

Promoter Optimisation of Lentiviral Vectors for Efficient Insulin Gene Expression in Canine Mesenchymal Stromal Cells: Potential Surrogate Beta Cells

Short title: Insulin expression in MSCs

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Abstract

Background: The lack of an ideal cell type that can be easily acquired, modified to produce insulin, and re-implanted has been a limitation for *ex vivo* insulin gene therapy. Canine diabetes is currently treated with human insulin and is a good model for human diabetes. Mesenchymal stromal cells (MSCs) are a promising candidate cell type for gene therapy. Here we optimised insulin production using lentiviral transduced canine MSCs aiming to evaluate their ability for use as surrogate beta cells.

Method: Canine MSCs were derived from bone marrow and validated by measuring expression of MSC lineage specific markers. Lentivirus vectors encoding the proinsulin gene (with or without a Kozak sequence) under the control of SFFV, CMV, EF1 α and SV40 promoters were generated and used to transduce primary cMSCs and a hepatocyte cell line. The insulin producing capacity of transduced primary canine MSCs was assessed by measuring the concentration of C-peptide produced.

Result: Primary canine MSC could be readily expanded in culture and efficiently transduced using lentiviral vectors encoding proinsulin. Increasing the multiplicity of infection from 3 to 20, led to an increase in C-peptide secretion (1700 pmol/l to 4000 pmol/l). The SFFV promoter conferred the strongest transcriptional ability.

Conclusion: Our results suggest that optimised lentiviral transduction of the insulin gene into primary canine MSCs renders these cells capable of secreting insulin both short- and long-term, in sufficient quantities *in vitro* to support their potential use in insulin gene therapy.

Introduction

Type 1 diabetes is characterised by T cell-mediated destruction of insulin producing pancreatic beta cells and associated with a reduction in life expectancy, early morbidity and a diminished quality of life [1]. Current treatment with exogenous insulin therapy greatly increases diabetic life expectancy but still does not fully prevent complications of long-term hyperglycaemia. A significant proportion of patients with Type II (non auto-immune) diabetes also require exogenous insulin. New approaches are needed that provide more efficient delivery of physiological levels of insulin to achieve optimal blood glucose control and prevent diabetic complications.

Current approaches to alternative therapies to exogenous insulin injections can be broadly divided into cell-based and gene therapies [2, 3]. Gene therapy, defined as transfer of therapeutic genetic material to specific target cells for the prevention or cure of a particular disease, has significant potential for treatment of a diseases like diabetes, where treatment involves restoration of functional levels of a protein. Insulin production in non-beta cells is being attempted by exogenous insulin gene expression using either viral or non-viral methods of gene delivery [4, 5]. Their natural glucose-sensing capability and the ability to undergo glucose-stimulated transcription, make hepatocytes attractive as surrogate beta cells. Although insulin gene therapy using hepatocytes has shown considerable promise in murine models of diabetes, with some studies even reporting a cure, lasting correction of blood glucose has not yet been reported in a preclinical model [6, 7]. Furthermore the use of autologous hepatocytes for clinical application may not be easily scalable for clinical use [8]. Skeletal muscle cells have been targeted by direct injection of modified adeno-associated virus serotype 1 (AAV1) encoding both the insulin gene and a glucokinase gene, into the thigh muscles of diabetic dogs as an *in vivo* insulin therapy [5]. *In vivo* insulin gene therapy has its benefits but it also presents safety concerns regarding potential off-target cell transduction and the risk of irreversible hypoglycaemia from excessive insulin production [9-11]. *Ex vivo* gene therapy using retroviruses has been used for successful treatment of severe combined immunodeficiencies (SCIDs) for over a decade and represented a significant milestone in gene therapy, although a number of patients developed leukaemia due to insertional mutagenesis [9, 11-13]. *Ex vivo* insulin gene therapy for the treatment of diabetes has, until now, been limited by the availability of an appropriate cell lineage for insulin production.

Mesenchymal Stromal Cells (MSCs) are multipotent cells able to differentiate into cell types of mesodermal origin such as osteoblasts, chondroblasts and adipocytes [14]. MSCs can be readily isolated from the bone marrow based on their ability to adhere to plastic culture dishes and identified by their spindle-shape morphology [15]. They can be easily re-implanted after genetic modification and show long-term engraftment following transplantation, thereby proving a good potential candidate cell type for use in *ex vivo* gene therapy. [16]; they have attracted considerable attention as a potential tool for therapeutic gene transfer [17-21].

Lentiviral vectors based on human immunodeficiency virus (HIV) type 1 are emerging as vectors of choice for *ex vivo* and *in vivo* gene therapy in a number of scenarios. HIV 1 vector based gene delivery depends on using the HIV packaging signal and other viral *cis*-acting sequences to encapsidate the desired gene to be delivered via a lentiviral particle [22-25]. Their advantages include a large gene capacity of up to 8 kb, the ability to infect dividing and non-dividing cells, the absence of inflammatory/immune response induction, and long-term transgene expression. In addition pseudotyping lentiviral vectors with vesicular stomatitis virus envelope broadens the cell tropism increasing the number of potential target cell types.

We sought to carry out a proof-of-principle study for cell based gene therapy applicable to Type I or insulin-requiring Type II diabetes. Diabetes is a relatively frequent condition in dogs and human insulin is the standard therapy. Complications occur as in human diabetes and difficulties with repeated needle usage are well recognised in both pets and by their owners. Cell based insulin gene therapy with autologous canine MSCs would provide a highly workable treatment and provide a stepping stone to this approach in humans.

