 positive cells; blue areas are DAPI positive nuclei.*

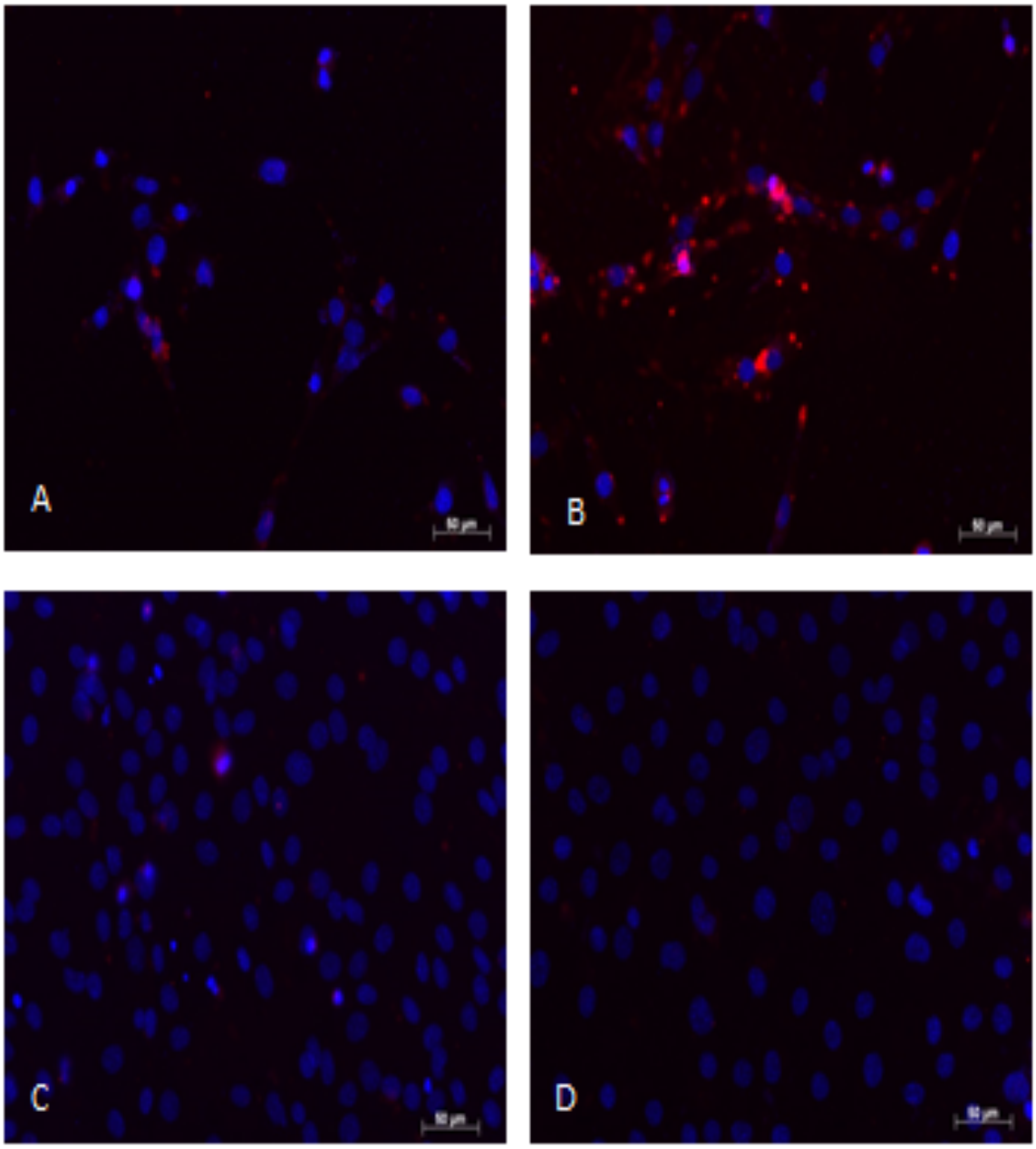


Figure 4-5 – Time course of plasmid transfection using GLP-1 in C2C12 myoblasts*, x20mag, 100ms. Cells were grown on fibronectin coated coverslips (to support the cell matrix) and removed at day 1 (A), day 2 (B), day 5 (C) and day 7 (D), post transfection. Cells were fixed in fresh 10% formalin and stained with GLP-1 primary antibody. Red fluorescence areas indicate GLP-1 positive cells; blue areas are DAPI positive nuclei.*

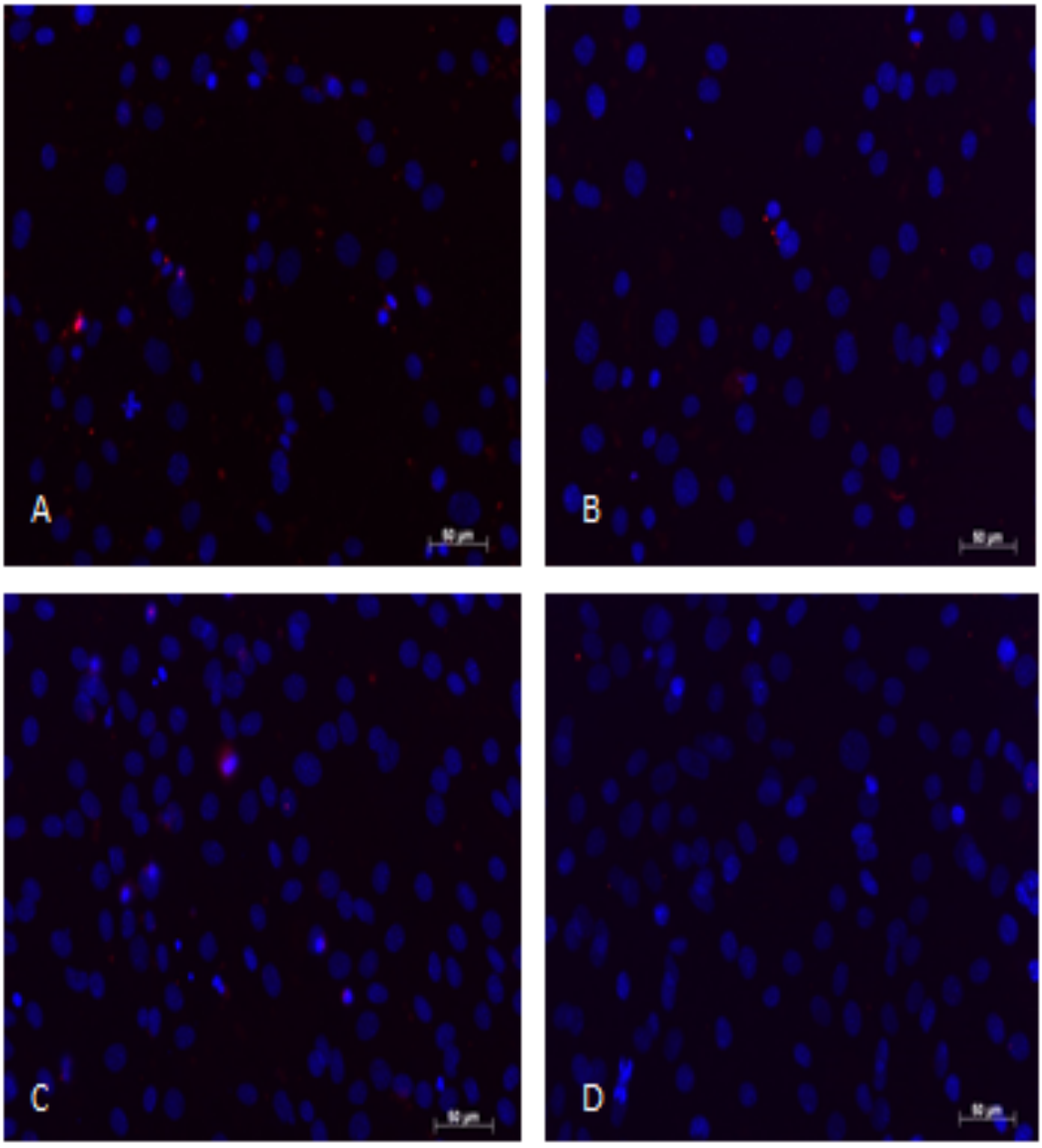


Figure 4-6 – Time course of AAV infection using GLP-1 in C2C12 myoblasts*, x20mag, 100ms exp. Cells were grown on fibronectin coated coverslips (to support the cell matrix) and removed at day 1 (A), day 2 (B), day 5 (C) and day 7 (D), post transfection. Cells were fixed in fresh 10% formalin and stained with GLP-1 primary antibody. Red fluorescence areas indicate GLP-1 positive cells; blue areas are DAPI positive nuclei.*

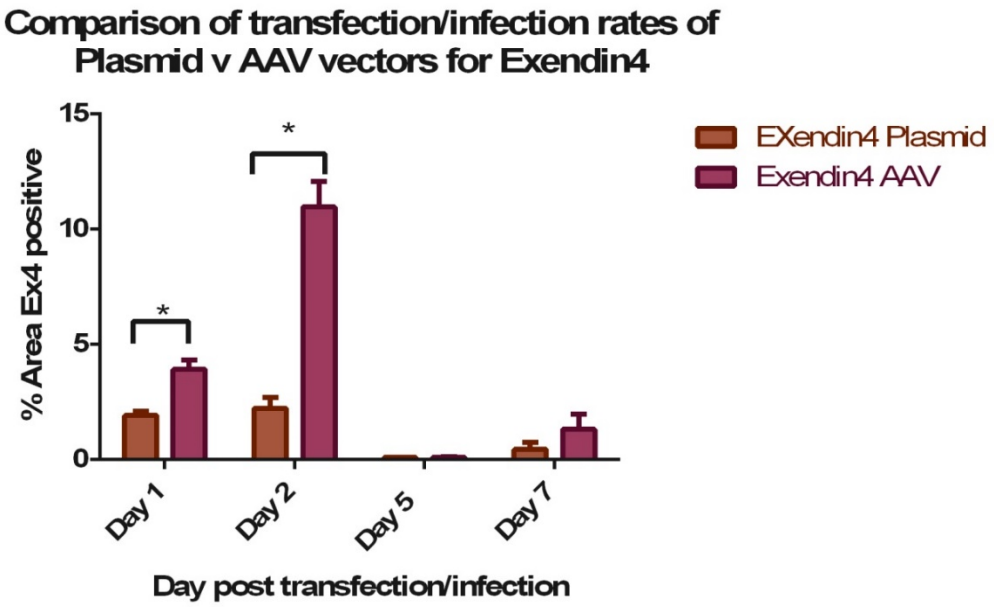


Figure 4-7– Exendin-4 expression data for plasmid v AAV in C2C12 myoblasts. *% area as observed using fluorescence microscopy. (n=6, *p<0.05). Data shows a peak in expression from AAV infection at day 2, and with a 6 fold increase in that observed from plasmid transfection.*

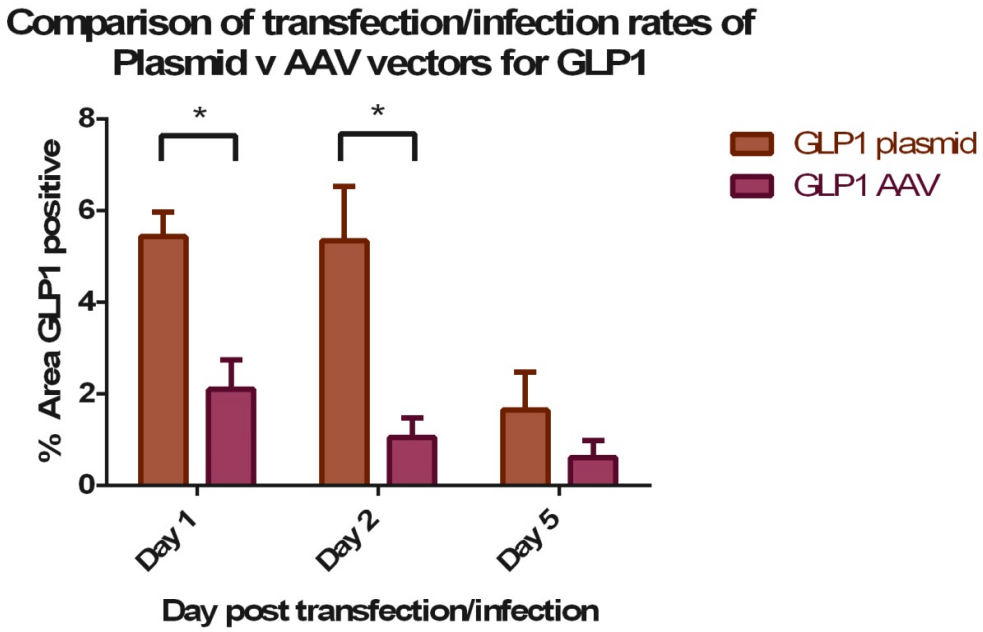


Figure 4-8 – GLP-1 expression data for plasmid v AAV in C2C12 myoblasts. *% area as observed using fluorescence microscopy (n=6, *p<0.05). Data shows a peak in expression from plasmid transfection at day 1 and 2, and with a 3 fold increase in that observed from AAV infection.*

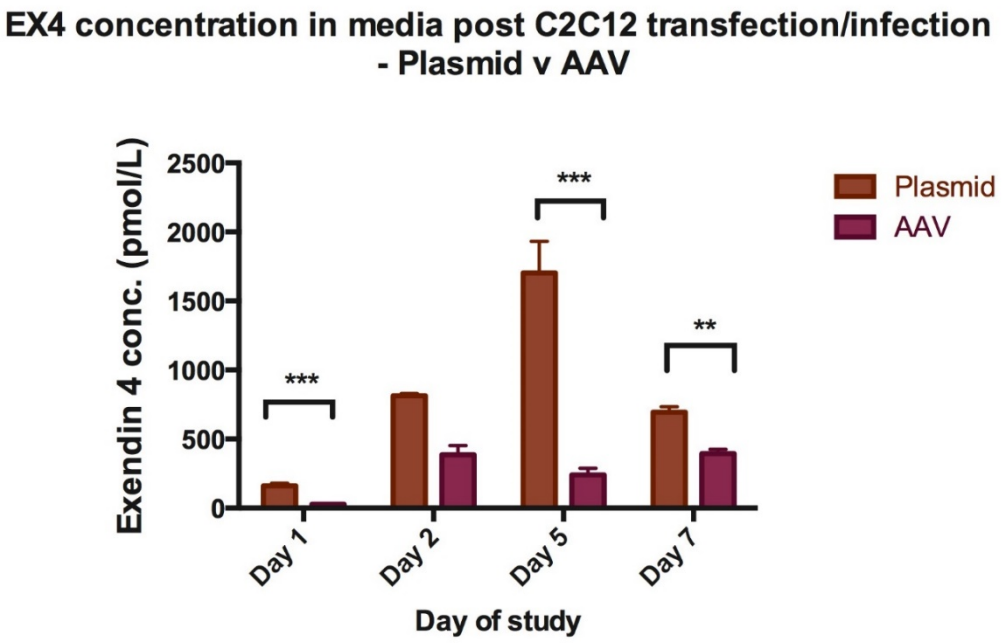


Figure 4-9- Exendin-4 concentration in media from C2C12 *myoblasts transfected with Plasmid/infected with AAV. (n=6, **p<0.01, ***p<0.001)*

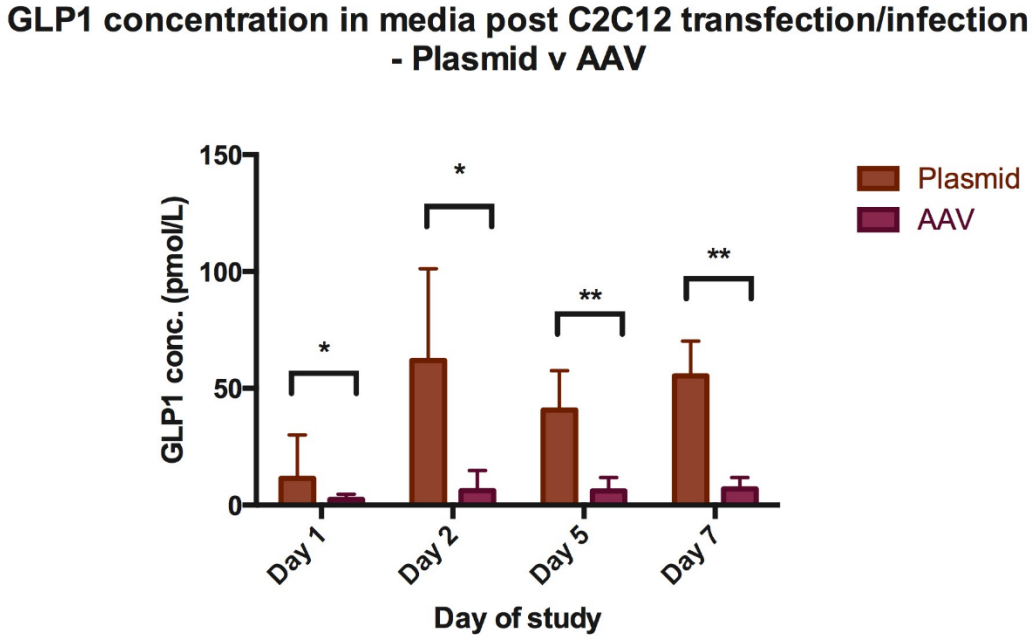


Figure 4-10- GLP-1 concentration in media from C2C12 cells *transfected with plasmid/infected with AAV (n=6, *p<0.05, **p<0.01)*

4.4 Discussion

The *in vitro* experiments to establish GLP-1 and Exendin-4 expression after C2C12 myoblast transfection confirmed the proof of concept that this system warrants further evaluation. Transfection resulted in detectable medium protein concentrations, as well visible cellular expression.

Chemical transfection was successful and yielded results, albeit variable, that confirmed expression of Exendin-4 and GLP1 via histochemical and ELISA/EIA analysis.

Some literature suggests DPP-4 inhibitors do not prevent all GLP-1 degradation, therefore the concentrations observed throughout this study may not be truly representative of the actual secretory levels that may be achievable (Mahmoud, 2010). Inhibitor concentration optimisation studies should also be considered.

The current studies showed variable results with respect to which delivery method was most successful. Detection of Exendin-4 expression by histological analysis determined AAV infection yielded superior results to plasmid transfection (Figure 4-7), however, all other studies suggested that plasmid was the most effective method for protein secretion in both histological analysis and media concentration (Figures 4-8 through 4-10.

Published data suggests that the timeline for AAV based secretion to become evident is greater than that for plasmid based secretion. Based on this evidence, the 7 day timeline that was prohibited by the ability of C2C12 cells to remain viable was simply not long enough for the AAV study (Grieger and Samulski, 2005).

It should also be noted that both GLP-1 and Exendin-4 are secreted from C2C12 myoblasts almost immediately after synthesis due to these particular cells lacking a storage facility. Any positive staining is a good indicator of protein synthesis and expression, but in hindsight, may not have been the best method to use for attempting to evaluate the efficacy of the delivery system. Other studies have effectively used RT-PCR to determine protein secretion, specifically GLP-1, in both C2C12 and HEK-293 cells (Lee *et al.*, 2006; Mahmoud, 2010).

Further evaluation of the secretory properties versus storage facilities may have yielded further insight into the systems. For example, cell lysate analysis would have allowed a

quantitative determination of the concentration of both GLP-1 and Exendin-4 that had not yet been secreted. This has somewhat been determined in previous works (Mahmoud, 2010) but no direct comparison between plasmid and viral vectors, using the same transgene sequences, has yet been performed.

The optimisation of transfection/infection may be of interest in further studies, although it should be borne in mind that optimisation during *in vitro* studies does not necessarily translate into optimisation of *in vivo* results. However, the general proof of concept has been established and it is worthwhile to move this system into an *in vivo* model. Nevertheless, the possibility of including other methods, such as electroporation to enhance delivery and RT-PCR for further analysis, could also be considered, in conjunction with altered vectors to gain an even greater efficiency of expression (Léger *et al.*, 2011). As with all alterations to a system, this must be trialled *in vitro* before moving into an *in vivo* model.

In conclusion, the *in vitro* studies have provided sufficient confidence that the plasmid vectors could produce sustained expression of the transgenes. The results yielded from the AAV *in vitro* studies showed some expression of transgenes, however, due to the limitations of the lifespan of the myoblasts/myotubes, an *in vivo* study is required to provide a more stable, longer term evaluation.

5 Chapter 5 – Assessment of constitutive expression of Exendin4 and GLP1 via both plasmid and AAV administration *in vivo*

5.1 Introduction

Skeletal muscle as a route of delivery for gene therapy has many advantages and has been used repeatedly as a successful method in many disease states (Manno, 2003; Mingozzi, 2009; Qiao *et al.*, 2011).

Skeletal muscle is a stable tissue with an excellent vascular supply. The muscle fibres provide a stable matrix for gene transcription and therefore confer long-term protein production, which is secreted directly into the blood stream via the large vascular supply to the fibres. Moreover, skeletal muscle fibres are thought to persist for the lifetime of the subject, under normal circumstances, which means the limitations of the gene transcription are not hindered by cell turnover (Qiao *et al.*, 2011).

Transfection into skeletal muscle is most effective when used with various adjuvant techniques, which help the transgenes integrate into the fibres more effectively. In this study, the use of hyaluronidase and electroporation were employed to enhance the transfection efficiency of the plasmid DNA in to the rodent skeletal muscle. No adjuvant techniques were used with AAV infection (Mathiesen, 1999).

Hyaluronidase helps to increase tissue permeability in the skeletal muscle matrix by lowering the viscosity of hyaluronan, via hydrolysis, which is found in the connective tissue of the matrix. This creates a temporary permeability of the connective tissues to allow the plasmid, carrying the transgenes, easy access to the muscle fibres themselves. (Mennuni *et al.*, 2002).

Electroporation is again, another external method of increasing tissue permeability, by creating a localised electro-magnetic field across the injection site to create a temporary pore in lipid bilayer portion of cell membranes. This pore then allows larger, charged molecules (such as DNA) to pass through the otherwise impermeable membrane (Mathiesen, 1999; Mir *et al.*, 1999; Wells, 2010).

Both of these techniques can be used together to provide a ‘best-chance’ scenario, with hyaluronidase being administered prior to DNA introduction, and the electroporation technique being employed post DNA introduction.

Several studies have shown this combination of enhanced transfection has provided increased transfection efficiencies and therefore higher levels of transgene expression.

(Aihara and Miyazaki, 1998; Rana *et al.*, 2004; De Vry *et al.*, 2010; Wells, 2010).

Some studies have already investigated the potential of GLP-1 and/or Exendin-4 expression *in vivo*. However, none of these studies (as determined at the onset of this study) have used the direct skeletal muscle route, performed a direct comparison of plasmid and AAV vectors using the same transgenes or assessed the longevity of the expression over this time frame.

5.2 Objectives

The objective of this study was to assess the constitutive expression of GLP-1 and Exendin-4 in non-diseased and diseased models via administration using plasmid and AAV technologies, with the use of adjuvant techniques in the case of plasmid transfection.

- Establish *in vivo* gene transfer protocol for longer-term studies with Exendin-4 and eGFP reporter genes, confirmed via regular blood sampling and *in situ* reporter gene imaging.

- Comparison of GLP-1 and Exendin-4 plasmids in a longer-term study in an *in vivo* model of insulin resistance and type 2 diabetes.

- Comparison of GLP-1 and Exendin-4 AAV vectors in a longer term *in vivo* study.

5.3 General notes

All mice were housed in flat bottom cages, with a maximum of 6 mice per cage. Mice were approximately 3weeks at arrival and weight assessment at arrival indicated no significant differences; therefore, mice were assigned groups randomly. They were allowed free access to food and water, unless a specific part of the protocol required restricted access to food, at which point the mice went no longer than 16 hours without food, but generally were subject to no longer than 12-14 hours with no food access. At no point were the mice restricted access to water. The housing area was subject to 12hour light/dark cycles and all mice had a 1 week acclimatisation period in their assigned groups before any protocols were activated. During the protocols, mice were monitored daily for clinical scoring and weight gain or loss. Any health and welfare issues were discussed in full with the area technicians and escalated to the NVS/NACWO where necessary.

Prior to administration, all injectable material was subject to endotoxin level assessment. No injectable material was prepared with solutions if the endotoxin level was found to be >0.25 EU/ml, in-line with the USP guidelines for endotoxin limits for water for injection (*U.S. Pharmacopeia Sterile Water for Injection*). No final injectable solution was used if the total EU level was found to be >5.0 EU/kg, which is in line with the FDA guidelines for non-intrathecal administration (*FDA Technical Guides - Bacterial Endotoxins/Pyrogens*). Endotoxin testing was carried out using the EndosafePTS and associated cartridges (Charles River Laboratories International Inc, MA, US).

There were no health issues with the CD1 females and no intervention or special measures were implemented at any point. However, with the db/db females, weight gain was rapid and obvious when compared to the standard black 6 mouse, whose strain is the background for the db/db (figure 5-1) which resulted in small skin tears in the shoulder region.

Figure 5-2 indicates the location and size of the injury (inside red circle). These were monitored closely for any signs of infection and treated with standard green clay (no active pharmacological ingredients which may impact the study results). The wounds

healed quickly and cleanly and no further measures were needed. No other issues were observed.

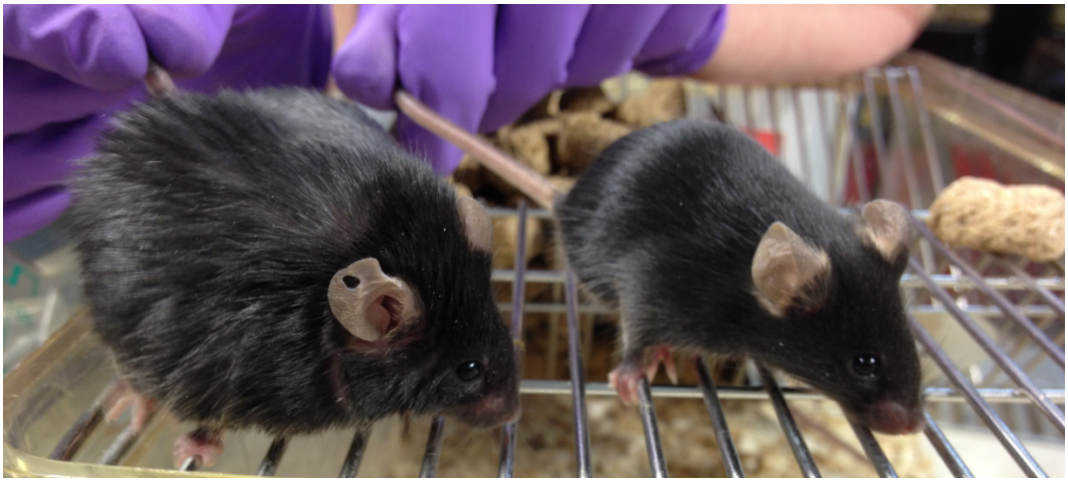


***Figure 5-1 – An example of a dbdb mouse and black 6 (C57BL/6) of the same age**. Dbdb mice have a black 6 background and are bred with a spontaneous point mutation of the leptin receptor gene.*

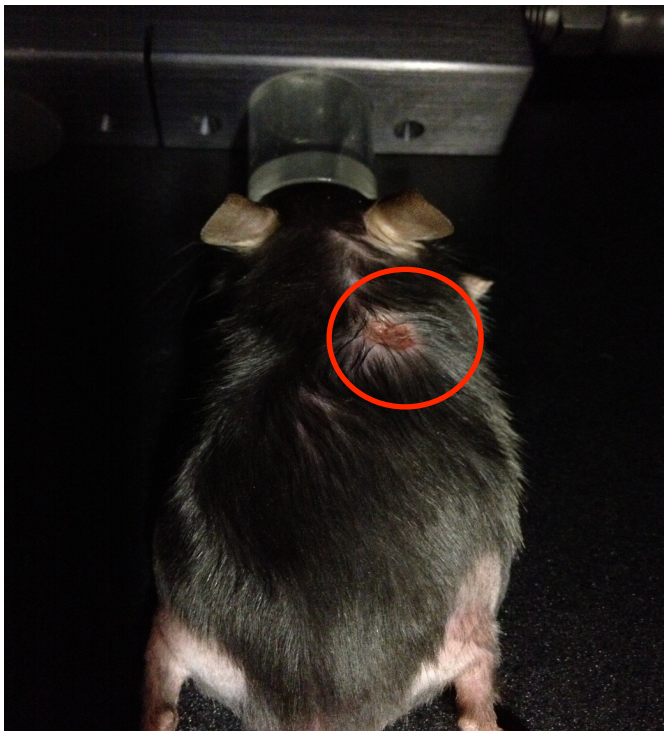


***Figure 5-2 – Image showing area and average size of tearing observed in db/db females at 4-6 weeks old.** During the early phase of rapid weight gain, the skin of the mice was not given enough time to stretch to accommodate the increase in size. This resulted in small tears, localised to the shoulder area. In all cases, the wounds healed quickly and cleanly and no intervention was required. The red circle indicates the localised area of injury. No other injury locales were noted.*

5.3.1 *Vector Administration and adjuvant techniques*

Plasmid administration was coupled with hyaluronidase and electroporation as a method to increase the efficiency of vector uptake and subsequent gene expression.

For plasmid administration, the mouse was anaesthetised lightly to prevent movement and reduce pain and stress via inhalant anaesthetic isoflurane. The analgesic carprofen was administered subcutaneously into the scruff of the mouse (dose 50 mg/kg, total volume 0.1 ml). Hyaluronidase was administered into the muscle in the location of the subsequent plasmid administration, at a concentration of 0.4 U/µl in a total volume of 25µl prior to plasmid injection, which was at a concentration of 1 µg/µl in a total volume of 50 µl. Post plasmid injection, the leg was subjected to electroporation (settings used: 200 V/cm, eight 20 ms pulses). The calliper electrodes were stainless steel plates and ECG gel was applied to the plates prior to electroporation taking place. All injections were made percutaneously to both tibialis anterior and gastrocnemius muscles, perpendicular to the central portion of the muscle.

AAV vectors were administered under the same conditions, without the use of the hyaluronidase or electroporation. The AAV vectors were administered in a total volume of 50 µl in the following titre of vector genome (vg) (as determined appropriate from published literature (Zincarelli *et al.*, 2008):

- Exendin 4 - 1.776×10^{11} vg/mouse
- GLP1 - 1.784×10^{11} vg/mouse
- eGFP - 1.745×10^{11} vg/mouse

Whole blood samples were obtained using standard tail venepuncture, as larger volumes (approximately 100 µl, depending on the weight of the animal) were required.

The exact volume obtained was kept within the NC3R guidelines for blood sampling (which was also reflected within the PPL - https://www.nc3rs.org.uk/blood-sample-volumes)

5.3.2 Completion of study

On completion of the study (see individual sections for duration), mice were euthanized as per schedule 1 appropriate protocol, cervical dislocation, or where larger volumes of blood samples were needed, cardiac puncture was used and therefore mice were subject to non-recovery anaesthesia. Cervical dislocation post cardiac puncture confirmed death.

Pancreas and muscle samples (anterior tibialis and gastrocnemius region) were harvested immediately after death using a consistent standardised method of dissection. Pancreas samples were immediately placed in 10% formalin for FFPE processing. Muscle samples were placed in a Buffer A mixture (see chapter 2, section 2.2.12) or snap frozen in liquid nitrogen or dry ice cooled isopentane depending on processing requirements (snap frozen for sectioning/ICC, buffer for homogenisation for analysis via ELISA/EIA)

5.4 In Vivo assessment of secretory properties and pharmacodynamics of Exendin 4 in CD1 mice via plasmid administration, with an accompanying eGFP control group

This assessment took place over the course of 30 days with CD1 male mice as the rodent subjects.

5.4.1 Weight Evaluation

Monitoring the weight gain (and/or loss) of the animals was one of the main indicators of general health. Weight measurements were taken daily, during which time, a general assessment of the animals were made including activity observation and skin/fur checks, paying particular attention to the injection and electroporation sites on the hind legs.
All mice showed normal weight gain and normal activity levels during the course of the study (figure 5-3). However, fighting was observed between some of the subjects, which resulted in segregation for some or all of the remainder of the study.
No significant differences were observed between the groups over the course of the study. All mice gained weight, which is part of the acceptance criteria of the clinical assessment score chart for general animal health (see Appendix 1 (8.1), page 164).

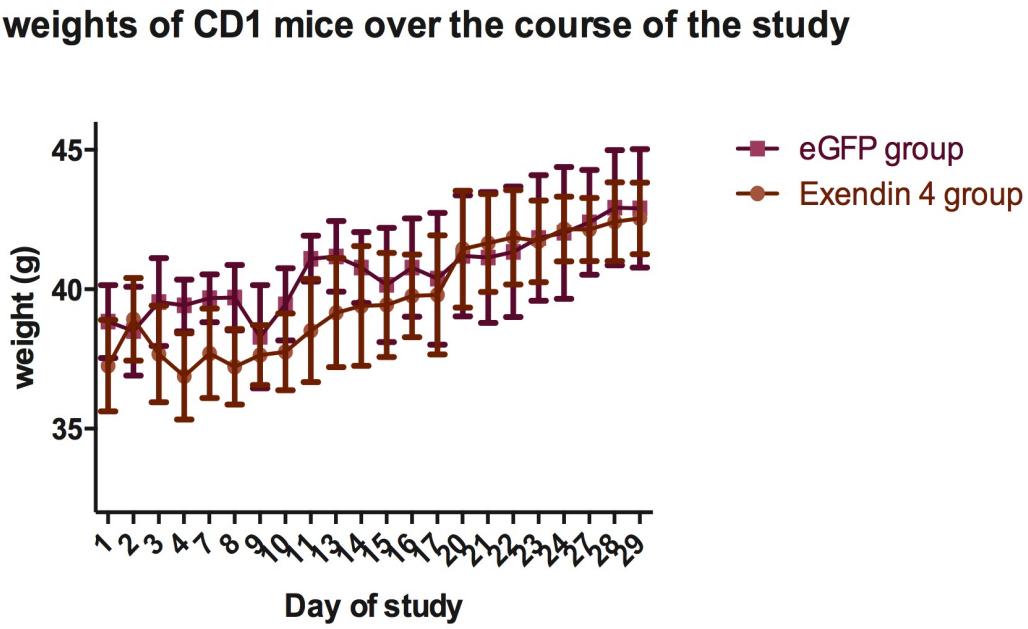


***Figure 5-3 – Mean weights observed for all mice over the course of the study**. No significant difference was observed between the two data sets over the 30 day period (n=5; p.0.05).*

5.4.2 IPGTT and mean fasted glucose

Fasted IPGTTs (intraperitoneal glucose tolerance tests) and fasted glucose measurements were taken at various time points throughout the study. Mice were allowed free access to water only for approximately 12-14 hours before being subjected to the tests. For glucose measurements only, blood samples were taken using an animal specific glucometer and strips (the AlphaTRAK2 system (Zoetis, NJ, US)), which has been specifically validated for rats and mice, using <5µl whole blood (the meter only needs a minimum of 0.3 µl to make a reading) via a small capillary sample obtained from the tail. For the purpose of the IPGTT an initial glucose reading was taken (T0) prior to administration of glucose. Subsequent glucose readings were taken at specific time points after administration of 2 g/kg glucose via intra-peritoneal injection and before allowing free access to food (T= 15, 30, 60, 90 minutes post glucose administration). Results are shown in figure 5-3, 5-4 and table 5-1. No significant differences were observed for mean fasted glucose or IPGTT data over the course of the study.

Figure 5-4 – Mean fasted glucose observed for all mice over the course of the study. *Fasted glucose levels were recorded as part of the IPGTT process. No significant difference was observed between the two data sets over the 30 day period (n=5; p.0.05).*

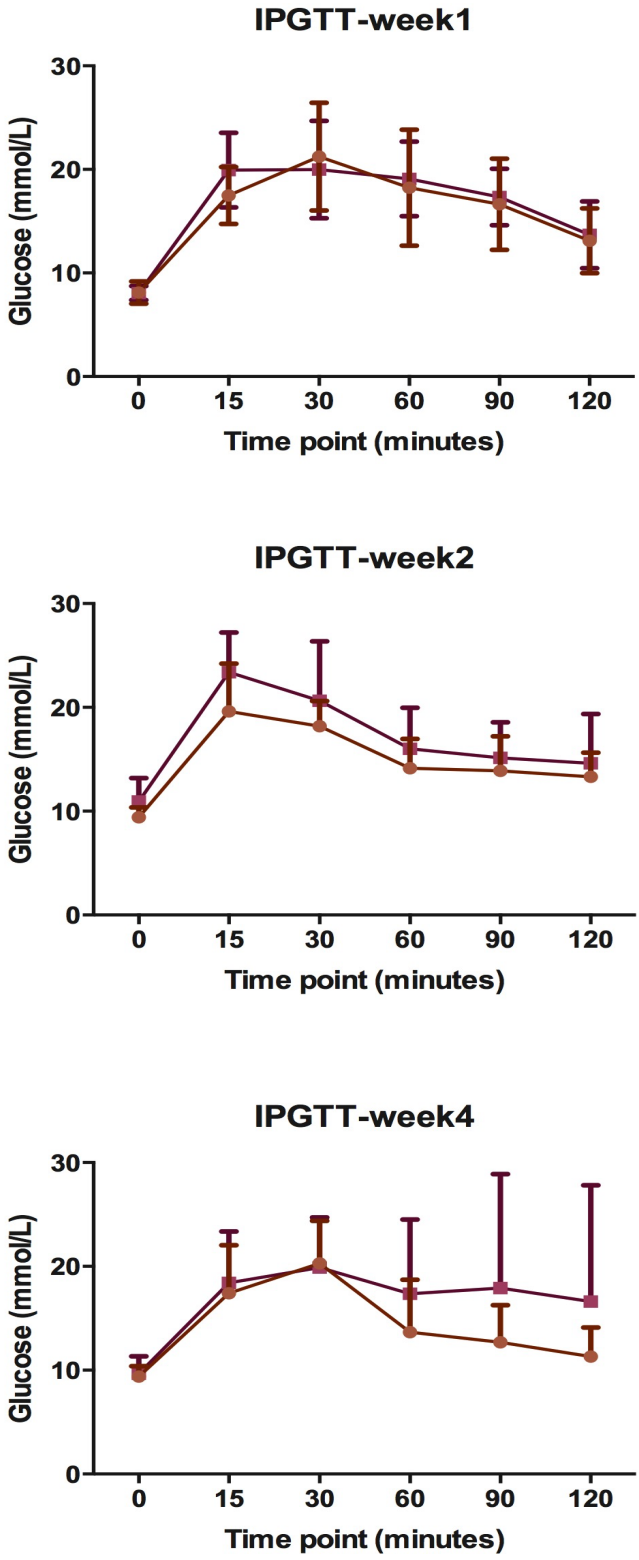


Figure 5-5– IPGTT data*. IPGTT analyses were obtained at 3 points during the study. The curves indicate a normal response to IP glucose, as expected in a normal model animal (n=5).*

DATE	GROUPING	REPEATED MEASURES - BONFERRONI
IPGTT DATA OVER ALL 4 WEEKS	Ex4 vs. GLP1	1.000
	Ex4 vs. eGFP	1.000
	GLP1 vs. eGFP	1.000

***Table 5-1 - Statistical analysis of IPGTT data**. Using repeated rANOVA and Bonferroni correction shows no significant difference between any of the groups (n=5; p>0.05).*

5.4.3 eGFP expression as evaluated via IVIS

The IVIS spectrum is a non-invasive method of determining the location and intensity of fluorescent (or luminescent) biomarkers without needing to sacrifice the animal.
Each animal is lightly anaesthetised purely to prevent any movement whilst the scans are taking place. Any and all auto-fluorescence is detracted from the expression detected by the transgene by the software associated with the equipment. The specific intensity of each individual region of interest is then calculated, and the corresponding images can be used to assess locale.
All mice were subjected to IVIS scans on a weekly basis. This evaluates the eGFP expression found in a rodent, independent of any auto-fluorescence that may be found in all mammals.
The data obtained showed an increase in eGFP expression, from day 8 up to day 30, with a significant difference during this time frame ($p>0.05$), suggesting the uptake of plasmid and successful transcription is taking place.