Specifically, we aimed to a) establish the feasibility of culturing primary cMSCs, b) provide *in vitro* proof of lentiviral transduction and insulin secretion by cMSCs, c) compare the ability of different viral and mammalian constitutive promoters to drive insulin gene expression in cMSCs, and d) investigate the effect of long term culture on viral promoter expression in transduced cells. We envisaged that implantation of autologous insulin producing cMSCs could provide an *in vivo* source of constitutive basal insulin secretion to prevent hyperglycaemia between meals and at night.. This study would provide the precursor to the much more challenging goal of glucose responsive gene therapy which has yet to be achieved.

Materials and Methods

Cell culture

The human hepatocellular carcinoma cell line, Hep3B and human embryonic kidney cells, 293T, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, U.K) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). Cells were maintained at 37°C with a humidified atmosphere of 5% CO₂.

Canine MSCs were derived from bone marrow cells isolated from the tibia or femur of otherwise healthy dogs, with owner consent, during open surgery (for example hip replacement or tibial plateau levelling osteotomy). The collection and generation of primary cMSC cell lines was approved by the institutional Ethics review committee and management board of Dick White Referrals Ltd. All the dog owners (or other legally acceptable representatives) provided a written informed consent to allow the use any cells obtained as part of routine work in the clinic for "research to the benefit of animal welfare". The bone marrow cells acquired were always obtained as part of routine and recognized diagnostic testing or treatment procedures and the procedure caused no unnecessary distress or harm to the dogs (i.e.: samples were never to be taken for research-only reasons and excessive sampling was also not permitted). Cells were cultured in custom made MSC media; 33% [v/v] low glucose DMEM, 67% [v/v] complete MSC media (Life Technologies, Paisley, U.K), 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare Life Sciences, Logan, Utah), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Adherent MSCs adopted a spindle-shape morphology in culture and were expanded for up to 21 days. Once the cells reached over 80% confluence in a T175 flask, the cells were frozen at a concentration of one million cells per ml in freezing media (90% FCS, 10% DMSO v/v).

Lentiviral vector construction

The lenti SFFV Ins(F) IRES GFP vector encodes the human proinsulin transgene modified to allow alpha and beta chain processing from the proinsulin transgene effected by the ubiquitously available cellular protease furin together with an enhanced GFP reporter gene under the control of the spleen focus forming virus (SFFV) promoter [26]. This was a kind gift from Dr Gan Shu Uin (National Institute of Singapore, Singapore). The vector SFFV Kozak Ins(F) IRES GFP containing a Kozak consensus sequence immediately upstream of the insulin gene was generated by PCR amplification of

the Kozak proinsulin(F) DNA insert using a forward primer encoding a *Bgl*III restriction site followed by a Kozak consensus sequence upstream of the proinsulin gene, and a reverse primer encoding an *Xho*I restriction site. The lenti SFFV Ins(F) IRES GFP plasmid vector was double digested using *Bam*HI and *Xho*I restriction enzymes (New England Biolabs, Hitchin, U.K), treated with Shrimp alkaline phosphatase (SAP) (Promega, Southampton, U.K) and ligated with the PCR amplified insert to generate the lenti SFFV Kozak Insulin(F) IRES GFP vector. The primers used were as follows: Kozak Insulin Forward, 5' GCAAGAAGATCTGCCGCCACCATGGCCCTGTGGATGCGC^{3'}; Kozak Insulin Reverse 5' TGGTCGACCTCTTGATGACGTTGATCGAGCTCAGAACG^{3'}.

The lenti Kozak Insulin(F) vector was constructed as follows. Lenti SFFV Kozak Insulin(F) IRES GFP vector was double digested using *Xho*I, *Not*I restriction enzymes (New England Biolabs), for removal of the “IRES GFP” genes. The restricted plasmid vector was subsequently SAP dephosphorylated and gel purified. A linker DNA encoding 5' *Xho*I-*Bst*XI-*Not*I^{3'} restriction sites was generated by annealing forward and reverse primers. Subsequently, the linker DNA insert was ligated with the prepared vector to generate lenti SFFV Kozak Insulin(F) vector. The primers used were as follows: forward-linker 5' GGCCGCCCATGGTTGTGGC^{3'} and reverse-linker 5' CGGGTACCAACACCGAGCT^{3'}.

The lenti CMV Ins(F) IRES GFP vector was constructed by replacing the SFFV promoter with a CMV promoter in the lenti SFFV Ins(F) IRES GFP vector. Briefly, the plasmid vector was double digested with *Bcl*II and *Bsi*WI restriction enzymes and the resultant promoter-less vector was gel purified and SAP treated. The CMV promoter insert was generated by PCR amplification from the pcDNA3.1 plasmid. The CMV promoter insert containing a 5' *Bcl*II site and a 3' *Bsi*WI site was ligated to the promoter-less vector to generate the lenti CMV Ins(F) IRES GFP vector. The primers used were as follows: forward-CMV 5' AAATGATCAGCGCGGTTGACAT^{3'}; reverse-CMV 5' ATTCGTCTCGAGAGGCATGCAAA^{3'}. Likewise, the lenti SV40 Ins(F) IRES GFP vector was constructed by sub-cloning the SV40 promoter into the prepared promoter-less plasmid vector. The SV40 promoter was PCR amplified from plasmid pRL SV40 plasmid using a forward primer incorporating a *Bcl*II restriction site and a reverse primer incorporating a *Bsi*WI restriction site and ligated with the promoter-less vector. The primers used were as follows: forward-SV40 5' AAATGATCAGCGCAGCACCATGG^{3'}; reverse-SV40 5' AAACGTTTTTCGAAGCATGCGCG^{3'}. The lenti EF1 α Ins (F) IRES GFP vector was constructed by ligation of the EF1 α promoter DNA insert into the *Bcl*II, *Bsi*WI site of the prepared promoter-less vector. PCR amplification was carried out using plasmid pEF3.1 as a template; a forward primer incorporating a *Bcl*II site and a reverse primer incorporating a *Bsi*WI site. The EF1 α promoter was subsequently ligated into the prepared promoter-less lenti “X” Ins(F) IRES GFP plasmid vector to generate lenti EF1 α Ins(F) IRES GFP vector. The primers used were as follows: forward-SV40 5' GCATGATCAGGCAATTGAACCGG^{3'}; reverse-SV40 5' ATCGCAAA TTTGAAGCATGCCGACGA^{3'}. The mock control plasmid, lenti SFFV

GFP, was a kind gift from Prof. John Sinclair (Dept. of Medicine, University of Cambridge). All the plasmid vectors generated were sequenced by GATC Biotech Ltd.

Virus production

Replication deficient HIV-1 based lentiviruses were produced by transient cotransfection involving a three plasmid system [27]. 293T packaging cells were transiently cotransfected with plasmids encoding the gene of interest flanked by a self-inactivating (SIN) viral 3' long terminal repeat (LTR) and a 5' *psi* sequence (packaging signal); the vector pCMVdelta8.91 encoding the *gag/pol* and *rev* genes; and pMD2G encoding the VSV-G envelope protein. Briefly, 6×10^6 293T cells were seeded in a T75 flask in complete DMEM medium 24 hours prior to transfection with plasmid DNA. A lipid based transfection reagent (Effectine; Qiagen, Manchester, U.K) was used according to the manufacturer's instructions to produce lentivirus particles. Viral harvest was carried out at 48 hours and 72 hours post transfection. The harvested medium was subsequently filtered to remove cell debris using a 0.45 μ m Millex-HA filter (Millipore, Watford, U.K). Virus was concentrated by centrifugation at 64000g for 2 hours at 4°C in a Beckman Coulter Optima™ L-100 XP ultracentrifuge. Lastly, the virus pellet was re-suspended in 300 – 500 μ l of DMEM media with no additives and stored at -80°C in 70 μ l aliquots. The multiplicity of infection (MOI) of lentiviruses produced from each of the vector was determined by titrating the viruses on 293T cells.

Lentiviral transduction

24 hours prior to lentiviral transduction Hep3B hepatoma cells and primary cMSCs were each seeded at a density of 2×10^5 cells per well in 24 well tissue culture plates in 150 μ l of complete DMEM media (Hep3B) or custom MSC media (cMSCs). Viral transduction was carried out by adding concentrated lentivirus particles drop-wise to each well. Twenty-four hours post transduction the culture media was topped up to 250 μ l. The cells were then incubated at 37°C in 5% CO₂ for 5 -30 days depending on the experiment. For the time course and the promoter comparison experiments 50 μ l of supernatant was collected and 50 μ l fresh media was added every 24 hours post transduction. At the end of the experiment, 120 hours post transduction for cMSCs and 72 hours for Hep3B cells, the cells were harvested and GFP expression was analysed by flow cytometry (BD FACSCalibur). For the long term cell culture assay, the first sample (250 μ l supernatant) and cells were collected at 72 hours post transduction, then at days 14 and 21. The cells were passaged (1:2) and cultured in fresh 250 μ l of fresh media every 7 days.

Flow cytometry

A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) fitted with an argon-ion (488nm) and red diode laser (635nm) capable of four channels of fluorescent detection was used to carry out flow cytometry analysis in this study. Acquired data was analysed using FlowJo software (Tree Star Inc, Ashland, OR). Where GFP was the fluorochrome to be detected, cells were washed twice with PBS and re-suspended in either 300µl of PBS for immediate analysis or 300µl of 1% paraformaldehyde and stored at 4°C in the dark until data acquisition and analysis could be performed.

The analysis of positive and negative cell surface MSC markers was performed on untreated primary cMSCs and transduced cMSCs at 72 hours post transduction. The cMSCs were first blocked in 1% BSA/PBS and then stained for CD14 RPE:CY5 (mouse anti-human; Bio-Rad MCA2804C), CD29 PE (mouse anti-human; Abcam ab64629, Cambridge, U.K), CD34 Alexa Fluor®647 (mouse anti-dog; Bio-Rad MCA2411A647, Hertfordshire, U.K), CD45 APC (rat anti-dog; Bio-Rad MCA1042APC), CD90 PE (rat anti-dog; eBioscience 12-5900-42), CD44 FITC (rat anti-dog; eBioscience 11-5440-42, Hatfield, U.K), or corresponding isotype controls (IgG1 RPE:CY5, IgG1 PE, IgG1 Alexa Fluor®647, IgG2b, APC, IgG2b,k PE and IgG2a,k FITC respectively). Cells were subsequently washed twice with PBS, re-suspended in 300 µl of paraformaldehyde and acquired with FACSCalibur flow cytometer.

C-peptide measurements

C-peptide concentration in supernatant of transduced cMSCs or Hep3B cells was assayed using Liaison clinical diagnostic C-peptide ELISA assay kits [DiaSorin S.p.A, Italy; product code: 316171]. The C-peptide assays were performed by clinical diagnostic laboratories at Addenbrooke's hospital.

Statistical analysis

The data are presented as mean ± standard error of mean (SEM). Statistical significance of differences between groups was tested by one way analysis of variance (ANOVA) or, if there were two independent variables, by two-way ANOVA.