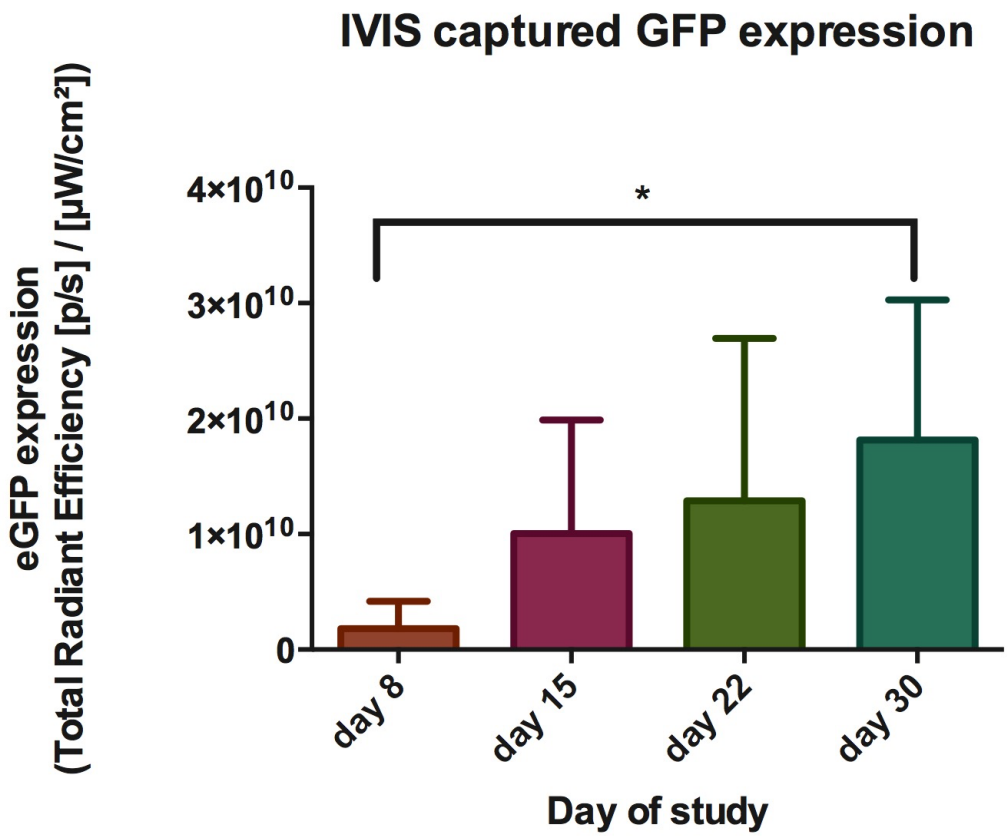


Figure 5-6 – eGFP expression as captured using the IVIS spectrum*. CD1 male mice in the study exploring the effects of plasmid introduction of Exendin 4 and eGFP over the course of 30 days (n=5; p>0.05).*

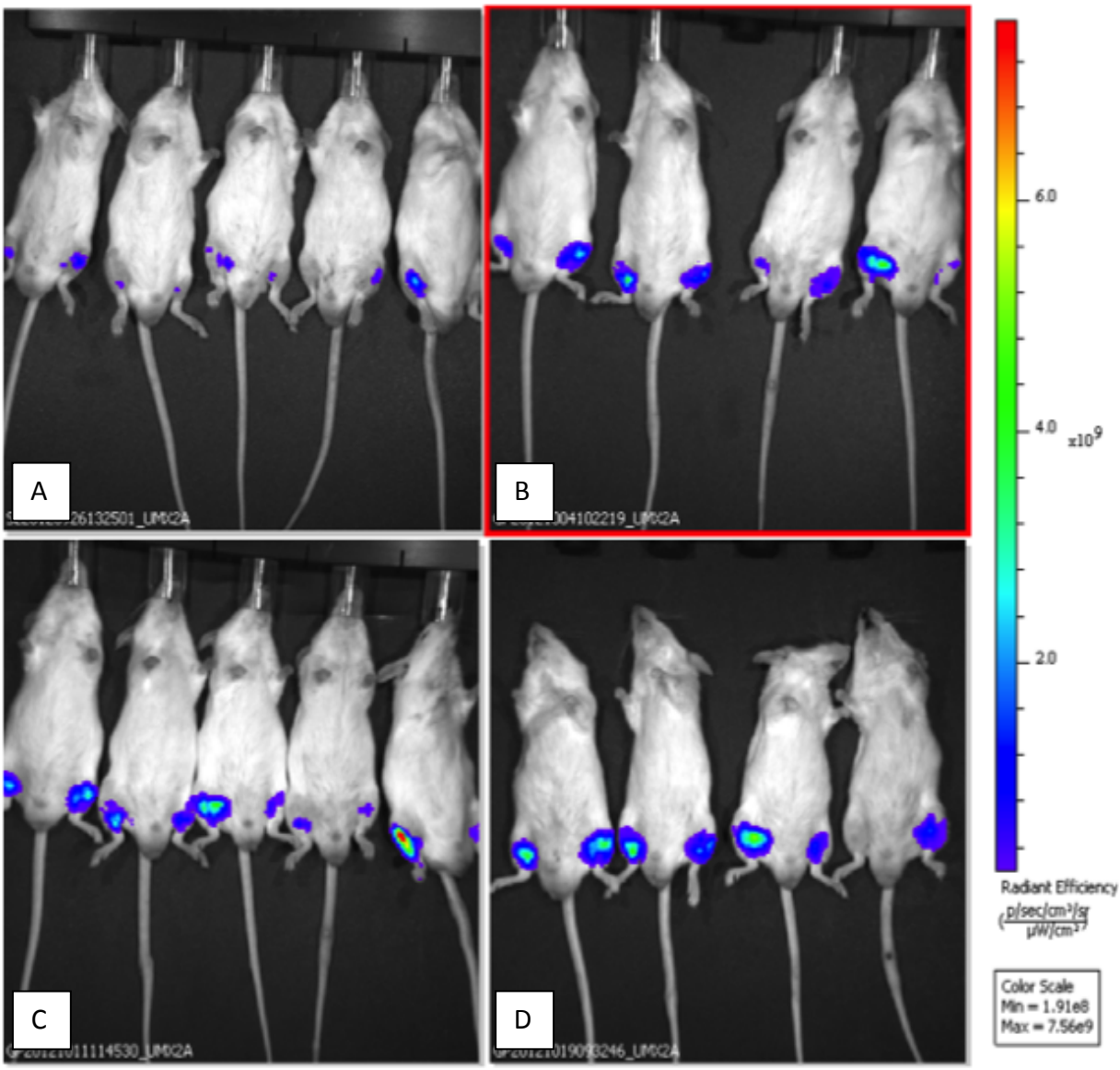


Figure 5-7 – Images taken from IVIS scanner showing the regions of expression obtained from pVR1012.eGFP.CMV, without auto-fluorescence*. One of the mice was subject to an IVIS/CT co-registration trial, hence not present in image B. The same mouse was sacrificed early due to unknown illness, therefore not present in image C. On post-mortem investigation, and consultation with the NACWO, the mouse appeared to have contracted a urinary tract infection; most likely as a result from a bite wound when fighting, males were not used for any further studies.*

5.4.4 *Evaluation of peptide concentration in blood plasma*

Blood samples were taken via venepuncture and collected in EDTA tubes. The plasma concentration of Exendin-4 was then evaluated via EIA analysis.

Samples were collected twice over the course of the study, on day 15 and day 30. Whilst the data suggest the Exendin-4 concentration was much higher at day 15 than day 30, due to the large standard deviation observed within the group at day 15, no statistically significant difference was found ($p>0.05$).

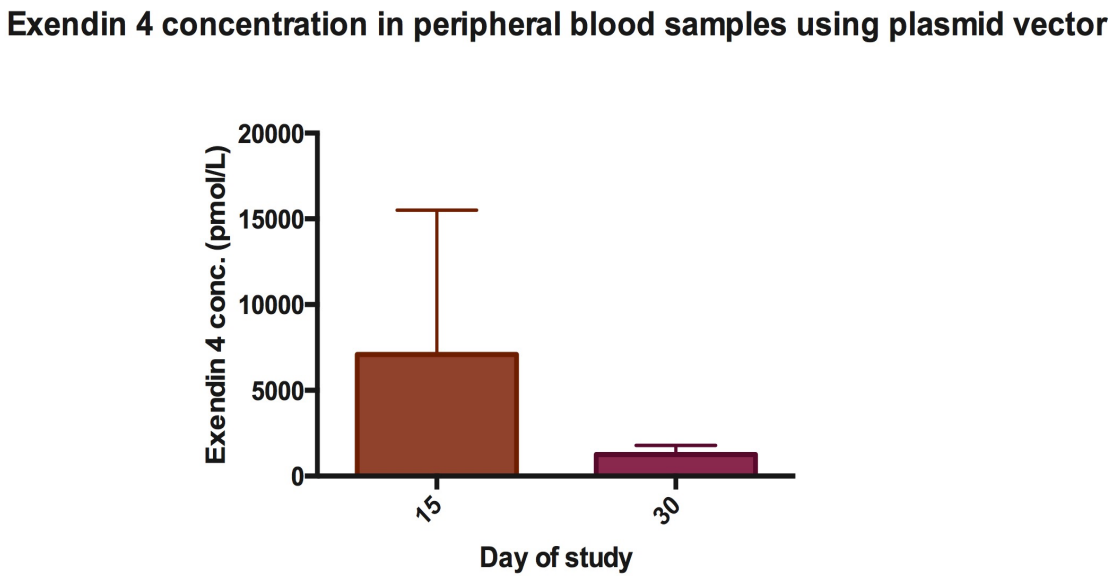


Figure 5-8 – Plasma/ serum Exendin-4 concentration in peripheral blood samples obtained via venepuncture and analysed via Exendin-4 EIA*. Due to the large variation seen in the group at day 15, no significant difference was observed (n=6; p>0.05).*

5.5 In Vivo assessment of secretory properties and pharmacodynamics of Exendin-4 and GLP-1 in db/db mice via plasmid administration, with an accompanying eGFP control group

The study consisted of a longer term assessment of both GLP-1 and Exendin-4 transgene expression with db/db female mice as the disease rodent model.

5.5.1 Weight Evaluation

Monitoring the weight gain (and/or loss) of the animals was one of the main indicators of general health. Weight measurements were taken daily, during which time, a general assessment of the animals were made including activity observation and skin/fur checks, paying particular attention to the injection and electroporation sites on the hind legs.
All mice showed expected significant rapid weight gain and low activity levels during the course of the study.
No significant differences were observed between the differently treated groups over the course of the study.

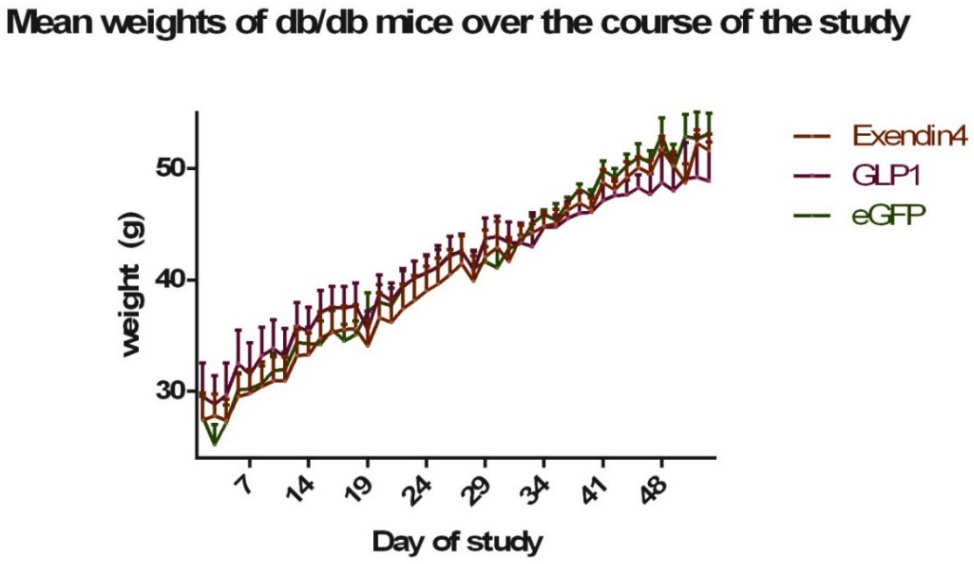


***Figure 5-9 – Mean weights as recorded throughout the study**. The mean weights ± SD for each group are shown. No significant difference was observed between the groups over the course of the study (n=6; p>0.05).*

5.5.2 IPGTT and mean fasted glucose

The methodology for fasted IPGTTs and fasted glucose measurements was the same as described above (5.4.2).

Fasted glucose levels showed statistically significant differences were observed between the Exendin-4 and GLP-1 groups ($p<0.05$), and the GLP1 and eGFP groups ($p< 0.05$). No significant difference was observed between the exendin4 and eGFP group ($p>0.05$). Additionally, repeated measures ANOVA analysis performed on the IPGTT data also proved a statistically significant difference in the glucose clearance capabilities of the Exendin-4 group (figure 5-10 and table 5-2) (n=6; $p<0.05$).

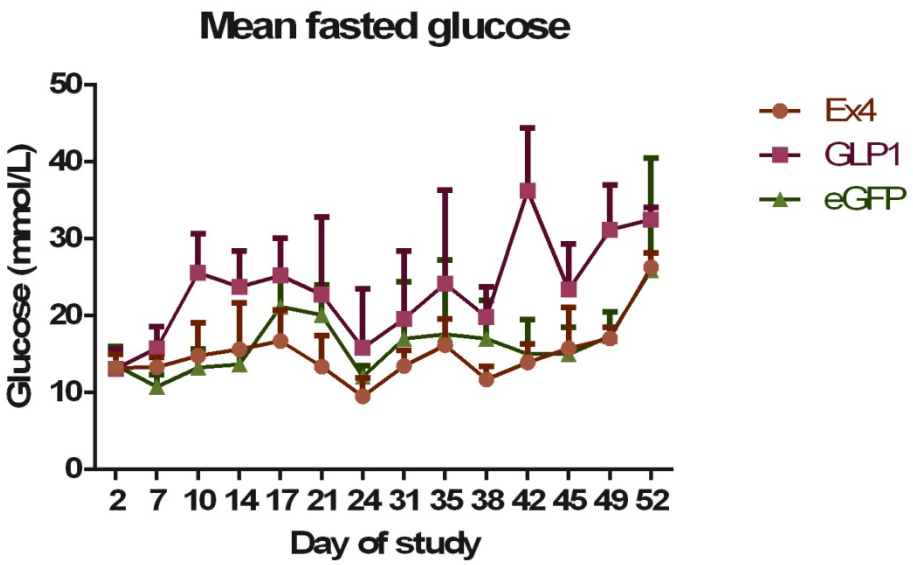


Figure 5-10 – Mean fasted glucose levels as recorded throughout the study*. Fasted glucose levels were recorded as part of the IPGTT process. The mean fasted glucose (n=6±SD) are shown. Significant differences were found between the Exendin-4 and GLP-1 groups (p<0.05), and the GLP1 and eGFP groups (p< 0.05). No significant difference was observed between the exendin4 and eGFP group (p>0.05).*

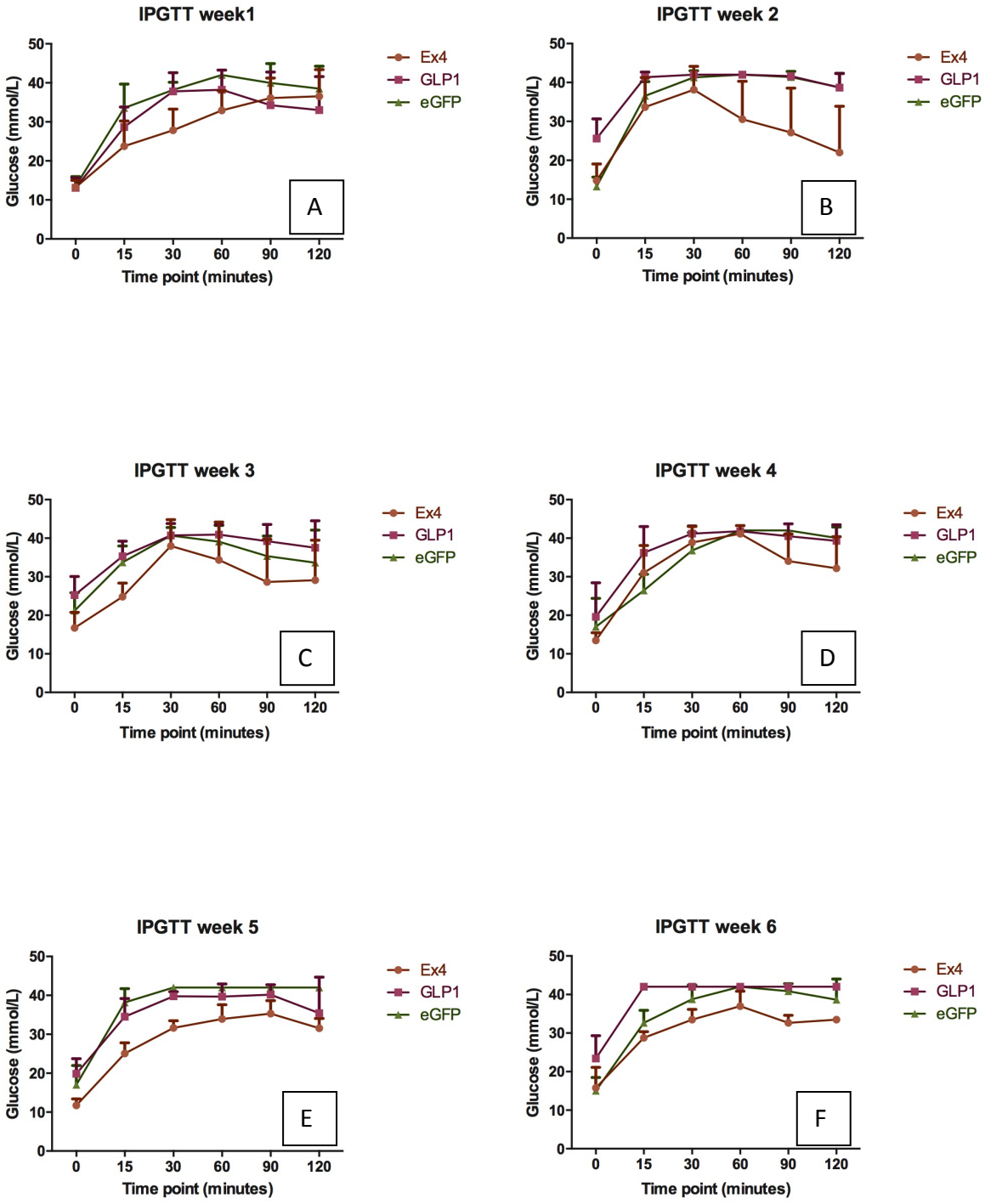


Figure 5-11 – IPGTT data; *(A) day 0 (pre-treatment), (B) day 7, (C) day 14, (D) day 28, (E) day 35 and (F) day 42. Statistical analyses shows there is a significant difference within the exendin4 group. (See table 5-2)(n=6).*

DATE	GROUPING	REPEATED MEASURES – BONFERRONI P-VALUE
IPGTT DATA OVER ALL 6 WEEKS	Ex4 vs. GLP1	**0.044***
	Ex4 vs. eGFP	**0.049***
	GLP1 vs. eGFP	1.000

Table 5-2 - Statistical analyses of the IPGTT data of mice treated with muscle-targeted gene therapy using pVR1012-EGFP, -Exendin-4 or –GLP-1, respectively gained throughout the study*. Using repeated measures ANOVA, which considers data over the whole time course, there is a statistical difference in favour of the exendin4 group (n=6; p<0.05).*

5.5.3 Evaluation of peptide concentration in both blood plasma samples and muscle homogenate

In order to further confirm transgene biosynthesis, analyses were performed on both blood plasma and muscle homogenate.