Results

Culture and characterisation of primary cMSCs

Canine bone marrow derived MSCs were distinguished phenotypically from other canine bone marrow lineages based on their adherent nature and the adoption of a spindle shape morphology in culture (Fig. 1a). The adherent MSCs were further characterised by staining for MSC lineage specific markers. Flow cytometry analysis showed that adherent marrow derived cMSCs express the MSC surface marker CD29, CD90 and CD44 but not the haematopoietic markers CD34, CD14 and CD45 (Fig. 1b) [28]

Lentiviral transduction of primary cMSCs

To assess the lentiviral transduction efficiency of primary cMSCs, lenti SFFV Ins(F) IRES GFP and lenti SFFV GFP (mock) vectors (Fig. 2a) were used to produce lentiviral vectors and transduce primary cMSCs. Mock vector expressing GFP but not insulin (lenti SFFV GFP) was used as a negative control for insulin secretion and a positive control for viral transduction. Physiologically, insulin is transcribed and translated as a pre-prohormone; a single polypeptide chain of 100 amino acids comprises, sequentially, of a signal peptide, the beta chain, a connecting (C-) peptide and the alpha chain [29]. Following translation, the signal peptide of pre-proinsulin is removed to form proinsulin. Proinsulin is further processed to form mature insulin, which consists of two individual alpha and beta chains joined together by two disulphide bridges. C-peptide is released in the process. Therefore, insulin and C-peptide are produced in a 1:1 ratio from proinsulin [29]. As the lenti- SFFV Insulin (F) IRES GFP virus encodes the proinsulin and the GFP gene, we used the concentration of C-peptide in the medium of transduced cells as well as expression of GFP to measure the transduction efficiency.

Fluorescence microscopy of cMSCs confirmed successful GFP reporter gene expression in lenti-SFFV Ins(F) IRES GFP transduced cells (Fig. 2b). As the multiplicity of infection (MOI) increased from 3 to 20, there was a statistically significant increase in the percentage of cells expressing GFP (60% to 80%) and an increase in the concentration of human C-peptide (1700 pmol/l to 4000 pmol/l) which was statistically significant at MOI 10 and 20 in the medium of the transduced cMSCs consistent with published literature (Fig. 2c) [31, 32].

A time-course experiment was performed to assess the accumulation of C-peptide in the medium of transduced cMSCs. Canine MSCs were either mock-transduced or transduced with lenti- SFFV Ins(F) GFP at a multiplicity of infection (MOI) of 10, or left untreated. The concentration of C-peptide in the medium was measured every 24 hours post transduction for 5 days (Fig. 2d). There was a significant increase in concentration of C-peptide in the medium with increasing time post transduction in cMSCs transduced with lenti- SFFV Ins(F) GFP. This observation of an exponential phase, a transition phase and a plateau phase in protein secretion after lentiviral transduction is consistent with published data [33].

Transcriptional activities of four constitutive promoters in primary cMSCs and Hep3B cells

Next, we evaluated if the SFFV promoter used was the optimal promoter for lentiviral transduction of primary cMSCs. We generated lentiviral vectors isogenic other than being driven by different constitutive promoters and compared them to the original vector driven by the SFFV promoter (Fig. 3a). Two of the three were virus-derived constitutive promoters (CMV, SV40); the third was a mammalian constitutive promoter (EF-1 α). All four are commonly used to express exogenous genes in mammalian cells. It was important to establish whether the amount of C-peptide produced by transduced cMSCs was comparable to the amount produced by naturally glucose sensing cells such as hepatocytes; thus the hepatocyte cell line, Hep3B was chosen for comparison. To compare the transcriptional activities of the four constitutive promoters, primary cMSCs and Hep3B cells were transduced at an MOI of 10. The percentage of cells expressing GFP and the concentration of C-peptide in the medium of the transduced cells was quantified 72 hours post transduction using flow cytometry and ELISA, respectively. The SFFV promoter proved to be the strongest driver for insulin and GFP gene transcription in primary cMSCs (Fig. 3b). The SFFV promoter was also the only promoter to evoke a statistically significant increase in C-peptide secretion. The amount of GFP detected was similar using the four constitutive promoters in both the cMSCs and the Hep3B cells, yet there was a large difference in the amount of C-peptide detected in the medium (Fig. 3c). This may be due to the difference in stability between the two proteins (the half-lives of GFP and C-peptide are 24 hours and 20-30 minutes, respectively) [30, 34]. Therefore, the difference in the percentage of cells expressing GFP between the different promoters may be masked by relative stability of expressed GFP protein. Nevertheless, the SFFV promoter within the lentiviral vector conferred the strongest transcriptional activity in cMSCs at 72 hours post transduction.

Optimisation of lentiviral vector mediated insulin expression by cMSCs

To further optimise insulin gene expression in cMSCs, lentiviral vectors encoding a Kozak consensus sequence (GCCGCCACC) directly upstream of the insulin gene transcription start site were generated. Kozak consensus sequences play an important role in initiation of protein translation [35]. Fig. 4b shows that addition of the Kozak consensus sequence produces a statistically significant ($P < 0.0001$) increase in the amount of C-peptide secreted by the transduced cMSCs. In addition, the percentage of cells expressing GFP was higher when cMSCs were transduced with a Kozak sequence containing viral vector although this difference was not statistically significant (Fig. 4b).

Future *in vivo* experiments would require the removal of the IRES-GFP reporter genes from the lentiviral vector. Therefore, to ensure that the removal of reporter gene does not lead to significant alteration in transduction efficiency, we generated a lentiviral vector without the IRES GFP gene (Fig. 4c). A similar amount ($P < 0.001$) of C-peptide was secreted by cMSCs when transduced with either the lenti SFFV Kozak Ins(F) or the original lenti SFFV Kozak Ins(F) IRES GFP viral vectors suggesting that the downstream IRES GFP gene does not affect expression and secretion of the upstream protein (Fig. 4c).