Bloods samples were taken via venepuncture at regular intervals during the study, in line with PPL statements and NC3R guidelines. Blood was collected in EDTA tubes and plasma was separated and/or muscle samples were homogenised and ELISA/EIA analysis was performed.

An increase in Exendin-4 concentrations were observed in peripheral blood plasma samples up to day 28 (figure 5-12). A seemingly steady decrease was observed at day 42 and further again at day 49. Whilst the final sample (day 49) was still much higher than that observed at day 14, due to the large variation within the group (and therefore large standard deviation), the only statistically significant difference was observed up to day 28.

In cardiac puncture samples, which represent the single time point at the closure of the study, Exendin-4 concentration was almost a quarter of what was observed a the peak of expression as determined via peripheral blood samples and similar but still lower than that observed from the last peripheral sample, taken only 1 day prior to cardiac puncture (figures 5-12 and 5-13 versus 5-14), however, the cardiac puncture samples were also analysed for cross-reactivity in the ELISA/EIA assay's. This confirmed the robustness and selectivity of the assay with negligible levels of Exendin-4 found in the GLP-1 and eGFP groups ($p<0.001$) (figure 5-14).

GLP-1 concentration in cardiac samples showed no significant difference in any of the groups ($p>0.05$) (figure 5-14).

Concentrations in muscle homogenate followed the same pattern. The Exendin04 concentration was significantly increased in the Exendin-4 groups, when compared to the GLP-1 and eGFP groups (as expected)($p<0.001$)(figure 5-15), however, the concentration found in the muscle homogenate was approximately 5 times reduced when compared to the cardiac puncture samples. There was no statistical significant difference in the GLP-1 concentration in muscle homogenate between any of the groups ($p>0.05$) (figure 5-15).

Additionally, plasma insulin and fructosamine were evaluated in the terminal samples obtained through cardiac puncture. The increased insulin concentrations validated the phenotype of the db/db mice, all showing hyperinsulinemia; a difference in insulin concentration or fructosamine, reflecting the average plasma glucose over the course of the study, was not detected between the groups (figures 5-16 and 5-17).

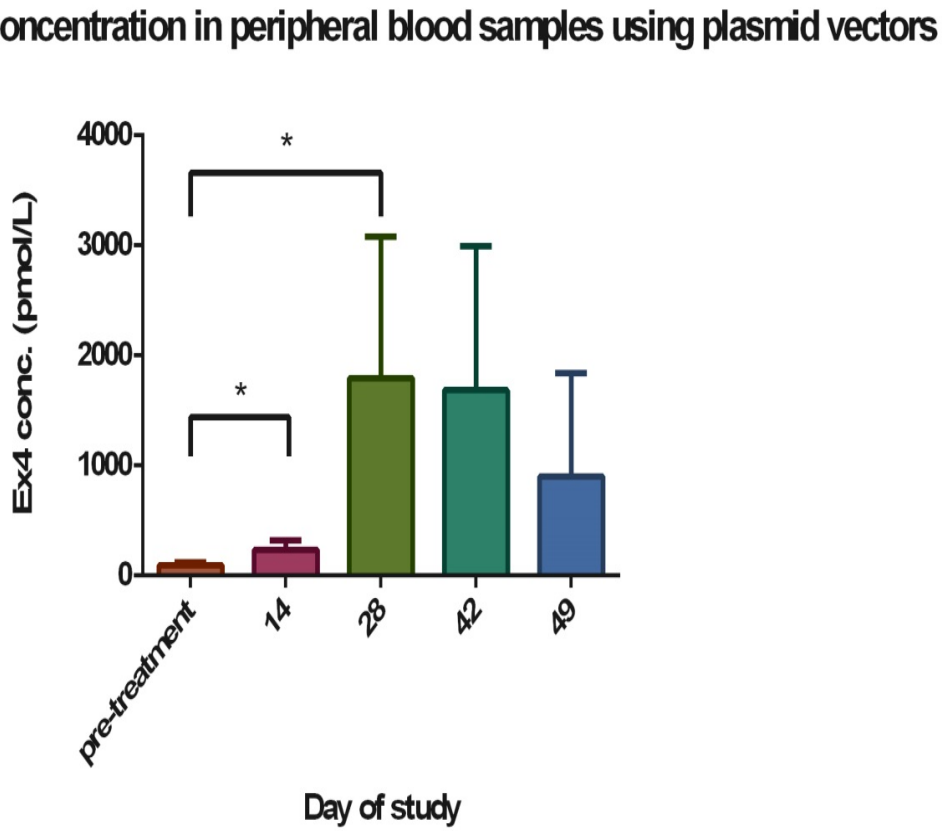


Figure 5-12 –Exendin-4 levels in peripheral blood samples. *Exendin-4 concentration was measured in peripheral blood samples (venepuncture) at various intervals throughout the study, via Exendin-4 EIA. All times points showed a significant rise from pre-treatment levels, however the only statistically significant difference was observed up to day 28 due to the large standard deviations observed at days 42 and 49 (n=6; p<0.05)*

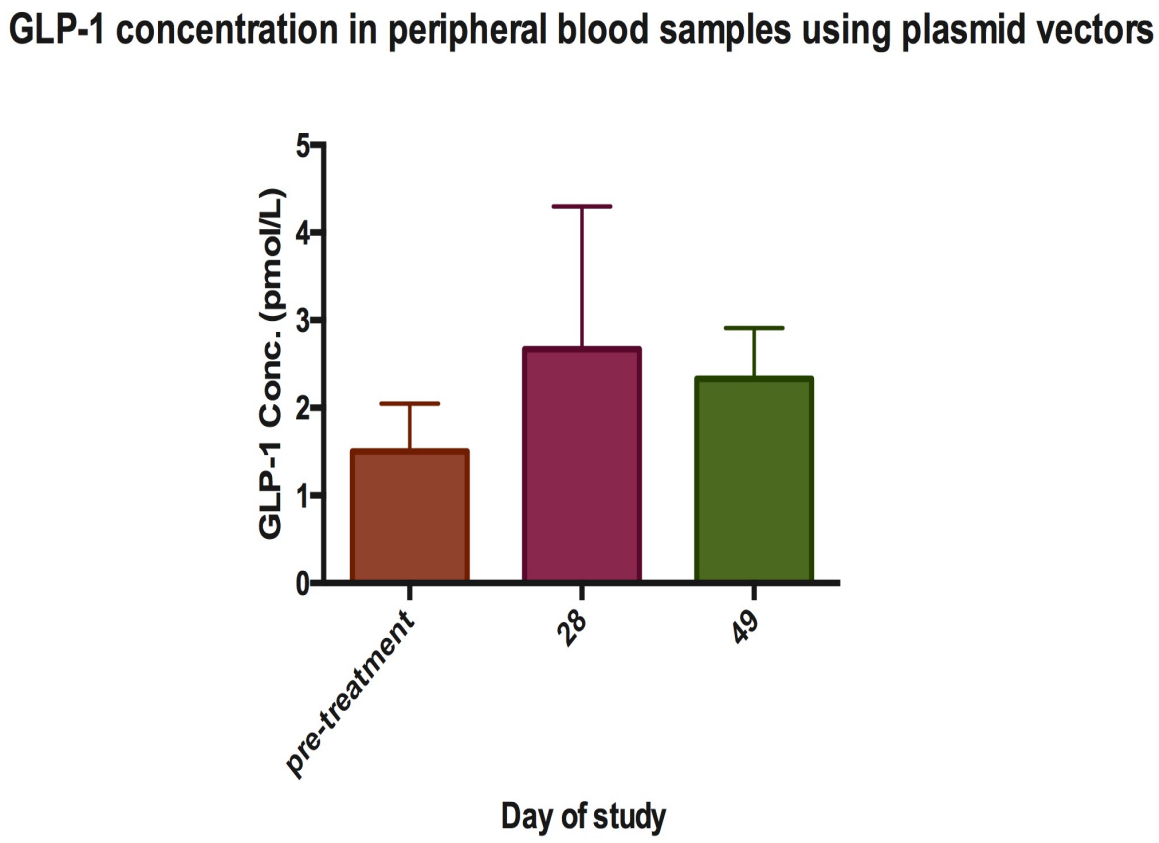
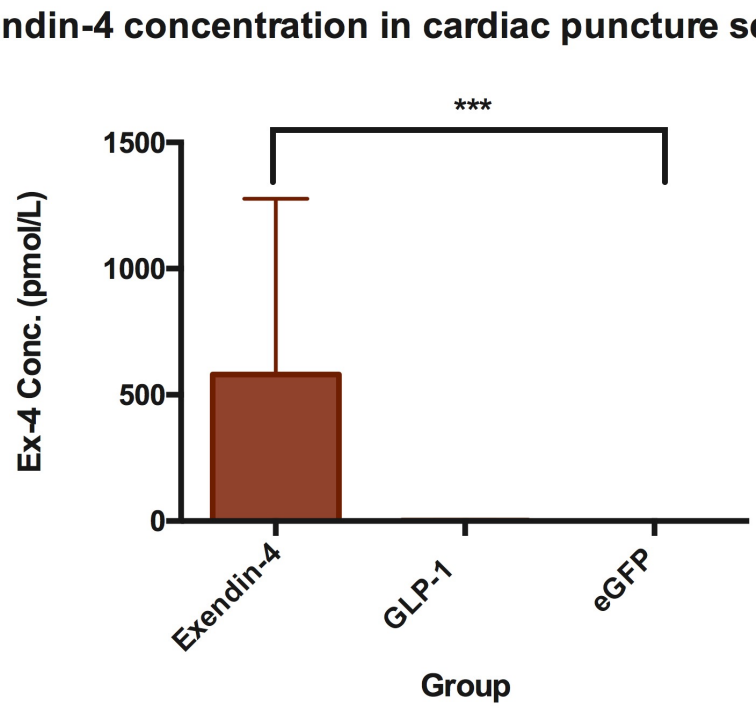


Figure 5-13 – GP004 GLP1 levels in peripheral blood samples*. GLP-1 concentration was measured in peripheral blood samples (venepuncture) at various intervals throughout the study. There was no significant difference in concentration at any time point (p>0.05). The intervals were not as frequent as those collected for Exendin-4 concentration determination due to the volume of serum required for analysis and the restrictions in blood volume collection in place on the PPL/PIL.*



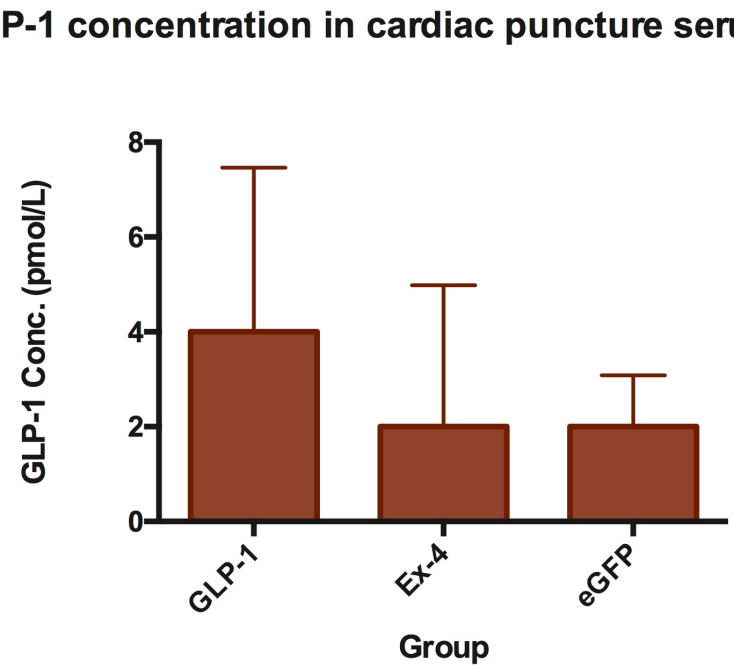
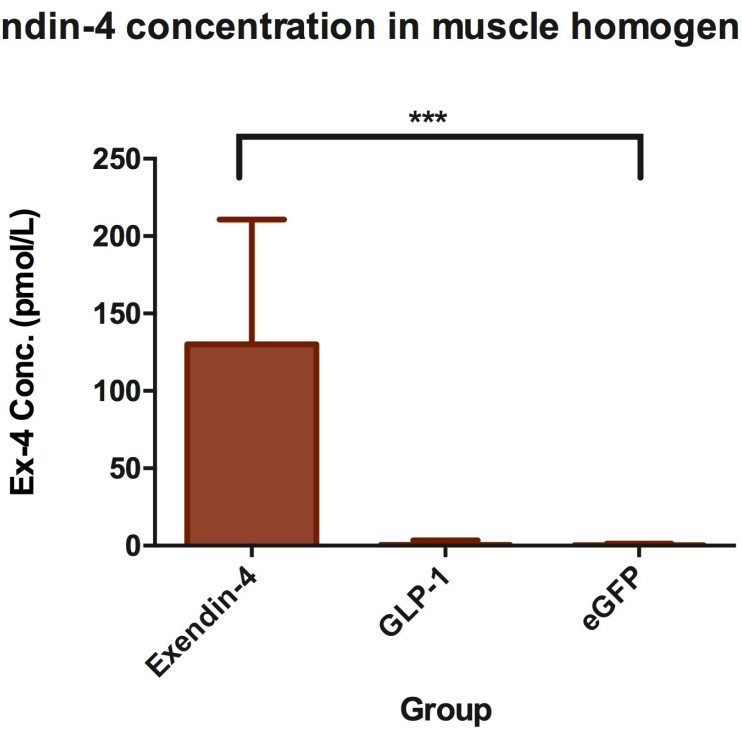


Figure 5-14- Peptide concentration in cardiac puncture serum samples. *Exendin-4 concentration in cardiac puncture serum samples from each group shows negligible levels in the GLp-1 and eGFP groups, as expected, and significantly higher levels in the Exendn-4 group (n=6±SD; p<0.001). Additionally, this confirms the legitimacy of the Exendin-4 EIA test. The GLP-1 concentrations obtained from the same groups did not show any significant difference between any of the groups (n=6; p>0.05)*



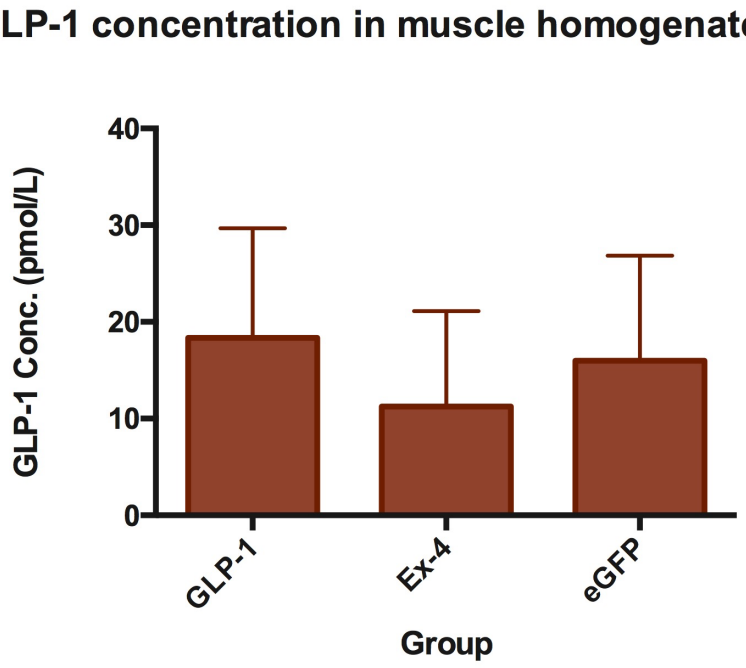


Figure 5-15 – Peptide concentration in muscle homogenate. *Exendin-4 concentration in muscle homogenate samples from each group shows negligible levels in the GLp-1 and eGFP groups, as expected, and significantly higher levels in the Exendn-4 group (n=6; p<0.001). Again, this confirms the legitimacy of the Exendin-4 EIA test. The GLP-1 concentrations obtained from the same groups did not show any significant difference between any of the groups (n=6; p>0.05)*

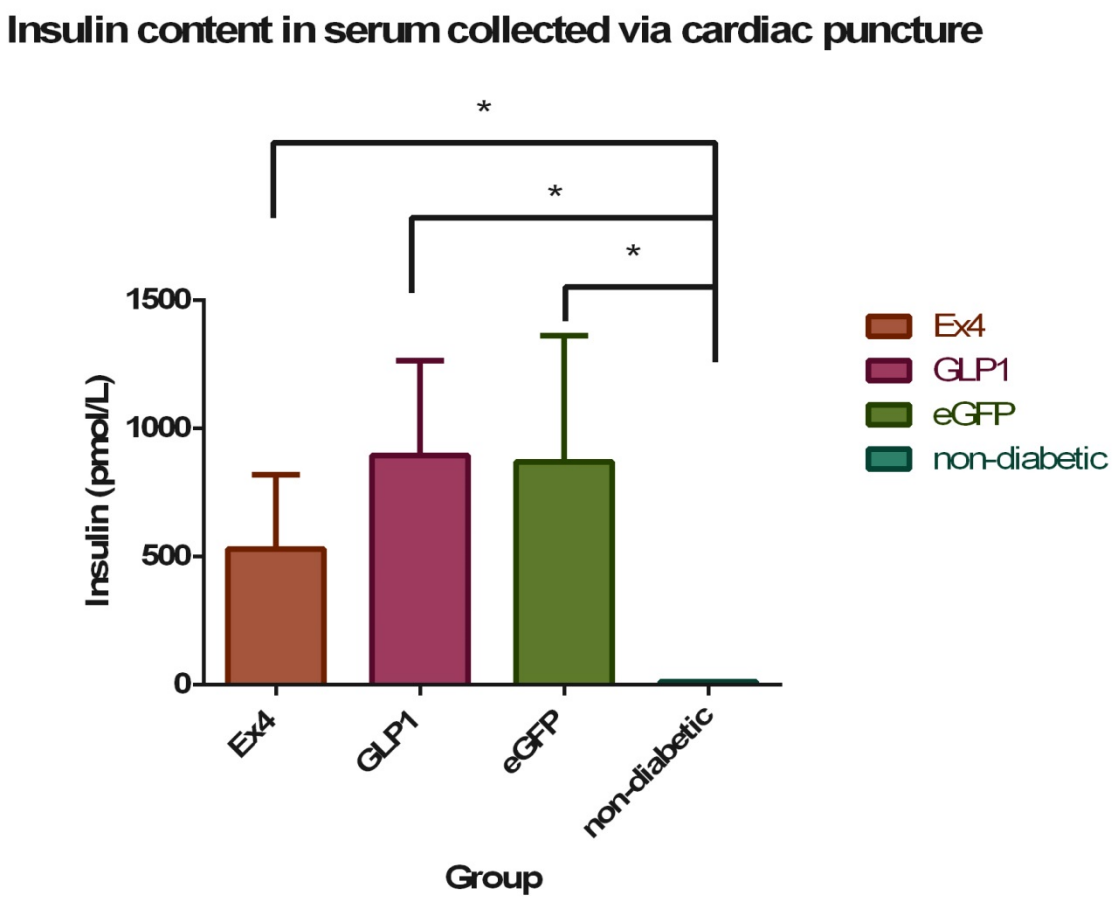


Figure 5-16 – Cardiac puncture insulin analysis*. Blood taken via cardiac puncture was analysed for insulin content in each of the groups. There was no significant difference between the groups though each group showed a marked increase in concentration when compared to a non-diabetic standard mouse serum control (n=6; p<0.05)*

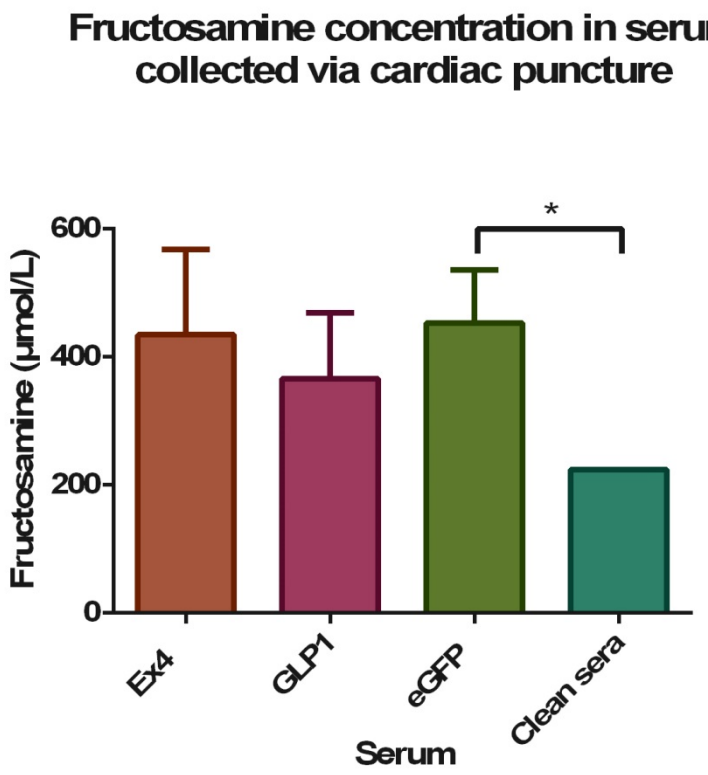


Figure 5-17 – Fructosamine assay on cardiac puncture samples. *A fructosamine assay was conducted on blood from cardiac puncture from each group. The only significant difference observed was between the eGFP group and the clean sera control (n=6; p<0.05).*

5.5.4 *Immunohistochemical analysis of pancreas*

The entire pancreas was harvested from each animal at the time of death. This was immediately placed in a 10% formalin solution prior to FFPE processing.
Sections were obtained from each tissue block and analyses were performed to establish the general health of the pancreas and an attempt to establish both insulin and glucagon content.
No obvious differences were observed between each of the 3 groups under standard bright field H&E microscopy (figure 5-17).
Sections were then stained for insulin and glucagon and software (http://imagej.nih.gov/ij/) utilised to calculate and compare the area staining positive for both (figure 5-18). Image J analysis is further explained in method section 2.2.16.

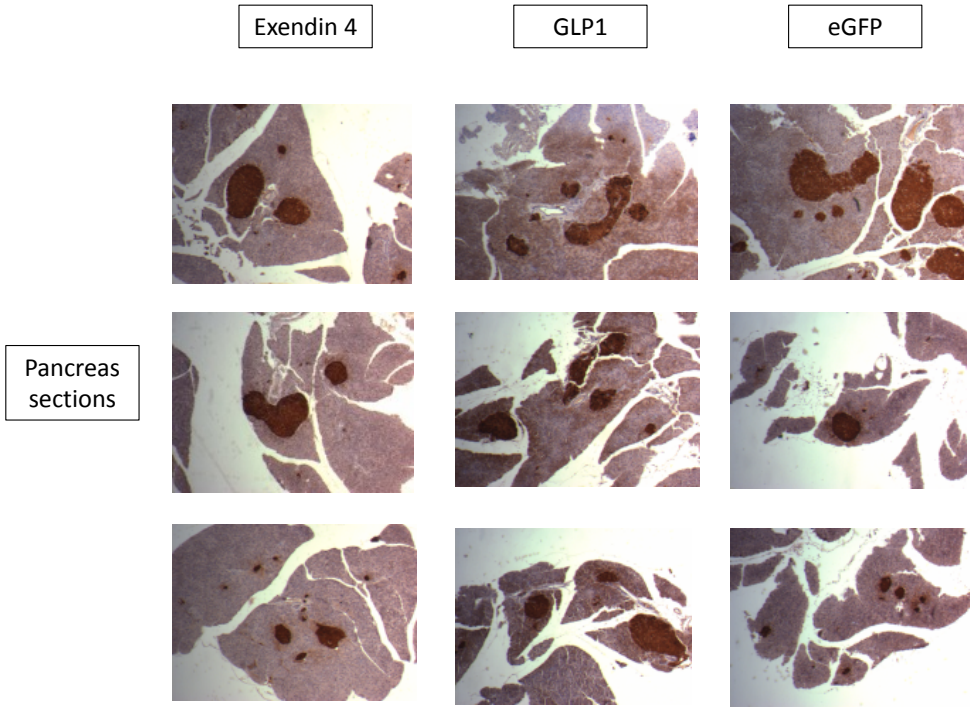


***Figure 5-18 – Examples of FFPE pancreas samples**. FFPE pancreas samples were sectioned (4µm) and double stained (DAB and nickel respectively) for insulin and glucagon. The resulting images were used to assess islet area for the pancreas. X400 magnification.*

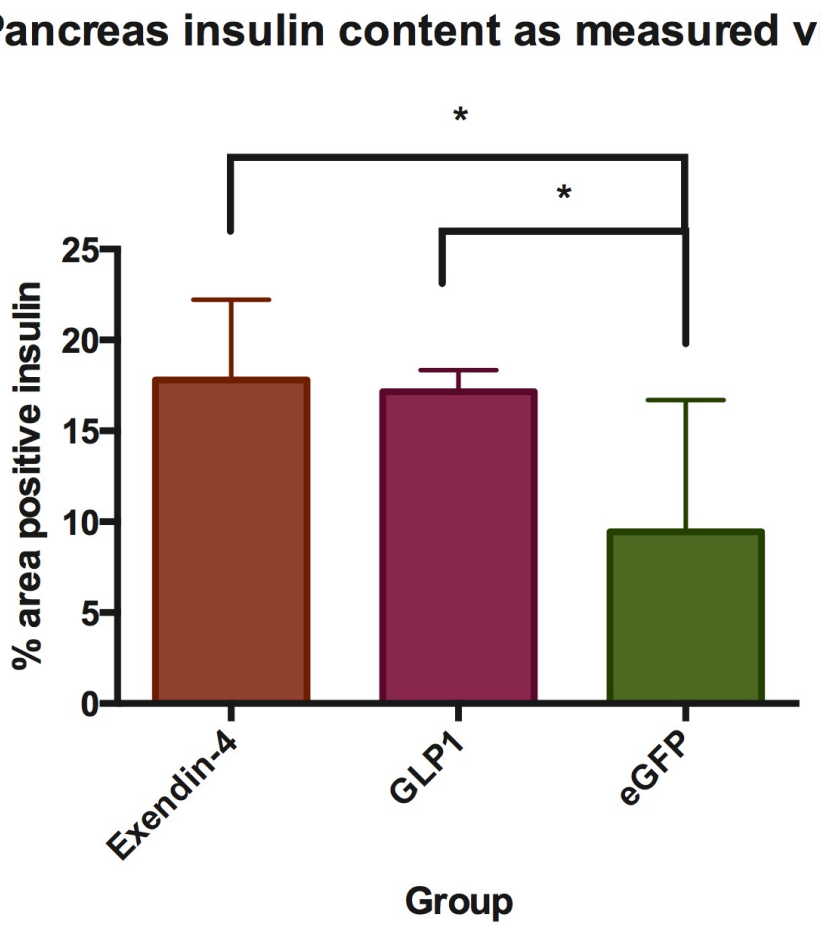


***Figure 5-19 – Insulin content in pancreas samples as measured via IHC staining**. IHC and the resulting image analysis was used to calculate the % area of positive insulin stain using colour deconvolution processing. A significant difference ($p>0.05$) was observed between the Exendin 4 group and the eGFP group, and the GLP1 group and the eGFP group. No significant difference was observed between the Exendin-4 and GLP1 group.*

5.5.5 *Assessment of eGFP expression as measured via IVIS-spectrum*

IVIS scans were performed, as described on 5.4.3.

A minor update to the PPL/PIL allowed increased IVIS scans to take place for this study. Three out of the 5 mice of the eGFP group died before the completion of the study. The uptake of the vector had already been confirmed in the previous study (see section 3.4), however, this group was still included in the study protocol as the expected longevity of this study was greater than previous. Using ANOVA to determine statistical significance, it was determined that the expression gained in this study was also significant ($p<0.0001$) indicating the phenotype of the animal (db/db) did not hamper the transgene biosynthesis.

Figure 5-20 shows the localised area of eGFP expression during the study. The resulting quantitative data obtained is shown in figure 5-21, the group was split between the 2 mice that survived the entire duration and the 3 that were deceased part way through. Peak expression was observed early in this study at day 10, which is in contrast to the expression trend observed in the previous study which was still increasing at day 30 (figure 4-5).

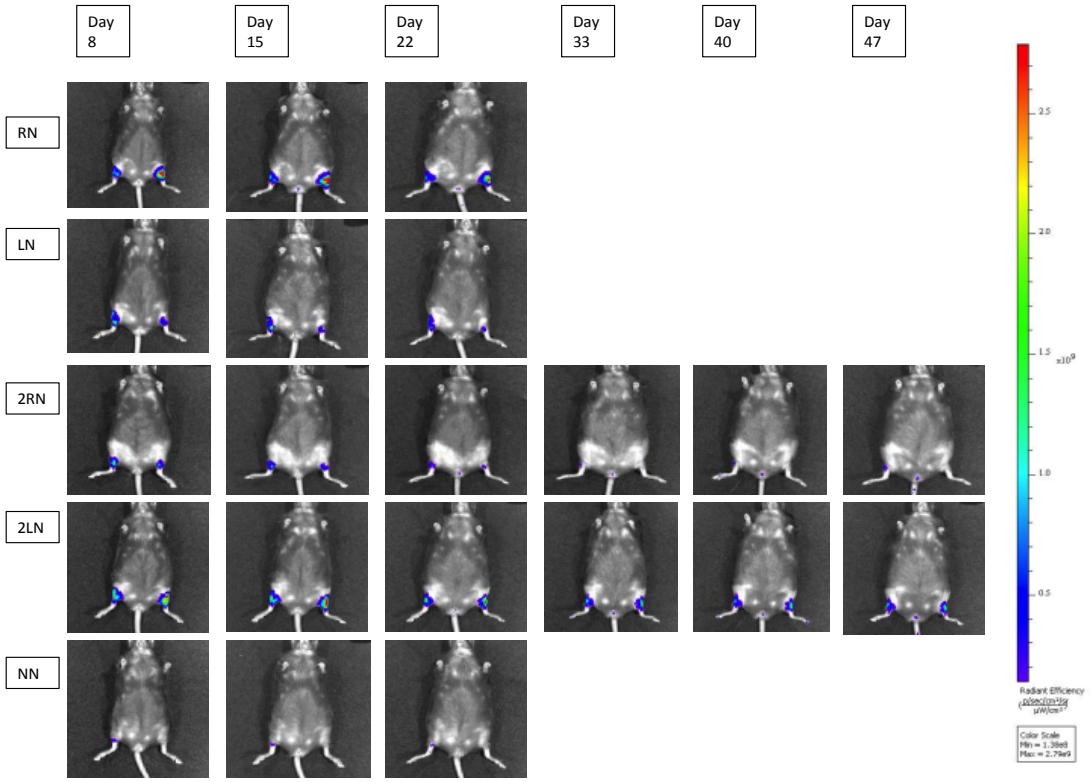


Figure 5-20 - Images of eGFP intensity in individual mice over the length of the study. *Localised areas of expression are observed in all mice, although expression seen in mouse NN is marginal compared with the others in the group. These images correlate with the data shown in Figure 5-21*

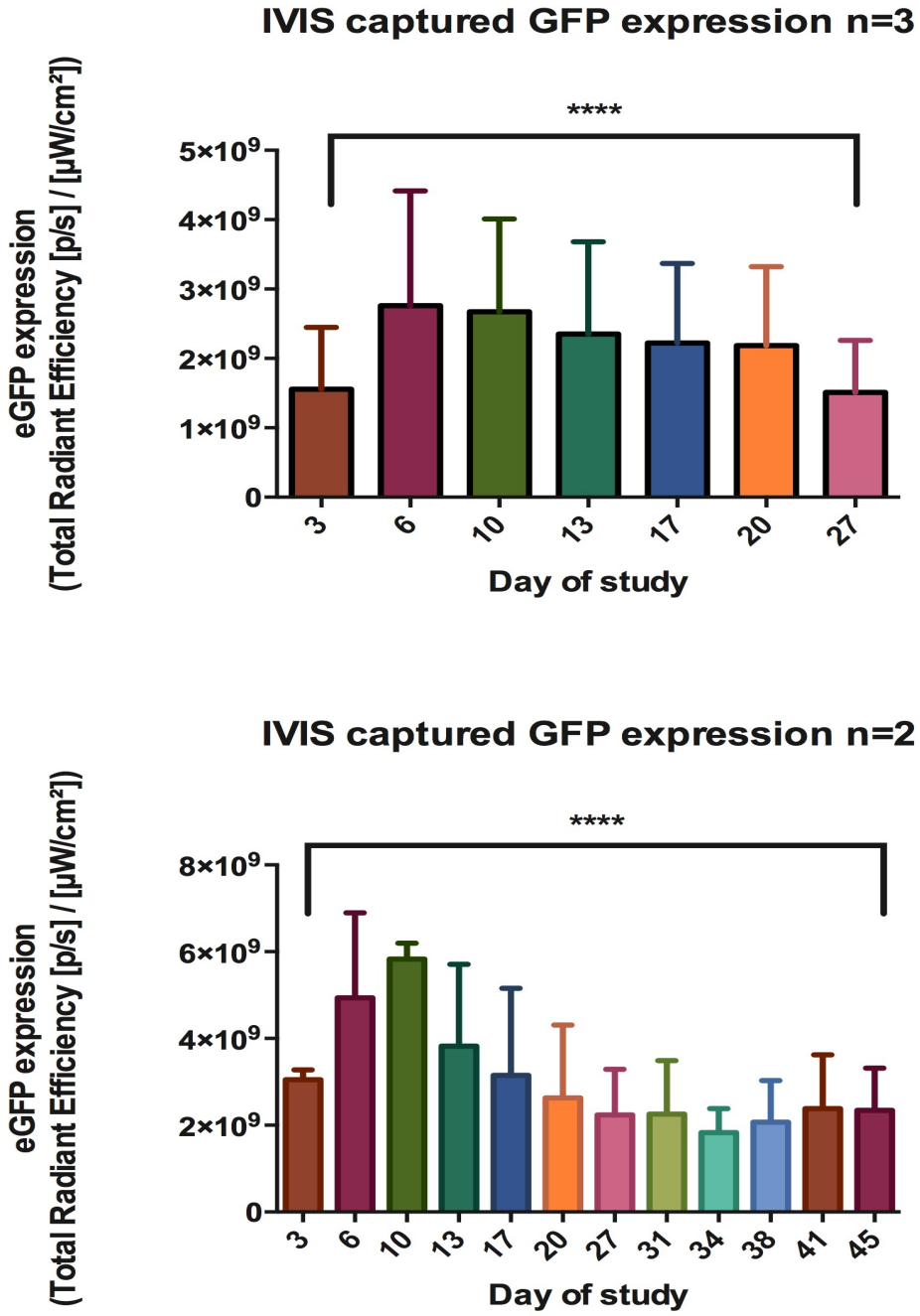


Figure 5-21– Longitudinal eGFP expression in mice transfected using pVR1012-eGFP, was analysed using IVIS Spectrum instrumentation and software (Perkin Elmer). *Only 2 of the mice survived the full course of the study. Graph A shows the 3 mice who survived up to day 27 scanning whilst Graph B shows the 2 mice which survived up to the 45 day scanning. The mean eGFP intensity peaks at an early time point (between days 6-10) for both data sets. The longer time course indicates a slight second may be occurring post day 40, however this cannot be sufficiently substantiated by the current data.*

5.6 In Vivo assessment of secretory properties and pharmacodynamics of Exendin 4 and GLP1 in CD1 mice via viral (AAV) administration, with an accompanying eGFP control group

Much of the literature available suggests AAV vectors have an initial lag in expression time, although AAV is not as slow as other viral based vectors (for example retrovirus), the ssDNA still needs to be converted to dsDNA, meaning any expression obtained from the transgene will be slower than that obtained with plasmid DNA. Due to this, the AAV studies were deliberately scheduled over a longer period of time than the plasmid studies.

5.6.1 Weight Evaluation

All mice were monitored as per above. All mice showed normal weight gain and expected activity levels during the course of the study.
No significant differences were observed between the groups over the course of the study. All mice gained weight.