Transcriptional activities of promoters have been shown to decrease with time in permanently transduced cells [33, 36]. Additionally, we have previously reported that early GFP protein 'expression' seen in transduced cells can be due to virally translated GFP protein in the vector producer cell rather than representing the true levels of transgene integration and cellular GFP protein synthesis [37]. Therefore, cMSCs transduced with each of the four constitutive promoters were kept in culture and the amount of C-peptide and GFP expressed was measured at days 3, 7 and 21 after transduction. This assay demonstrated that despite the SFFV promoter inducing the highest amount of C-peptide secretion and GFP expression among the four promoters when compared at day 3 post transduction, at days 7 and 21 the level of protein expression by the SFFV promoter was similar to that of SV40 and EF1 α promoters ($P < 0.0001$, Figs. 5a, b). The percentage of GFP expression in cMSCs transduced with lentivirus containing the viral constitutive promoter CMV and the mammalian constitutive promoter EF1 α were slightly lower at day 21 than that seen at day 3 post transduction (Figs. 2b and 5b). Analysis of MSC surface markers showed that the cells were positive for the MSC marker CD29 and CD 90 and negative for the haematopoietic markers CD34, CD14 and CD45 despite long term culture (Fig. 5c). In addition, the expression level of the surface markers from these cells were similar to that observed in untreated cells (Figs. 1a and 5c), suggesting that long term

culture of transduced cMSCs did not alter their phenotype. Overall, these assays demonstrate that the SFFV promoter is an optimal promoter at driving transgene expression in cMSCs short- and long-term (up to 21 days) and emphasises the stability of phenotype of transduced cMSCs.

Discussion

MSCs are promising candidate cells for *ex vivo* insulin gene therapy. They are easily obtained from bone marrow, are readily transduced with lentiviruses and can be reimplanted autologously into donor animals [17, 19]. Here we show that expression of proinsulin in cMSCs allows these cells to serve as surrogate beta cells *in vitro*. Transduction of primary cMSCs using lentiviral vector encoding the furin cleavable proinsulin and GFP genes resulted in insulin production (measured as C-peptide secretion in the medium) and GFP expression in primary cMSCs.

C-peptide measurements are considered to be considerably more reliable indicators of insulin secretion than direct insulin measurements [30] in part because, unlike mature insulin, there is no reassociation of C peptide with producer cells. In addition the half life of secreted insulin *in vivo* may be as low as 4 minutes whereas that of C peptide is generally considered to be at least 5 times as long. Thus the time window for accurate measurement of C peptide makes it a far better reflection of secreted insulin than attempting to factor in the rapid degradation of insulin that occurs between sampling media and freezing for diagnostic assay. C peptide is also far more stable at room temperature than is insulin [38]

The SFFV promoter conferred the strongest transcriptional ability in primary cMSCs at 72 hours post transduction (as judged by C-peptide production and GFP expression). Expression from CMV, SV40 and EF1 α promoters at an identical MOI were less efficient. The CMV promoter conferred the weakest transcriptional ability in primary cMSCs. This is a somewhat surprising finding as this is widely reported as a strong promoter [39, 40]. However the strength of gene expression is variable and dependent on the cell type [39-41]. Furthermore, the percentages of cells expressing GFP were similar in cells transduced with all four different promoter-containing vectors in both cell types. This verifies that the observed difference in concentration of C-peptide produced by cells transduced with lentiviral vectors with four different internal promoters is not due to differences in transduction efficiency. We observed a greater than 1.5 fold increase in both C-peptide and GFP expression when a Kozak consensus sequence was added upstream of the proinsulin gene. This result is consistent with published data [42]. The Kozak consensus sequence has become increasingly valuable as a tool for improving gene expression using viral constructs since its discovery in 1987 and has been

incorporated in multitude of expression studies. Removal of the downstream IRES GFP from the lentiviral vector did not influence C-peptide secretion.

Observation of the time course of expression post transduction (Fig 2) demonstrates that secretion of insulin increases steadily to a plateau over the first five days. The rise in value of supernatant C peptide does not reflect simple accumulation of peptide as the half-life is too short. This typical picture of expression rising to a peak and plateauing is an encouraging indicator that stable expression can be expected from this approach. This was further validated by the observation that primary cMSCs sustained their level of transgene expression when kept in culture for up to 21 days post transduction. There was no evidence of silencing of any promoter in cMSCs when tested at day 21. This is consistent with our previous findings that once a lentiviral vector has integrated and is expressing a transgene, it is rarely switched off [43]. Additionally, there was no difference in expression of the MSC marker CD29 and stem cell marker CD34 before transduction, at 72 hours post transduction or at day 21 post transduction suggesting that the MSCs maintained their state of differentiation in culture and post transduction.

In humans only 10% of the physiological circulating insulin level is required to prevent life threatening diabetic ketoacidosis [44]. Thus a constitutive basal level of insulin secretion would be expected to increase glycaemic control and the quality of life of diabetic patients. Insulin gene therapy that provides a basal level of insulin could also serve as a substitute that currently provided via the exogenous administration of long-acting insulin. Continuous low-level insulin secretion by *ex vivo* modified MSCs as presented here, is therefore also a promising alternative approach to continuous subcutaneous insulin infusion therapy. The latter has been shown to successfully reduce glycosylated haemoglobin A1c with a concomitant decrease in the rate of hypoglycaemic events and without excessive weight gain [45, 46].