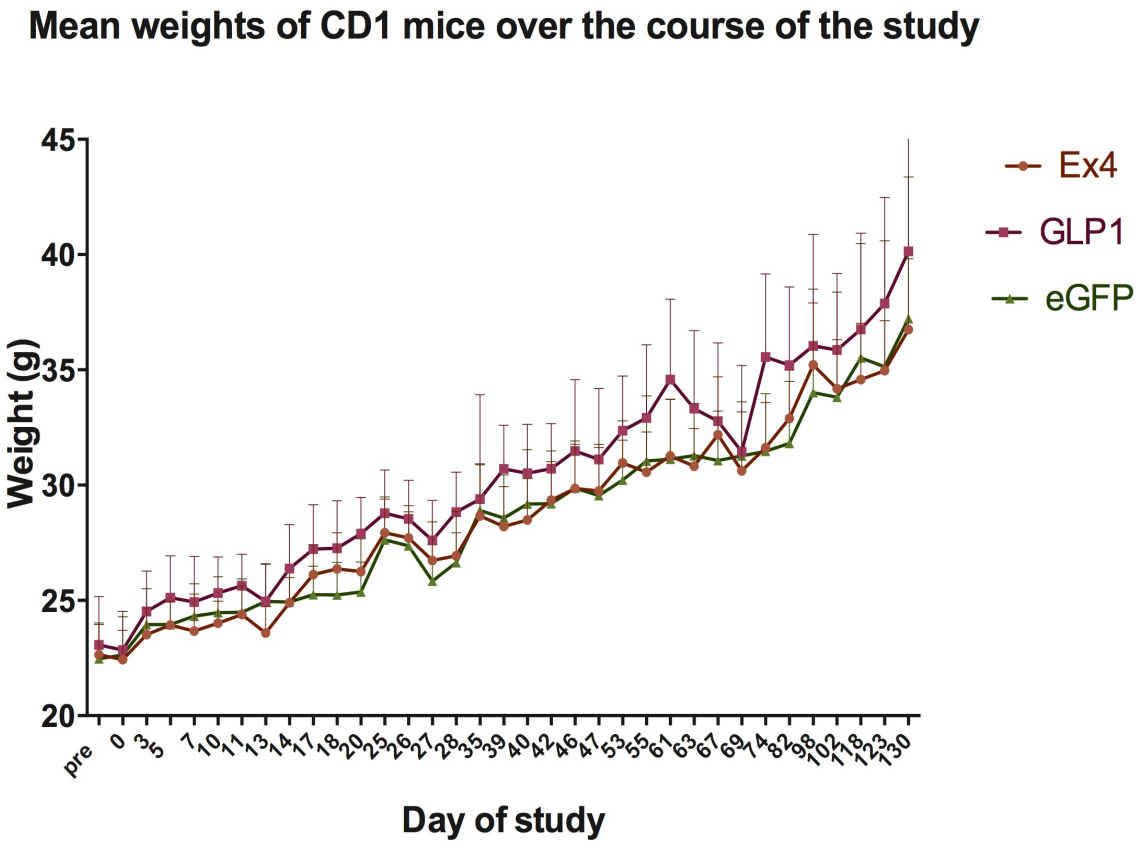


Figure 5-22 – Mean weights of mice throughout the study. *Mice were monitored daily and the weights obtained showed no significant difference between the 3 groups (n=6; p>0.05).*

5.6.2 IPGTT and mean fasted glucose

Measurements were performed as per previous studies.

No significant difference was observed between any of the three groups with regards to mean fasted glucose (figure 1-23)(n=6; p>0.05).

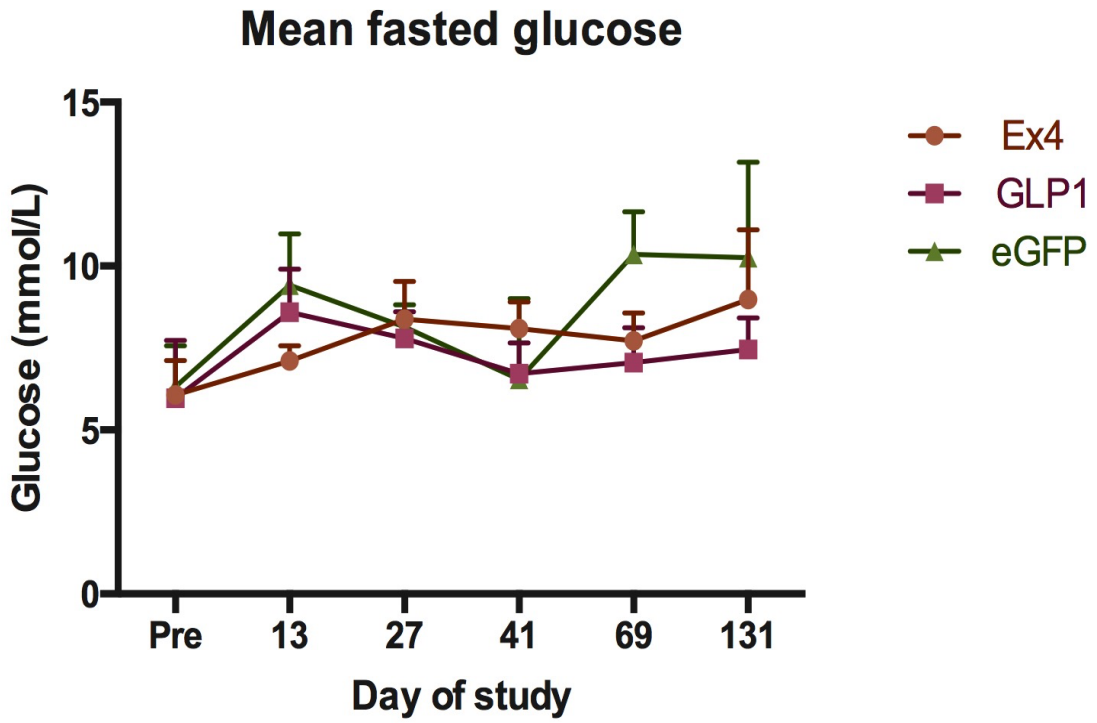


Figure 5-23 – Mean fasted glucose*- Fasted glucose levels were recorded as part of the IPGTT process. No significant difference was observed between any of the three groups (n=6; p>0.05)*

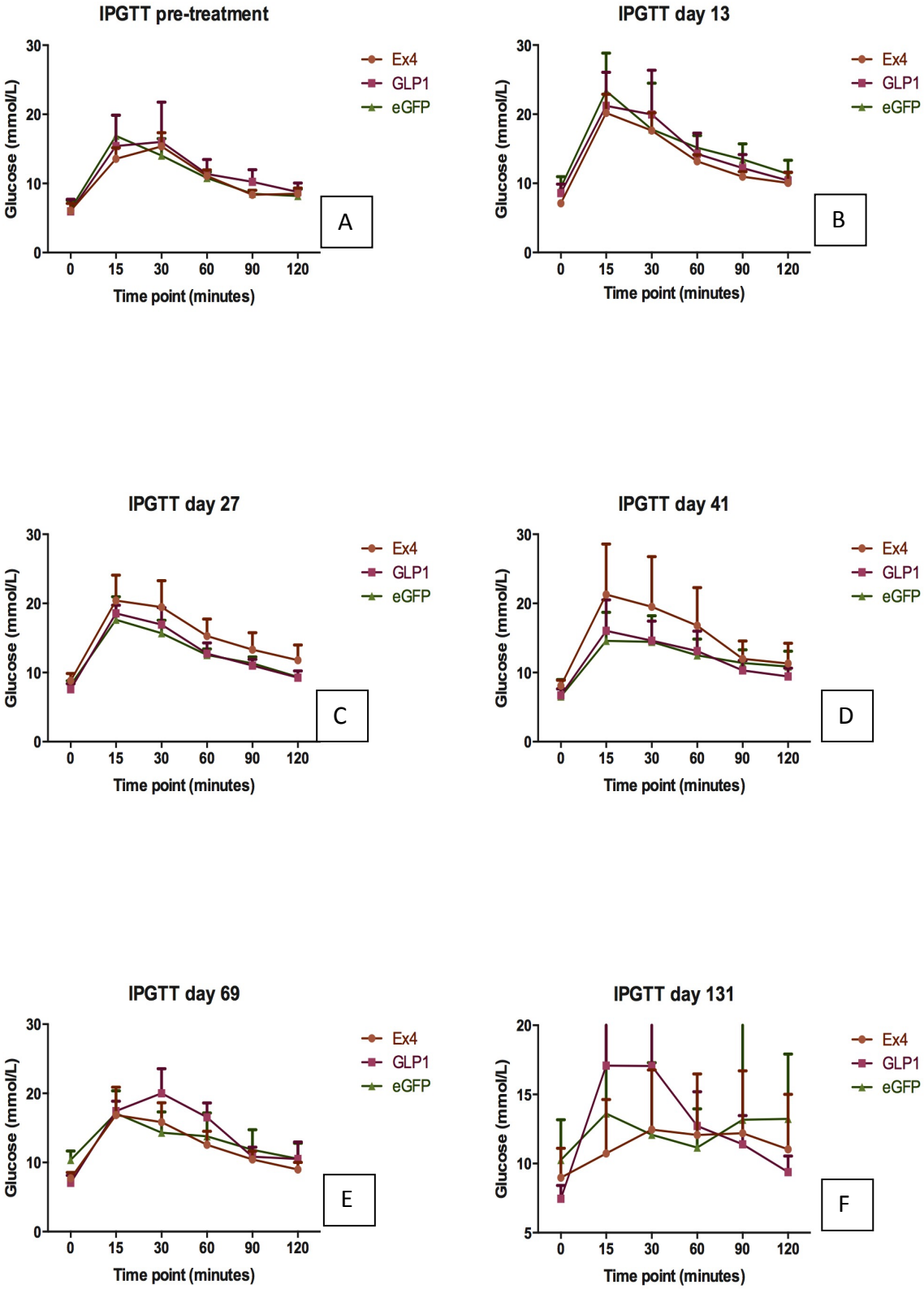


Figure 5-24 – IPGTT data. *(A) day 0 (pre-treatment), (B) day 13, (C) day 27, (D) day 41, (E) day 69 and (F) day 131. Statistical analyses (see table 1-3) shows there is no significant difference within the exendin4 group (n=5;p>0.05)*

DATE	GROUPING	REPEATED MEASURES - BONFERRONI
IPGTT DATA OVER ALL 19 WEEKS	Ex4 vs. GLP1	1.000
	Ex4 vs. eGFP	1.000
	GLP1 vs. eGFP	1.000

***Table 5-3 – statistical analyses of IPGTT data**. No significant difference when analysed using rANOVA with Bonferroni correction (n=6; p>0.05).*

5.6.3 Evaluation of peptide concentration in blood plasma samples

In order to further confirm transgene biosynthesis, analyses were performed on both blood plasma and muscle homogenate.
Bloods samples were taken via venepuncture and regular intervals during the study, in line with PPL statements and NC3R guidelines. Blood plasma was separated and/or muscle samples were homogenised and ELISA/EIA analysis was performed.

An increase in Exendin 4 levels were observed in peripheral blood plasma samples were observed up to day 74, albeit a variable trend. A seemingly steady decrease was observed post day 74. Whilst the final sample (day 102) was still higher than that observed at day 11, The statistical significance was great, despite the large standard deviations, due to the large increase in circulating concentration at day 74 (almost 1500 fold increase compared with pre-treatment) (Figure 5-25) (n=6; $p<0.0001$).
The increase in circulating GLP-1 concentration was also found to be statistically significant (figure 5-26, n=6; $p<0.05$) and followed the same wave like pattern as observed with the Exendin-4 levels, although the GLP-1 trend is more pronounced. The statistical significance is less due to the large standard deviations, the higher GLP-1 levels pre-treatment and the smaller increase from pre-treatment to peak concentration at day 74.

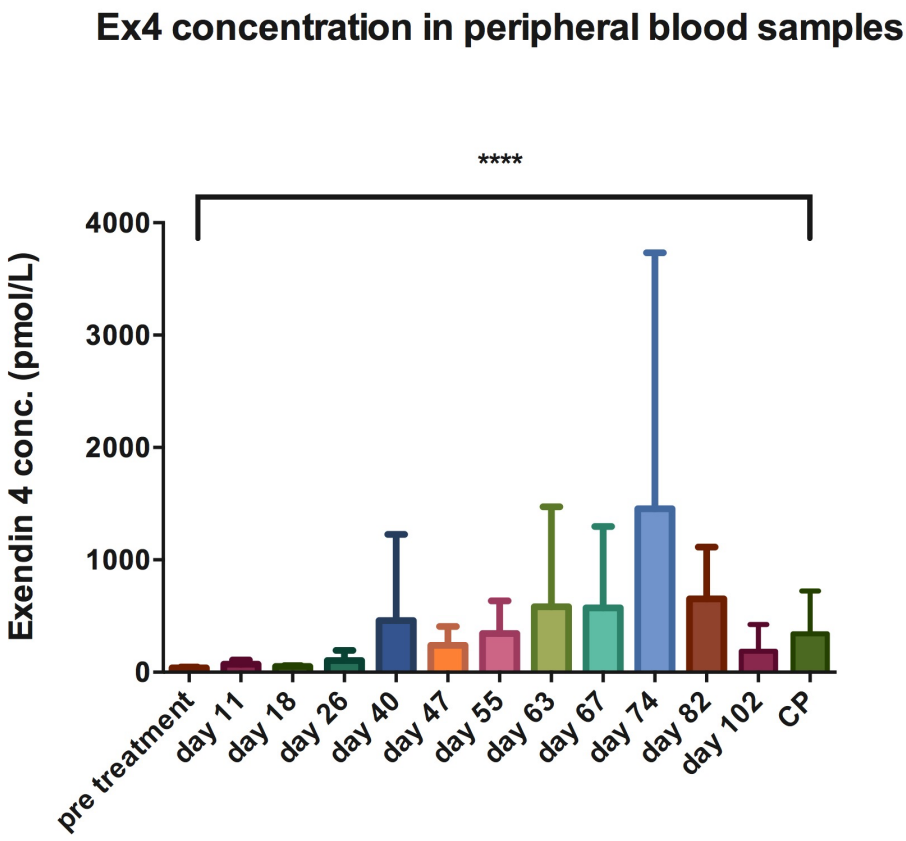


Figure 5-25 – Exendin 4 concentration in peripheral blood samples as determined by EIA. *An increase in Exendin 4 levels were observed in peripheral blood plasma samples were observed up to day 74, albeit a variable trend. A seemingly steady decrease was observed post day 74. Whilst the final sample (day 102) was still higher than that observed at day 11, The statistical significance was great, despite the large standard deviations, due to the large increase in circulating concentration at day 74 (almost 1500 fold increase compared with pre-treatment) (Figure 1-25) (n=6; p<0.0001).*

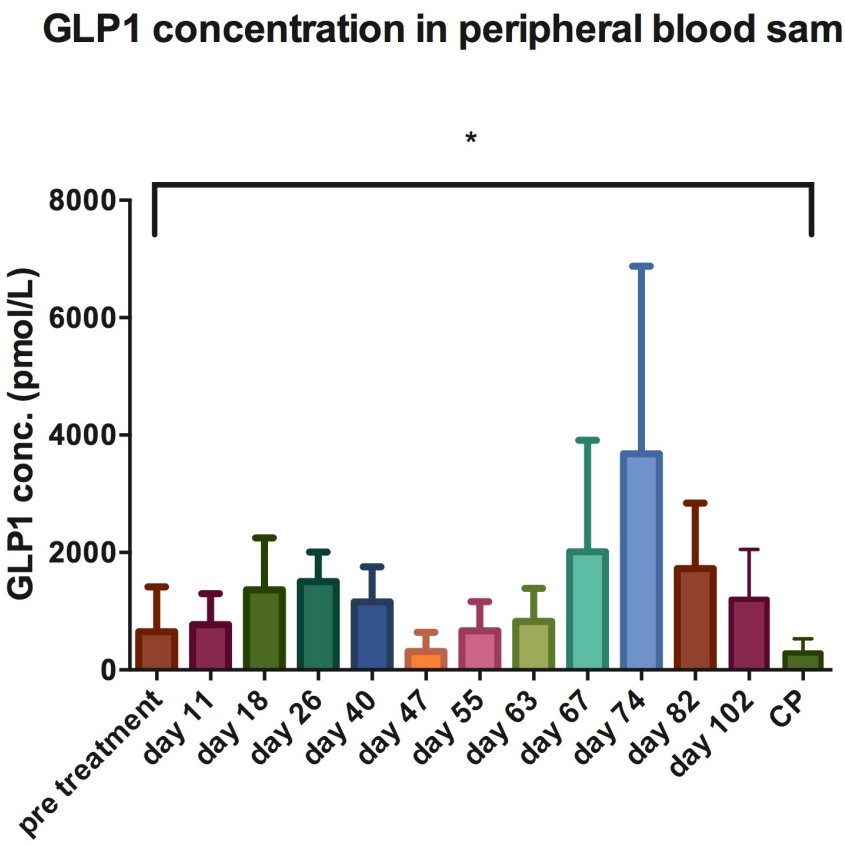


***Figure 5-26 – GLP1 concentration in peripheral blood samples as determined by ELISA**. The increase in circulating GLP-1 concentration was also found to be statistically significant (figure 1-26, n=6; p<0.05) and followed the same wave like pattern as observed with the Exendin-4 levels, although the GLP-1 trend is more pronounced. The statistical significance is less due to the large standard deviations, the higher GLP-1 levels pre-treatment and the smaller increase from pre-treatment to peak concentration at day 74.*

5.6.4 *Immunohistochemical analysis of pancreas*

As with the plasmid studies, the entire pancreas was harvested from each animal at the time of death. This was immediately placed in a 10% formalin solution prior to FFPE processing.

Sections were obtained from each tissue block and analyses were performed to establish the general health of the pancreas and an attempt to establish both insulin and glucagon content.

No obvious differences were observed between each of the 3 groups under standard bright field H&E microscopy.

Sections were then stained for insulin and glucagon and software utilised to calculate the area, which stained positive for both, and thus establishing a percentage content per sample.

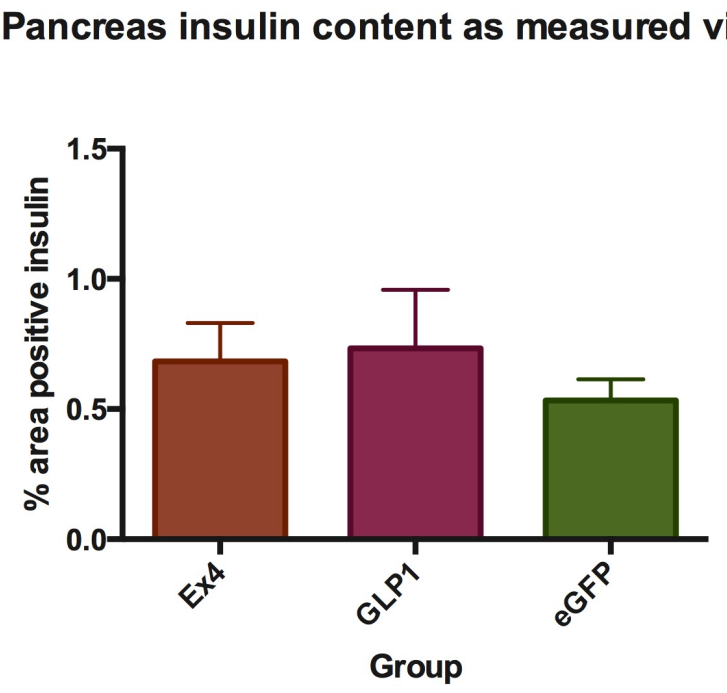


Figure 5-27 pancreas section staining Insulin content in pancreas samples as measured via IHC. *Staining and image analysis was used to calculate the % area of positive insulin stain using colour deconvolution processing. No significant difference was observed between any of the 3 groups (n=6; p>0.05).*

5.6.4.1 Assessment of eGFP expression as measured via IVIS-spectrum

IVIS scans were performed, as described on 5.4.3.

Unfortunately, the IVIS scans returned no evidence of transgene expression in any of the mice at any time point over the duration of the study, as shown in figure 5-28.

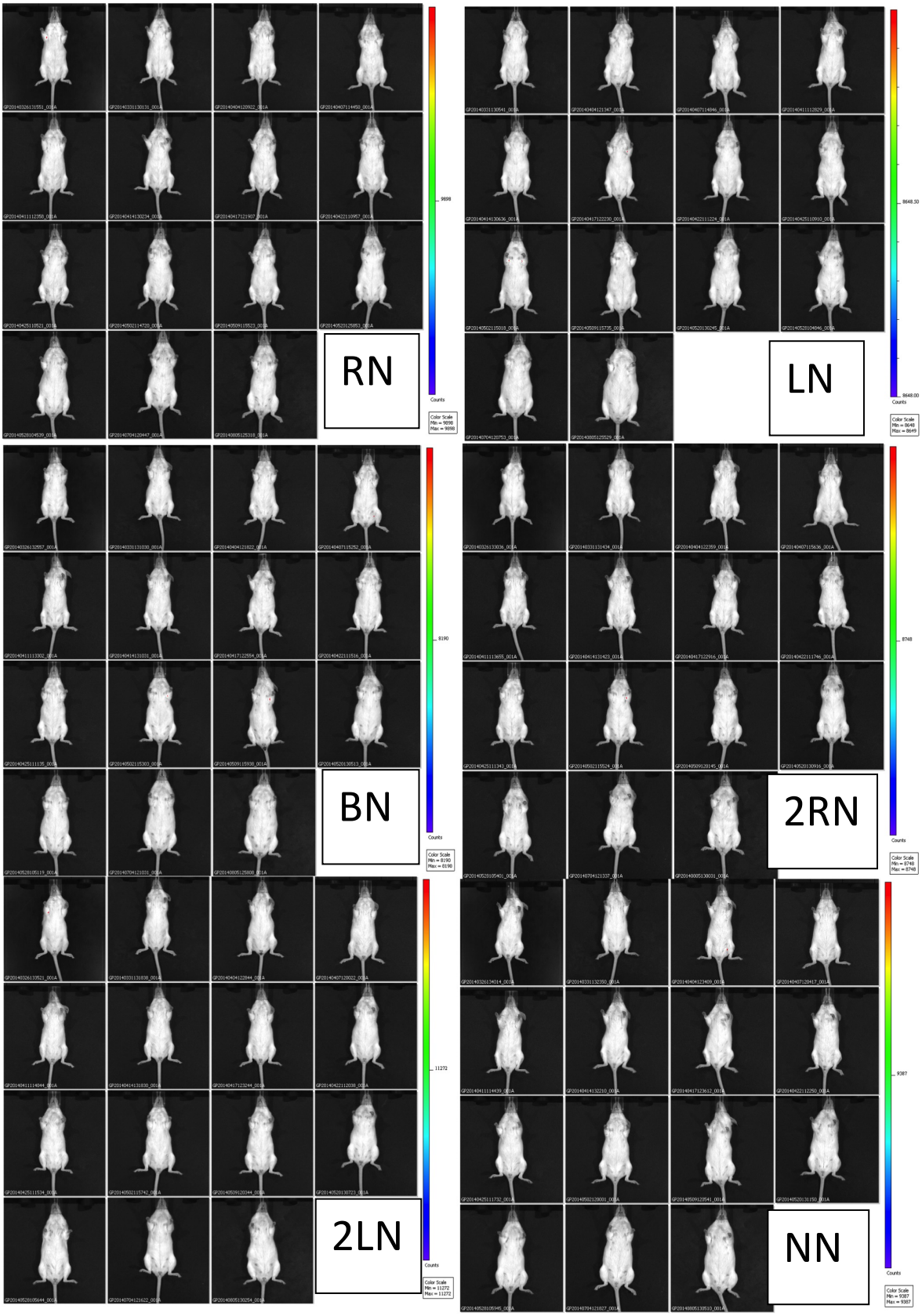


***Figure 5-28- IVIS image sequences**. No expression was observed in any animal over the entire duration of the study.*

5.7 Discussion

5.7.1 In Vivo assessment of secretory properties and pharmacodynamics of Exendin 4 in CD1 mice via plasmid administration, with an accompanying eGFP control group

Males continuous fighting resulted in bite wounds to the tail on several of the mice. None appeared infected but on recommendations from the NVS no procedures involving venepuncture were allowed until the fighters had been separated and the wounds healed. This is the reasoning behind the large gaps in data acquisition. As a result, the following studies were all conducted in female mice.

One of the mice from the GFP group was involved in a co-registration trial between the IVIS spectrum and CT scan. The mouse was anaesthetised using 0.3 ml ketamine/medatomidate (50 mg/kg and 0.5 mg/kg respectively) via IP injection with 0.5 ml antisedan reversal post procedure. Unfortunately this particular mouse died, and after minor post mortem investigation, it appeared to be caused by an internal infection (enlarged bladder containing both blood and urine). Again, after consultation with the NVS, this could have been caused by a bite wound when the mice were fighting.

The PPL/PIL was altered slightly after this study to allow for further blood samples to be taken. All was in accordance with the recommended volume in the NCR 3R guidelines. This allowed slightly larger volumes to be taken via venepuncture for ELISA/EIA and for more IPGTT tests to take place.
We also altered the number of times animals were allowed to be anaesthetised to allow more IVIS scans to take place. This was deemed acceptable an appropriate as the anaesthesia required for IVIS is quick and light. The animals do not undergo any painful treatments (IVIS is non-invasive, anaesthesia is only required to keep the animals still whilst they are being scanned) and recovery time is less than 1 minute once removed from the chamber.

This protocol was successful in establishing a sustained delivery method resulting in eGFP expression for up to 30days, as observed throughout via IVIS scan. Unfortunately the Exendin-4 expression did not reflect the sustained increase in eGFP expression. Exendin-4 concentrations peaked at day 15, and whilst there was no statistical significance due to the large standard deviations, the drop at day 30 was large. Further investigation into the reasoning behind this would be of interest.

5.7.2 In Vivo assessment of secretory properties and pharmacodynamics of Exendin 4 and GLP1 in db/db mice via plasmid administration, with an accompanying eGFP control group

The db/db females (changed from males to females due to the fighting observed previously and the potential of not being able to obtain data due to animal welfare). Animals receiving the control plasmid (eGFP) were separated into 2 groups of 3 rather than the standard group of 6 due to the expected large weight gain and polydipsia (and therefore polyuria), and was determined the mice would be more comfortable in smaller groups.

The rapid weight gain seen in these mice resulted in small tears appearing in the skin, close to the shoulder area. This healed quickly with no incidences of infection. The animals did not appear to be in distress and green clay was used as a non-medicinal soothing agent. Using the scorecard (appendix 8.1) there was no call for any procedure modifications.

The db/db phenotype is typically known to be a severe model for type 2 diabetes. Information obtained from The Jackson Laboratory database (jaxmice.jax.org) indicates the following; Death will typically occur within 10months of age, during which time, the phenotypic indicators linked with diabetes start at a very early age. Plasma insulin concentrations begin to rise at 10-14 days, they are classed as obese at 3-4 weeks and blood sugar levels start to rise uncontrollably at 4-8 weeks (exogenous insulin will not control this rise). Classic diabetic signs are seen (polyphagia, polydipsia and polyuria) and there is a depletion of active beta-cells. Wound healing is delayed and some forms of myocardial disease are detectable.

As the onset of symptoms is early and dramatic, it was important to start the protocols at an early age. The mice were brought in to the research facility at 3 weeks and after the 1 week acclimatisation, the protocol began.

Circulating GLP-1 levels were not collected as consistently as those for Exendin-4 due to the volume of plasma required for GLP-1 ELISA. The Millipore ELISA used required 100µl serum for analysis. Dilution was permitted, as indicated in the protocol provided by the manufacturer, however it recommended no more than 50%, which meant that 50µl was still required. Due to the restrictions in place for blood volume collection in the PPL/PIL, this was not able to be gained on a weekly basis.

The IVIS Spectrum uses, in this case, epi-illumination to visualise any fluorescent source in vivo. The accompanying software allows 'spectral unmixing' of the resulting image which differentiates any auto-fluorescence from that of any other fluorescent source. It can be clearly seen that some animals show greater expression than others. This suggests the uptake and subsequent over expression of the plasmid and gene relies quite heavily on a good delivery technique. It has been speculated that the age, gender and species of an animal can alter the DNA uptake and therefore transgene expression. However, as these animals were all of similar age, same gender and species, it may also be a function of the diabetes that these animals are more lethargic than others and the muscle mass not increasing may have implications on the transgene expression also. This assumption can be somewhat validated by the size of the muscles observed during harvesting, which were considerably smaller than those in previous studies. The muscles were unfortunately not weighed to more objectively establish this.
Clinical scoring to assess overall health was difficult due to the size of the dbdb mice. The rapid weight gain forced skin to stretch, and as a result the coat was more pronounced than a 'normal sized' mouse (see figure 1-1). As one of the measures of clinical health is observation of coat (known as 'starey'), the dbdb mice seemed to have this, but this was due to the afore mentioned skin stretching and the coat not lying correctly against the skin. This was therefore not included in the clinical scores for these mice.

Again, this protocol was successful in establishing a well tolerated delivery method in a diseased model. The results from the db/db study (figure 5-19) show significantly higher levels of insulin in the Exendin-4 and GLP-1 groups, suggesting the hormones are having some effect on the β-cells by increasing the volume of insulin secreted in an attempt to lower blood glucose levels. This pattern is also demonstrated in the IPGTT data, where the Exendin-4 and GLP-1 groups are both showing a greater capacity for glucose reduction over the eGFP group.

Attempts were made to analyse the glucagon content alongside the insulin content in the pancreas samples via β-cell mass determination. However, the colour deconvolution function could not distinguish between the glucagon positive areas and some areas of background, rendering the analysis inconclusive, and was therefore not included in the final analysis.

When comparing the results of the insulin content in the dbdb mice to the CD1 mice, the differing results suggest a good correlation between the expected levels of insulin in a diseased and non-diseased model. The dbdb mice are clearly showing increased levels of insulin (approx. 15%), obviously in attempt to achieve glucose homeostasis, whereas the non-diseased model is showing much lower levels in comparison (approx. 0.6%)

5.7.3 In Vivo assessment of secretory properties and pharmacodynamics of Exendin 4 and GLP1 in CD1 mice via viral (AAV) administration, with an accompanying eGFP control group

CD1 females were used to assess the efficacy of the AAV vectors using Exendin-4 and GLP-1 as genes of interest.

The circulating peptide levels for both GLP-1 and Exendin-4 peaked at day 72 (Figures 5-25 and 5-26), providing further confirmation that AAV based viral vectors require a longer time to synthesise and subsequently secrete the optimal concentrations of transgenes, as evidence by the peak at anywhere from day 10 to 30 when using plasmid vectors (Figures 5-8 and 5-12). However, the circulating levels of GLP-1 obtained from

viral administration was far greater than that obtained via plasmid at almost 1000 fold increase (Figures 5-14 and 5-26).

Peptide concentration in muscle homogenate was un-retrievable due to an error in the storage of the harvested tissue which resulted in the degradation of the protein. This would have been interesting data considering the observations made in the circulating concentrations of peptide.

5.7.4 General Conclusion

These studies suffered from biological variation which led to there not being statistical differences where there potentially could have been. This is commonly encountered in muscle gene transfer studies (Davis *et al.*, 1993).

The concentration determination of GLP-1 was not consistent. This could be due to the method of collection, sample preparation and analysis or this could have generated higher levels of endogenous DPP4. One aspect that was overlooked during this study was any determination of DPP4 concentration in the GLP-1 group. Constitutive overexpression of this peptide may not increase the circulating levels of GLP-1 and may only trigger the release of increased DPP4, therefore still rendering the half-life of GLP-1 extremely short at 2-3 minutes and not providing any clinical benefit.

The data obtained from the plasmid studies in the diseased (db/db) model suggested promising results with regards to glucose clearance with Exendin-4, as shown in the IPGTT data and statistical analysis. Dose response studies and further expanded longitudinal studies may increase the effect seen in this study. One major aspect that I believe needs attention is the determination of blood glucose levels during the IPGTT test. The AlphaTRAK 2 system used has a ceiling of 40 mmol/L, which was reached several times during IPGTT data acquisition. Had the true concentration of blood glucose been determined, the function of the Exendin-4 may have been greater.

Additionally, due to the loss of the muscle samples and the lack of eGFP expression observed in the AAV studies, there is only the circulating peptide data to reflect on. However, the data from both Exendin-4 and GLP-1 indicates an increase in circulating levels for both peptides was obtained. This in itself is worth repeating the AAV studies in a normal model.

In short, both formats of vector have provided evidence of transgene biosynthesis and subsequent expression, with plasmid based Exendin-4 administration providing some evidence of increased glucose clearance in a diabetic model and a viral based GLP-1 administration providing far superior circulating concentrations in a healthy model.
A long term study in a milder diseased model would be a very interesting next step.

6 Chapter 6 – General Discussion

The impaired/reduced GLP-1 secretion seen in T2DM patients makes GLP1 replacement an extremely attractive treatment methodology. The ability to replace endogenous compounds immediately has the benefit of reducing the possibility of initiating any sort of immune response and generating unwanted and unnecessary side effects. However, the short half-life of GLP-1 still remains the most restricting factor.

There have been many methods investigated to try and overcome the short half-life of GLP-1, including amino acid substitution (as the DPP4 targets only a specific loci in the endogenous GLP-1 sequence – see figure 1-4), DPP4 inhibitors to prevent the degradation altogether or the use of GLP-1 mimetics, such as Exendin-4. However, all current incretin therapies or incretin related substitutions have still only managed to prolong the need for injectable material to once per week, with 3 GLP-1 RA's currently available (exenatide extended release, albiglutide and dulaglutide) (Karagiannis *et al.*, 2015) .

The need for such regular injections may lead to issues such as hypertrophy/lipoatrophy, which not only can affect the patient by hindering the application of the therapy, but may also affect them psychologically. The potential for extended therapeutic action which may be achievable through gene therapy is still an extremely attractive target for many researchers, despite the advances seen in current injectable treatments and the potential issues associated with gene therapy delivery routes.

In order to provide successful and long term gene expression, a suitable delivery system is key. The use of skeletal muscle to uptake the gene vectors is a logical choice, due to the ease of access and the muscle fibres themselves having many attractive qualities, such as a good vascular system. Studies have been conducted on evaluating the benefits of skeletal muscle against other quiescent based tissue (such as liver), on the whole, the studies yielded extremely similar results, and certainly not enough benefit to warrant the much more invasive procedure of using liver as the target tissue (Léger *et al.*, 2011).

The vectors themselves must be well tolerated and not have any adverse side effects which would outweigh the benefits to whatever amelioration the treatment provides. The most popular vector types in current gene therapy trials are viral based, with lentiviral and AAV based (several different serotypes) topping the list, for a wide range of disease, including Haemophilia B, type 2 Leber congenital amaurosis and X-linked

SCID (Naldini, 2015). However, non-viral vectors are still very much in use, with a recent breakthrough in cycstic fibrosis treatment involving an inhalant mixture containing plasmid DNA and a lipid complex showing increased lung function against the placebo group (Alton *et al.*, 2015)

Previous chapters have described the process of evaluating the two versions of vector, plasmid and AAV, from analysis of reporter gene and promoter, through to *in vitro* proof of concept, and onto both diseased and non-diseased *in vivo* models. In each case, successful gene transfer and expression was obtained, albeit of variable quality.

6.1 *In Vitro* studies to establish both reporter (eGFP) and transgene (GLP-1 and Exendin-4) expression in a mouse muscle cell line (C2C12)

Proof of concept studies in the C2C12 cell line have shown transfection of the plasmid vector pVR1012 containing a CMV promoter region and the transgenes in question (eGFP, Exendin-4 and GLP-1) into myoblast-induced myotubes yielded successful results. All three genes were found to express over a period of at least 48 hours, and eventually up to 7 days (after moving to fibronectin coated coverslips), establishing that the length of expression in the cell line model was somewhat dependant on the stability of the fibres rather than the vectors themselves.

The C2C12 line was determined the most appropriate model. The myoblasts are derived from satellite cells, which exist in the muscle fibre matrix in between the sarcolemma and basal membrane structures. The myoblasts spontaneously differentiate into myotubes in reduced serum media (Burattini *et al.*, 2004). A mouse muscle cell line was the logical choice for *in vitro* assessment, taking into consideration the future plans for *in vivo* muscle targeted delivery. HEK293 cells were transfected alongside the C2C12 for comparative purposes for evaluation of AAV *in vitro* due to the reduced volume of AAV available. This cell line is known as being an 'easy to transfect' line and this reduced the risk of losing any AAV titre to loss of cells. HEK293 are derived from human embryonic kidney cells and originally cultured in late 1970's they have been extensively used for research purposes due to this very reason (Graham *et al.*, 1977). Although the HEK293

line did show a greater percentage of transfection area under the same conditions, the muscle model of the C2C12 line was used in subsequent studies as skeletal muscle is the end target tissue. A variety of transfection conditions were trialled, however the standard recommendations of Lipofectamine2000 yielded results of no statistical advantage or disadvantage, so these conditions were utilised. Transfection, and therefore subsequent expression of each of the transgenes was confirmed immunohistochemically via fluorescence staining of both myotubes and myoblasts, and the analysis of secreted expression via ELISA/EIA content in the cell media.

The percentage of myoblasts transfected was calculated via traditional means of counting DAPI stained nuclei and then counting the number of cells transfected (to give a positive 'green' eGFP), and gaining percentage transfected. However, when this method was used for calculating the percentage of myotubes transfected, it became clear that a different method was needed due to the tubes becoming multi-nucleated fibres and therefore the lack of clarity between what is classed as positive transfection. Therefore the percentage area of transfected fibres was calculated rather than a count of fibre. This was performed using the ImageJ image analysis software program (which is freely available for download) and the colour deconvolution function. This can be further manipulated to a binary image and the percentage of black versus white (white being non-transfected area) calculated. This was deemed the most appropriate method to take into account the variation of fibres per frame and the subjective nature of determining expression. This method was used for all other expression calculations for in vitro experiments.

Extracellular transgene protein was additionally detected in the cell media for both Exendin-4 and GLP-1, as determined via ELISA. ELISA detection for eGFP was also attempted, however this remained unsuccessful. The levels of GLP-1 were greatly reduced when compared to those of Exendin-4. This could be attributed to enzyme degradation, despite the addition of DPP4 inhibitor.

After transgene expression, and more importantly, extra cellular expression was confirmed, studies moved from *in vitro* to *in vivo*.

6.2 *In Vivo* studies to establish both reporter (eGFP) and transgene (GLP-1 and Exendin-4) expression in a mouse muscle cell line (C2C12)

In vivo studies were carried out in both CD1 (normal) and db/db (diseased) mice. These studies were originally conducted using males, however, due to un-controllable fighting, resulting in wounds which prevented protocols from taking place, we switched to females. This removed the issue of fighting and resulted in less stressed animals.
In all *in vivo* protocols, mice were housed randomly (due to the very young age of the mice, no variation in weight was seen so weight matching was not viable) and allowed 1 week to acclimatise in the CBC facility prior to any protocols commencing. Mice were monitored closely throughout each protocol and showed no signs of clinical issues, medical or behavioural.
All plasmid studies were performed using the hyaluronidase/electroporation method, in which 50 µl hyaluronidase was administered prior to plasmid injection (1 µg/µl), and the electroporation technique was performed post plasmid injection.
All AAV studies were standard injection volume of 50 µl, and slightly varying viral titre. No adjuvant delivery techniques were utilised for AAV.