Insulin supplementation could also be particularly attractive in treatment of insulin-dependent type II diabetes where the disease is not immune mediated and therefore autoimmune destruction of insulin producing transplanted cells is unlikely to occur. Furthermore, such a supplemental source of insulin from transduced MSCs would decrease the work load of the remaining beta cells in the pancreas, potentially allowing beta cell recovery. The remaining beta cells then would only need to provide the postprandial glucose dependent insulin secretion.

Further *in vivo* studies in diabetic animal models will be required to optimise the viral dose, to investigate the *in vivo* retention, mobilisation and stability of *ex vivo* modified insulin secreting MSCs and to assess the risk of an immune mediated response towards insulin producing transplanted cells. There is a potential risk of development of hypoglycaemia with constitutive insulin secretion, this can be mitigated by titration of the viral dose providing a means to regulate constitutive insulin production. Ideally a glucose-regulated insulin secretion system would be utilised but to date the regulated promoters which have been tested have been either too slow or not reliable in their responsiveness to glucose [4, 47, 48].

In conclusion, our data demonstrates that lentiviral transduction of the insulin genes into primary cMSCs derived from the bone marrow are a promising way forward for establishing secretion of insulin both short- and long-term.

Conflict of interest

The authors declare no conflicts of interest. The study was funded by the Lollipop Trust. Work in the laboratory is supported by the Biomedical Research Centre.

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Fig. 1

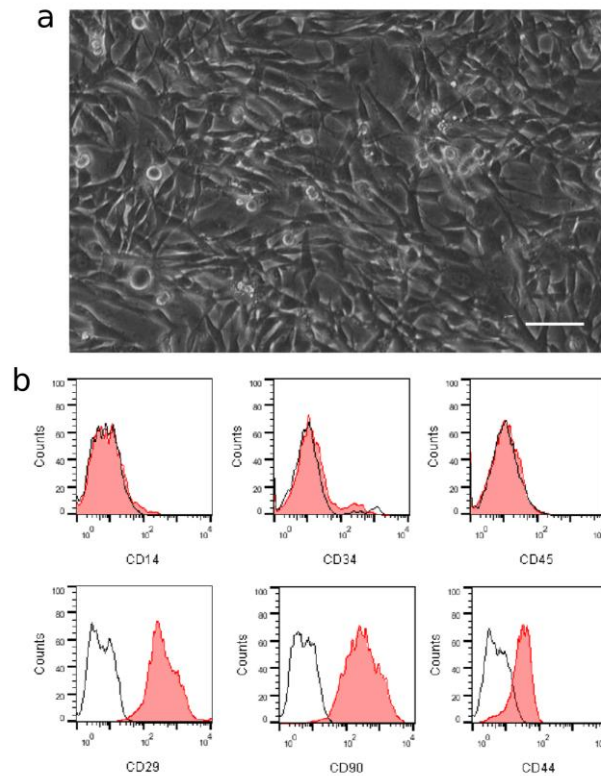


Figure 1. Characterisation of canine mesenchymal stromal cells. (a) Microscopy image of canine mesenchymal stromal cells at passage 0. Cells were derived from canine bone marrow aspirate and can be seen to have a fibroblast-like morphology. Scale bar represents 80 μ m. **(b)** Expression of mesenchymal cell specific surface markers by cMSCs CD29, CD90 and CD44 but not cell markers CD34, CD14 and CD45. Black lines indicate the isotype control.