The use of the CMV promoter was confirmed in an *in vivo* experiment as a more than acceptable choice, as determined by comparison of a muscle specific promoter DES (donated by Prof. H. Lochmuller). The CMV promoter, surprisingly, provided statistically significantly more positive transfection area than the vector containing the muscle specific promoter, MCK, over a 30 day period. Other muscle specific promoters are available and some studies have shown these do confer higher transfection efficiencies (Jonuschies *et al.*, 2014), however, due to the work already carried out within the group, and publications continuing to favour CMV promoters for targeting skeletal muscle over tissue specific promoters (Buchlis *et al.*, 2012), along with the preferable results yielded from the comparative experiments conducted within this study (3.4.2), the CMV promoter was used.

Further studies using the GLP-1 and Exendin-4 vectors were also performed in conjunction with eGFP reporter vectors, which not only acted as the control group but also the monitoring group for use with the IVIS system. The IVIS system determined the levels of eGFP expression in anaesthetised rodents. As the backbone of the vectors were identical, with the only variation being the transgene insert, it was suggested the level of eGFP expression could be extrapolated to determine the level of expression for both of the other transgenes. Attempts were made to utilise several eGFP ELISA kits to confirm this theory, by assessing the level of expression at time of death, as determined via IVIS, against the amount of eGFP present in muscle homogenate post mortem.
Unfortunately, the robustness of these eGFP ELISA kits were not sufficient to provide enough confidence in the precision of the kits, and therefore the data was not provided.
The software attached to the equipment, Living Image, has the capabilities to identify each individual region of interest and calculate the amount of eGFP expression without the interference of any auto fluorescence. This allowed the real time monitoring of expression without the need to sacrifice any animal's part way through protocols. The benefits of this were particularly noticed during the time of fighting between the CD1 males, during which time no blood samples were permitted due to the bite wounds on the tails of the mice.
High levels of eGFP expression were seen in the CD1 group, with some instances of expression in the gastrocnemius region being observable by the naked eye (figure 3-15).
Extracellular GLP-1 and Exendin-4 were detected in the blood stream when serum was analysed via ELISA (GLP-1) or EIA (Exendin-4), obtained from venepuncture blood samples. Final samples obtained via cardiac puncture tended not to provide directly comparable results to those obtained via venepuncture, even when the samples were obtained less than 24hours apart. This is an interesting note and one which, at time of writing, there was no published data which could explain this phenomenon.
As expected in normal mice, the IPGTT tests were also normal. However, the process of conducting the study in a normal model, and obtaining normal IPGTT results, determined that using both plasmid and viral vectors to administer pharmacologically active GLP-1 and/or Exendin-4, and the reporter gene eGFP did not have any adverse effect on normal, CD1 mice, up to 3months.

In the db/db (diabetic) mice, circulating levels of GLP-1 and Exendin-4 were also found. The initial, pre transgene delivery IPGTT confirmed abnormal glucose control. The circulating levels of transgene peptide were comparable to those found normal mice, however, the IPGTT results indicated the Exendin-4 administration via plasmid vector was exerting some activity over glucose control. Statistical analysis (repeated measures ANOVA) suggested a significant difference in the results obtained for Exendin-4 plasmid based delivery.

A drawback which should be noted is the limitation of the glucose meter used to measure blood glucose levels in mice. The AlphaTrak2 system is only capable of measuring blood glucose concentrations up to 40 mmol/L, the glucose levels often hit this limit meaning the actual difference in peak glucose concentration may have been even higher than recorded and furthermore, restricting the statistical significance the results yielded. This was deemed a major drawback of the protocol. Attempts to find alternative equipment were made, however none were found.

Even though variability in the AAV results in the CD1 mice was observed, the results indicated that successful biosynthesis and expression was gained. However, the severity of the db/db model coupled with the extra time needed for AAV to produce and release transgene peptide meant the study was not progressed into a diseased model study. This does not indicate we do not have confidence in the gene delivery system and resulting expression, rather the db/db model is not the appropriate disease model for this study. However, many successful studies have been reported. Buchlis *et al* (2012) has showed positive Factor IX expression was found in a deceased haemophilia B participant (un-related death) of a trial conducted 10 years previously with AAV mediated gene therapy (Buchlis *et al.*, 2012). The Fatima Bosch group (Barcelona) have seen somewhat more related success in treating diabetic beagle dogs with AAV1 gene therapy. A combination of insulin and glucokinase transgene expression has led to normal glucose homeostasis for over 4 years in the large animal model (Callejas *et al.*, 2013).

There is some concern that constitutive, over expression of GLP-1 may lead to adverse effects such as pancreatitis or even pancreatic cancer (Elashoff *et al.*, 2011; Deborah,

2013). However, recent studies have shown that GLP-1 may also have cardio-protective properties. Kinalska et al and Davidson have all shown that either GLP1 or Exendin-4 administration exerts some positive effect on various cardiac functions or conditions that may lead to adverse cardiac events (Davidson, 2011; Kinalska *et al.*, 2012). Unfortunately, there are as yet no definitive conclusions over the safety concerns or protective actions, and the use of GLP-1 and/or synthetic homologues, delivered by whatever means is still the most attractive and useful method for glucose homeostasis in individuals suffering from type 2 diabetes. No adverse effects were found in the pancreas during any of these studies.

6.3 Future work

The most immediate requirement would be to repeat the AAV study. Although most of the data was obtained, the error in sample storage meant the data surrounding the hormone levels in muscle sample homogenate was not obtained. This is an important step in determining the efficacy and longevity of hormone secretion in the targeted site of injection.

This study did show the variability of the expression with this system, and the early peak in activity seen in the plasmid system was not evident here. This is a known feature of AAV systems, extending the duration of the AAV study may show a clearer trend of increase/decrease in activity.

Analysis of other organs may help in identifying any possible adverse effects of prolonged, over expression of endogenous and synthetic compounds. For example, some reports of thyroid c cell carcinoma in certain studies suggest that prolonged exposure to high levels of GLP1/Exendin-4 may be harmful, however this has not been proven in human subjects (Aroda and Ratner, 2011; Shih *et al.*, 2012; Butler *et al.*, 2013). Whilst no signs of pancreatitis were identified in histological examination of the pancreas samples from any of the groups in this particular study, reports have been published that this may also be a side effect (Elashoff *et al.*, 2011; Deborah, 2013) .

A conversation held with Prof. Peter Flatt (University of Ulster) suggested that the db/db model used for the diseased animal model studies may have been an overly harsh form of the disease for a study of this type. The db/db model is clinically overt at an early age (4-8 weeks) resulting in profound hyperglycaemia and β-cell failure. This incredibly early onset of the phenotype and the practical time scale restrictions with working with this model in the UK (db/db mice are only available from an Italian supplier, after genotyping at 2-3 weeks, this, coupled with delivery time and 1 weeks acclimatisation meant the animals for this study were ordered with the specific requirements of early genotyping and expedited delivery to allow the 1 weeks acclimatisation and still meet the date requirements for initiating the study before the phenotype became too harsh and no treatment methodologies would have been evident) may have meant the time required for a gene therapy protocol to provide circulating peptide levels, and therefore exert any effect, may have been too long.

There have however been studies that show both GLP-1 and Exendin-4 can exert a blood glucose lowering effect in db/db mice, however the administration was via direct intraperitoneal injection and based on dose response experiments. Meaning the blood samples were collected at a maximum of 4 hours post injection (Young *et al.*, 1999). This is very different from assessing the potential of single dose gene therapy.
Other rodent models of diabetes which could be considered for future use include the ob/ob mouse or the Kuo Kondo mouse. The ob/ob mouse was the first rodent model of insulin resistance (1970's), as a result of a mutation on the leptin receptor gene. They gain weight rapidly and show hyperinsulinemia at approximately 3 to 4 weeks of age. However, the classic symptom of T2DM of declining plasma insulin levels does not really manifest until the mice are over a year old (Wang *et al.*, 2013). This is in stark contrast to the db/db mouse, whose life expectancy is only approximately 10 months. This would make this model much more suitable for a gene therapy based protocol. The Kuo Kondo mouse is also known to exhibit milder diabetic symptoms (which still include obesity, insulin resistance and hyperglycaemia), the milder symptoms may allow the determination of any effects the transgenes may exert, but, as with the ob/ob mouse, the longer life span would allow more in depth analysis (Wang *et al.*, 2013).

The use of CD1 mice as a control may not have given the most representative baseline control. Dbdb mice have a black 6 background, using this phenotype may have been beneficial especially with regards to IVIS analysis. It is widely accepted that skin colour does have some interference in the absorption of the light spectrum which is required for data acquisition. The white coat/pale skin of the CD1 mouse may yield different results to the dark coat/darker skin of the dbdb mouse. A small trial would be relatively easy to perform, however, this did not fall under the remit of our PPL and was therefore not conducted.

This study was also conducted with aid of RVC. The link with the RVC comes in the form of treatments for feline diabetes. The success of the trials in rodent model will lead to trials in a larger animal model, which in this case, would be cats.

Feline diabetes closely resembles the pathophysiology of the human form of T2DM in many ways and can be used as a good larger animal model for advanced therapy trials. Feline diabetes, as with the most common appearance in type 2 diabetes, occurs mostly in obese, middle age cats that have relatively low circulating concentrations of insulin at fast. As with humans, cats are also subject to various risk factors, which are largely the same as those described previously in section 1.2.1, mainly due to the fact that cats are companion animals and have been exposed to the same environment and lifestyle traits as their owners (O'Brien, 2002; Rand *et al.*, 2004; Henson and O'Brien, 2006).

One of the causes of decreased β-cell function is islet amyloidosis. This occurs in over 90% of FDM and T2DM patients and is one of the causes of loss of β-cell function in conjunction with approximately 50% loss in β-cell mass. Islet amyloidosis in only seen in a limited number of species. Rodents do not succumb to this form of β-cell destruction (O'Brien, 2002). These close links make cats an excellent model for studying and ultimately providing not only a treatment for human T2DM, but a treatment for feline diabetes also, which is an ever increasing problem for cats and their owners. Other large animal models have also been used. The Bosch group (Barcelona) have successfully used beagle dogs in a long term study which saw the dogs off insulin for up to 4 years (Callejas *et al.*, 2013).

There is also always the possibility of using newer and novel vectors, which are continuously being developed globally, not necessarily specifically for muscle targeted gene therapy of for diabetes, but any improvement in the safety and efficacy of transgene delivery is only going to improve the prospect for any disease modality with sub-optimal current treatments.

Treatments which target α-cells rather than β-cells (glucagon function rather than insulin) have been described. These could either target α-cells themselves (i.e. destroy the cells) or be based on the well-known incretin effect and target glucagon receptors on the α-cells (Gcgr). Antagonizing these receptors could be another route to reducing both postprandial and fasting hyperglycaemia in t2dm patients (Bagger *et al.*, 2011a; Christensen *et al.*, 2011).

Studies have shown that merging standard vectors together to produce new, novel structures have performed better in certain circumstances. The Samulski group has demonstrated that by fusing selected features of two or more different AAV serotypes, to form what they term 'hybrid serotypes', can generate higher efficiency vectors that can be tailored to the target end game (Choi *et al.*, 2005).

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8 Appendices

8.1 Appendix 1 – Clinical assessment score chart

Weight	Score	Eyes/Nose	Score	Skin tone	Score	Behaviour	Score	Coat	Score	Faeces	Score
Weight Gain	0	Normal	0	Normal	0	Normal	0	Normal	0	Normal	0
No change	0	sunken	1	Tents; 1-2 sec	2	Apathetic / inactive	2	Lack of grooming	1	Soft	1
<5% loss	2			Tents; persist	4	Immobile / non-responsive	4	Starey	2	Diarrhoea	2
<10% loss	4										
>15% loss	6										

Scoring:
≤ 6 – continue with protocol/monitoring
>8 – perform blood glucose to assess levels
>12 – perform blood glucose levels and obtain advice/assessment from NVS / NACWO

8.2 Appendix 2 – Example animal study protocol

Each animal study had a corresponding protocol which outlined each procedure, the date of the procedure and, where appropriate, the volume of blood taken, to ensure compliance with the PPL/PIL.

PPL:60/4374 Gene therapy and cell transplantation in diabetes

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Supervision: James Shaw (as above)

Animal husbandry: Chris Huggins and CBC

Muscle Targeted Gene Therapy Study – GP004

Diabetic mouse study utilizing exendin 4, GLP1 and eGFP (control) plasmids

License Reference: 19b3

Aim: To determine the effectiveness of the Exendin-4 and GLP-1 plasmids in a diabetic model

Animal Strain: db/db
Sex: Female
DOB: 19/12/12 - 4 weeks (at start of study after 1 week acclimatisation)
Number: 18
Animal supplier: Charles River

Group 1: n=6 (db/db) – pVR1012.CMV.Ex4
Group 2: n=6 (db/db) – pVR1012.CMV.GLP1
Group 3: n=6 (db/db) – pVR1012.CMV.eGFP

All animals will be subject to the following clinical scoring assessment;

Relative blood volume (see http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=419):

60ml/kg – under our current license guidelines; no more than 15% of the circulating blood volume can be taken in a rolling 28day period.

E.g. Mouse is 35g therefore ~2.1ml of circulating blood; @15%, ~315µl can be taken over a 28day period.

IVIS scanning can be performed up to 4 times in a 24hour period for a maximum of 5 days or twice per week for any studies lasting longer than 5 days.

Weights will be taken during the 1 week acclimatisation to obtain a baseline which will be used to monitor health (as well as other observations) during study.

Day	Date	Time	Procedure
Wed	16.01.13	09.00	• Weigh mice and clinical score • Perform pre-procedure IVIS • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
Thurs	17.01.13	09.00	• Weigh mice and clinical score • Obtain pre-procedure peripheral blood sample (for ELISA's) • Perform pre-procedure IPGTT (allow free access to food/water post procedure)
Fri	18.01.13	09.00	• Weigh mice and clinical score • Administer carprofen or metacam subcutaneously (at 50mg/kg or 1mg/kg respectively - diluted to make total administered volume 0.1ml). • Anaesthetise animals with isoflurane and shave both hind legs. • Inject all muscle groups perpendicularly percutaneously first with hyaluronidase (0.4U/ul in 25ul of normal saline) then with 50ug of plasmid (1µg/µl – total vol 50µl) in the central portion of the muscle (29 gauge needle 0.5ml insulin needle 3mm deep). Inject first in the left leg followed by the right. Injection duration is 10 seconds for each. Hold needle in place before withdrawing quickly. Muscles targeted are TA and

				gastroc. **<u>(total plasmid requirement = 200µl per mouse)</u>** • After injecting the second leg apply ECG gel to the first leg and electroporate transcutaneously (settings: eight x 20ms pulses of 200V/cm with intervals of 250ms applied through 2 x 1.5cm^2 stainless steel plate electrodes placed on each side of the leg). Electroporation is performed using BTX ECM830 firstly on the left, followed by the right leg. The plates should be placed in such a manner that each leg needs only one set of electroporation for both muscle groups. The plates should not restrict movement of the leg but should be close enough to form a circuit using the gel. • Place all mice into relevant boxes and place in the incubator for recovery.
	Sat	19.01.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
	Sun	20.01.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
	Mon	21.01.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
	Tues	22.01.13	09.00	• Weigh mice and clinical score • Perform fasting glucose measurement
	Wed	23.01.13	09.00	• Weigh mice and clinical score
	Thurs	24.01.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
	Fri	25.01.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
0	Sat	26.01.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
1	Sun	27.01.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
2	Mon	28.01.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
	T	29.0	0	• Weigh mice and clinical score • Perform fasting glucose measurement

3	ues	1.13	9.00	
4	Wed	30.01.13	09.00	• Weigh mice and clinical score
5	Thurs	31.01.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
6	Fri	01.02.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
7	Sat	02.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
8	Sun	03.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
9	Mon	04.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
10	Tues	05.02.13	09.00	• Weigh mice and clinical score • Perform fasting glucose measurement
11	Wed	06.02.13	09.00	• Weigh mice and clinical score
12	Thurs	07.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
13	Fri	08.02.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
14	Sat	09.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
15	Sun	10.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
16	Mon	11.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
17	Tues	12.02.13	09.00	• Weigh mice and clinical score • Perform fasting glucose measurement
	W	13.02	0	• Weigh mice and clinical score

8	ed	2.13	9.00	
9	Thurs	14.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
0	Fri	15.02.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
1	Sat	16.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
2	Sun	17.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
3	Mon	18.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
4	Tues	19.02.13	09.00	• Weigh mice and clinical score • Perform fasting glucose measurement
5	Wed	20.02.13	09.00	• Weigh mice and clinical score
6	Thurs	21.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
7	Fri	22.02.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
8	Sat	23.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
9	Sun	24.02.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
0	Mon	25.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
1	Tues	26.02.13	09.00	• Weigh mice and clinical score • Perform fasting glucose measurement
2	Wed	27.02.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
	T	28.0		• Weigh mice and clinical score

3	hurs	2.13		• Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
4	Fri	01.03.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
5	Sat	02.03.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
6	Sun	03.03.13	09.00	• Weigh mice and clinical score (CBC if possible please?)
7	Mon	04.03.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
8	Tues	05.03.13	09.00	• Weigh mice and clinical score • Perform fasting glucose measurement
9	Wed	06.03.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Fast mice from 17.00 (leave ½ food pellet per mouse in the cage) overnight
10	Thurs	07.03.13	09.00	• Weigh mice and clinical score • Obtain peripheral blood sample • IPGTT (allow free access to food/water post procedure)
11	Fri	08.03.13	09.00	• Weigh mice and clinical score • Image mice using IVIS spectrum • Terminate animals via cardiac puncture • Harvest muscles by stripping skin of hindlegs starting at the hock moving proximally, remove fascial layers and mark the area of injection on the right side by incision using a scalpel blade. Then harvest both TAs and gastroc groups by transecting muscle insertion proximally and distally. Cut along injection site into two pieces and snap freeze by placing directly into isopentane cooled via liquid nitrogen. Store in cryovials and store at -80^{0}C until needed**. AND/OR** Place each of the muscle groups into 1.5ml eppendorf tubes containing 1ml of Buffer A. • Muscles to be homogenised are to be placed, seperately, into Buffer A (containing protease inhibitors), after being weighed. • Muscles to be stained are to be snap frozen (iospentane) onto cork discs with coverslip support and placed in cryovials. • Remove pancreases, weigh and place into 10% formalin.

NOTES:

- Buffer A contains 10 mM HEPES pH 7·9, 10 mM KCl, 0·1 mM EDTA pH 8·0, 0·1 mM EGTA pH 8·0, 1 mM dithiothreitol and protease inhibitors (Roche, UK), 10µl/ml (to be added immediately before use)

- IVIS scans
    - Treatment groups to be scanned as groups of 3 (machine only capable of scanning a maximum of 5). Each eGFP group animal to be scanned individually (dorsal and ventral views) 2D and 3D.
    - Each leg needs to be shaven before each scan due to dark fur interfering with the light absorptions patterns.
    - Scan muscle once harvested? Need to think about logistics

- IPGTT – ask for assistance from CBC, with a view to handing procedure over in the future

- Peripheral blood samples (~100µl) – dates may need altering once maximum allowable blood volumes have been calculated.

- Muscle group method for snap freezing muscles
    - Make a surface incision in a 25mm cork disc – not cutting through the disc
    - Place coverslip in cut, perpendicular to the disc
    - Lay muscle in correct orientation against the coverslip
    - Cover muscle in OCT medium
    - Place entire sample into dry ice cooled isopentane to freeze.
    - Store at -80^{0}C until needed.