Fig. 2

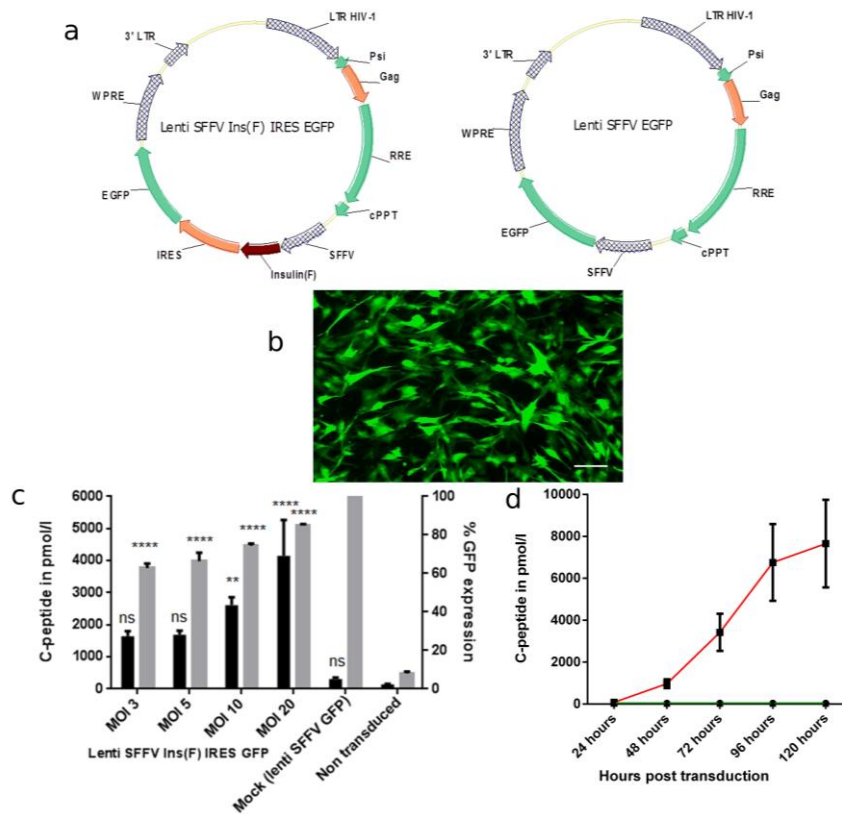


Figure 2. Lentiviral transduction of cMSCs. (a) Schematic representation of the lentivirus vectors encoding either the furin cleavable human proinsulin gene and an enhanced green fluorescent protein (GFP) reporter gene under the control of SFFV (top left) or the mock vector encoding enhanced GFP gene under the control of SFFV promoter (top right). (b) Expression of GFP reporter gene by the lenti SFFV Ins(F) IRES GFP transduced cMSCs. The GFP expression (green) illustrated successful transduction of the cMSCs by lenti SFFV Ins(F) IRES GFP viruses. (c) Bar graph illustrating the concentration of human C-peptide in the medium of transduced cMSCs (black bar; left axis) and percentage of GFP expressing transduced cMSCs (grey bar, right axis) 72 hours after transduction. Shown are cMSCs transduced with either lenti SFFV Ins(F) IRES GFP virus at MOI of 3, 5, 10 and 20; or with control virus (mock; lenti SFFV GFP); or left untreated (non transduced). Scale bar represents 80 μm. (d) Time-course of human C-peptide concentration in the medium of cMSCs after transduction. Canine MSCs were transduced at MOI of 10 with lenti-SFFV Ins(F) IRES GFP virus (red) or mock virus (blue) or untreated (green). Results are from three separate experiments, each in triplicate. The data are presented as mean ± standard error of mean (SEM). (NS) Non significant,

(* $P < 0.05$, (** $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$) by One way ANOVA compared to non-transduced cMSCs.

Fig. 3

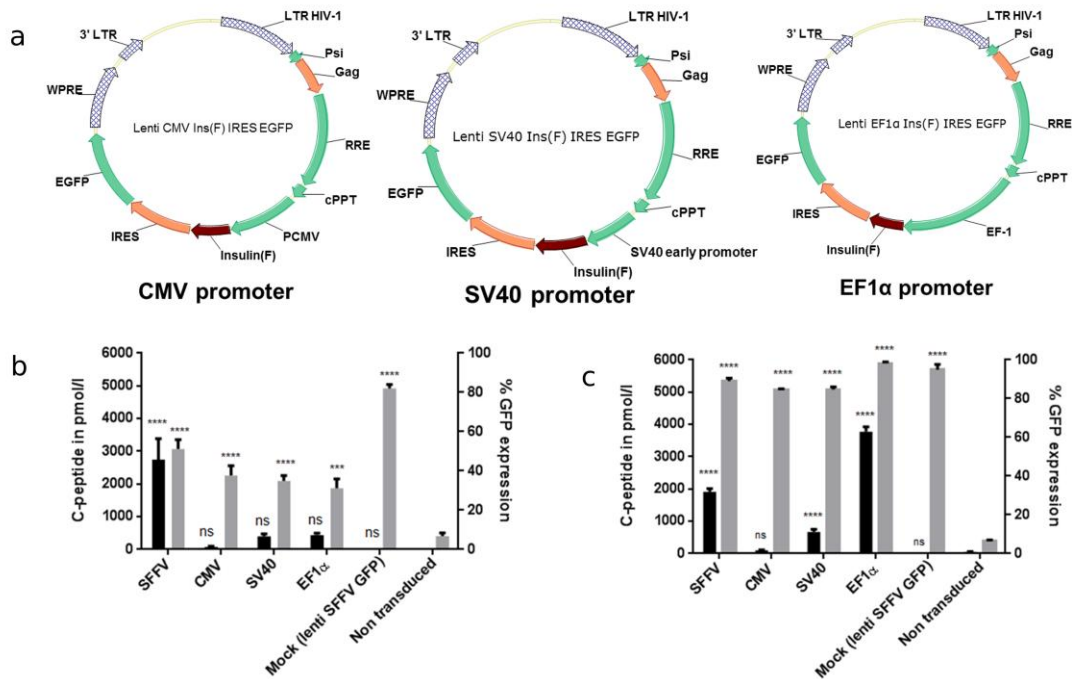


Figure 3. Comparison of promoters. (a) Schematic representation of lentivirus vectors encoding CMV, SV40 or EF1 α internal promoters (left to right) driving the transcription of furin cleavable proinsulin and GFP transgenes. The lentivirus vectors were identical to the lenti SFFV Ins(F) IRES GFP vector except for the promoters driving the transgene expression. (b) A bar graph illustrating the concentration of human C-peptide in the medium of transduced cMSCs (black bar; left axis) and percentage of GFP expressing transduced cMSCs (grey bar, right axis) 72 hours after transduction. Canine MSCs were transduced with lentiviruses with four different internal promoters driving transgene expression. (c) A bar graph illustrating the concentration of human C-peptide in the medium of transduced Hep3B cells (black bar; left axis) and the percentage of GFP expressing transduced Hep3B cells (grey bar, right axis) 72 hours after transduction with lentivirus vector encoding four different promoters. Shown are results of three (Hep3B) and four (cMSCs) separate experiments. The data are presented as means \pm SEM. (NS) Non-significant, (*)P<0.05, (**) P<0.01 (***) P<0.001, (****) P< 0.0001 by One way ANOVA compared to non-transduced cMSCs.

Fig. 4

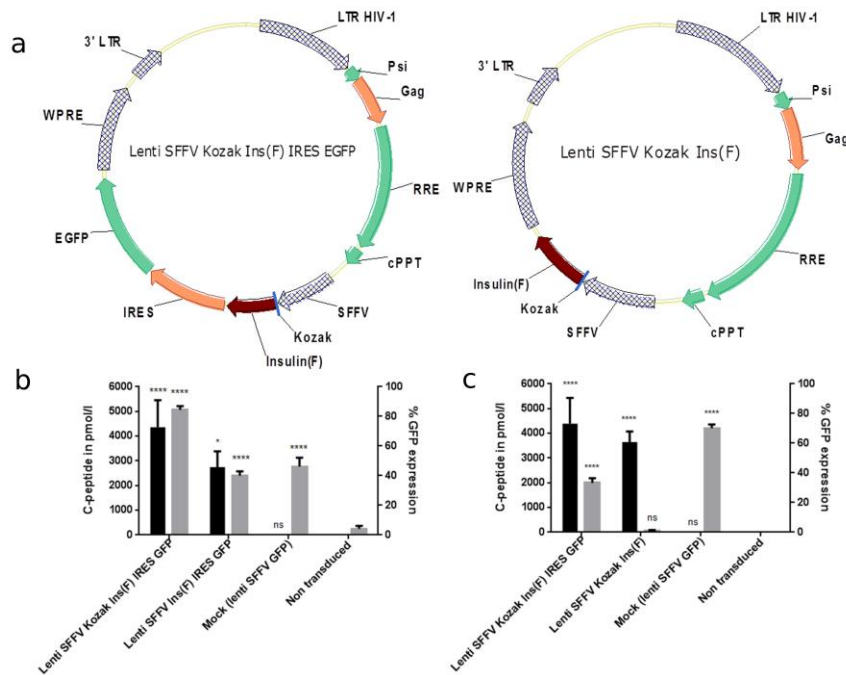


Figure 4. Optimisation of insulin production. (a) Schematic representation of lentivirus vectors generated to further optimise insulin production by transduced cMSCs. Shown are lenti Kozak Ins(F) IRES GFP with an additional Kozak sequence added upstream of the proinsulin gene (left) and lenti Kozak Ins(F) after the removal of downstream transgenes IRES GFP (right). (b) The concentration of C-peptide produced (black bar; left axis) and the percentage of GFP expression (grey bar, right axis) by cMSCs transduced with lentiviruses encoding or not encoding the Kozak consensus sequence directly upstream of the proinsulin transgene. (c) A bar graph illustrating the concentration of human C-peptide (black bar; left axis) and the percentage of GFP expression (grey bar, right axis) by cMSCs transduced with either the lentivirus vector encoding insulin transgene with or without the downstream IRES GFP transgene. Shown are results of three separate experiments, each in triplicate. The data are presented as means \pm SEM. (NS) Non-significant, (*) $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$ by One way ANOVA compared to non-transduced cMSCs.

Fig. 5

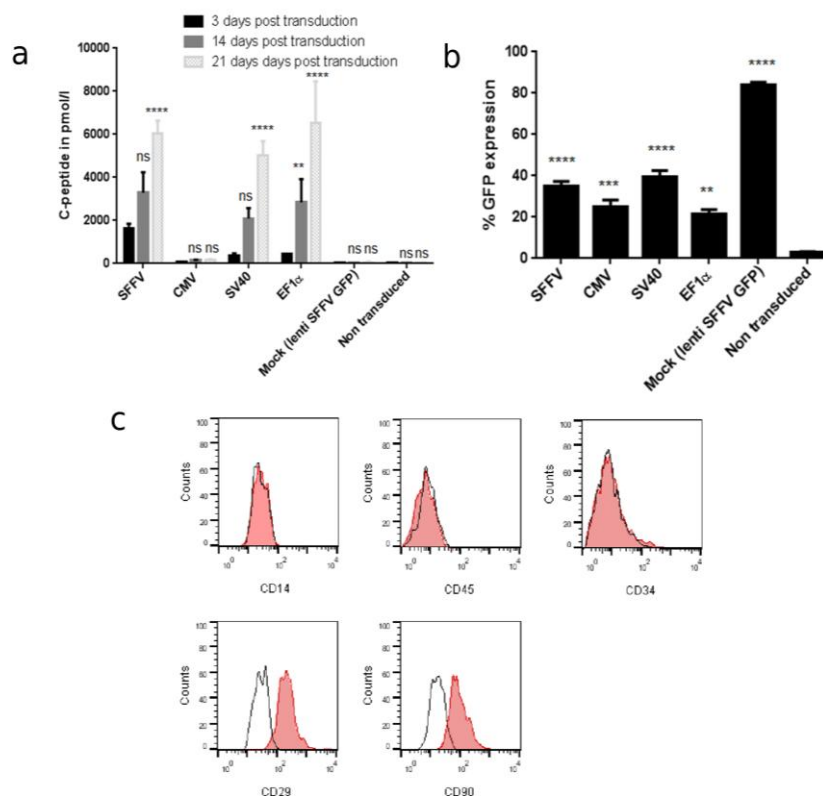


Figure 5. Long-term transcriptional activities of constitutive promoters in the lentivirus vector.

(a) Comparison of the amount of C-peptide produced at day 21 post transduction by cMSCs transduced with lentiviruses driven by the SFFV, CMV, SV40 or the EF1 α promoters. (b) Comparison of the percentage of cells with GFP expression at day 21 post transduction by cMSCs transduced with lentiviruses driven by the four different promoters. Shown are results of three separate experiments, each in triplicate. The data are presented as mean \pm SEM. (NS) represents non-significant, (*)P<0.05, (**) P<0.01 (***) P<0.001, (****) P< 0.0001 by Two way ANOVA compared to C-peptide production at day 3 by transduced cMSCs. (c) Expression of phenotypic MSC markers CD29 and CD90 by the cMSCs but not the haematopoietic markers CD34, CD14 and CD45. Black lines indicate the isotype control.