Received: 20 June 2016 | Revised: 2 August 2016 | Accepted: 25 August 2016

DOI 10.1002/jgm.2900

WILEY

RESEARCH ARTICLE

Promoter optimisation of lentiviral vectors for efficient insulin gene expression in canine mesenchymal stromal cells: potential surrogate beta cells

Pratigya Gautam[1] | Asha Recino[2] | Robert D. Foale[3] | Jing Zhao[1] | Shu Uin Gan[4] | Maja Wallberg[3] | Roy Calne[5] | Andrew M.L. Lever[1]

[1] Department of Medicine, University of Cambridge, Cambridge, UK

[2] Department of Pathology, University of Cambridge, Cambridge, UK

[3] Dick White Referrals, Station Farm, Six Mile Bottom, Suffolk, UK

[4] Department of Surgery, National Institute of Singapore, Singapore

[5] Department of Surgery, University of Cambridge, Cambridge, UK

Correspondence
A. M. L. Lever, Department of Medicine, Level 5, Box 157, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, UK
Email: amll1@medschl.cam.ac.uk

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. In the present study, we optimised insulin production using lentiviral transduced canine MSCs (cMSCs), aiming to evaluate their ability for use as surrogate beta cells.

Methods Canine MSCs were derived from bone marrow and validated by measuring the expression of MSC lineage specific markers. Lentivirus vectors encoding the proinsulin gene (with or without a Kozak sequence) under the control of spleen focus forming virus, cytomegalovirus, elongation factor 1α and simian virus 40 promotors were generated and used to transduce primary cMSCs and a hepatocyte cell line. The insulin-producing capacity of transduced primary cMSCs was assessed by measuring the concentration of C-peptide produced.

Results Primary cMSC 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 (from 1700 to 4000 pmol/l). The spleen focus forming virus promoter conferred the strongest transcriptional ability.

Conclusions The results of the present study suggest that optimised lentiviral transduction of the insulin gene into primary cMSCs renders these cells capable of secreting insulin over both the short- and long-term, in sufficient quantities *in vitro* to support their potential use in insulin gene therapy.

KEYWORDS

diabetes, gene therapy, insulin, lentivirus, mesenchymal stromal cells

1 | INTRODUCTION

Type 1 diabetes is characterised by T cell-mediated destruction of insulin-producing pancreatic beta cells and is 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 representing 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 such as 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 nonviral 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 pre-clinical 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 not only has its benefits, but 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 for over a decade and has represented a significant milestone in gene therapy, although a number of patients developed leukaemia as a result of 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 that are 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 also attracted considerable attention as a potential tool for therapeutic gene transfer.[17–21]

Lentiviral vectors based on 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 nondividing 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 by increasing the number of potential target cell types.

We aimed 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 dogss 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 (cMSCs) would provide a highly workable treatment and provide a stepping stone to this approach in humans.

Specifically, in the present study, we aimed to (i) establish the feasibility of culturing primary cMSCs; (ii) provide *in vitro* proof of lentiviral transduction and insulin secretion by cMSCs; (iii) compare the ability of different viral and mammalian constitutive promoters to drive insulin gene expression in cMSCs; and (iv) 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. The results of the present study would provide the precursor to the much more challenging goal of glucose responsive gene therapy, which has yet to be achieved.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human hepatocellular carcinoma cell line, Hep3B, and human embryonic kidney cells, 293 T, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Gibco). Cells were maintained at 37 °C with a humidified atmosphere of 5% CO_2.

Canine MSCs were derived from bone marrow cells isolated from the tibia or femur of otherwise healthy dogs, with the consent of the respective owners, 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 of the dog owners (or other legally acceptable representatives) provided their 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, UK), 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare Life Sciences, Logan, UT, USA), 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% dimethyl sulphoxide *v*/v).

2.2 | 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 green fluorescent protein (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 polymerase chain reaction (PCR) amplification of the Kozak proinsulin (F) DNA insert using a forward primer encoding a *Bgl*II 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, UK), treated with shrimp alkaline phosphatase (SAP) (Promega, Southampton, UK) and ligated with the PCR amplified insert to generate the lenti SFFV Kozak Insulin (F) IRES GFP vector. The primers used were: Kozak Insulin Forward, 5′-GCAAGAAGATCTGCCGCCACCATGGCCCTGTGGATGC GC-3′; Kozak Insulin Reverse 5′-TGGTCGACCTCTTGATGACGTTGA TCGAGCTCAGAACG-3′.

For construction of the lenti Kozak Insulin (F) vector, 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: forward-linker 5′-GGCCGCCCATGG TTGTGGC-3′ and reverse-linker 5′-CGGGTACCAACACCGAGCT-3′.

The lenti CMV Ins (F) IRES GFP vector was constructed by replacing the SFFV promoter with a cytomegalovirus (CMV) promoter in the lenti SFFV Ins(F) IRES GFP vector. Briefly, the plasmid vector was double digested with *Bcl*I 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*I 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: forward-CMV 5′-AAATGATCAGCGCGCGTTGACAT-3′; reverse-CMV 5′-ATTCGTCTCGAGAGGCATGCAAA-3′. Likewise, the lenti SV40 Ins(F) IRES GFP vector was constructed by subcloning the simian virus 40 (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*I restriction site and a reverse primer incorporating a *Bsi*WI restriction site and ligated with the promoter-less vector. The primers used were: forward-SV40 5′-AAATGA TCAGCGCAGCACCATGG-3′; reverse-SV40 5′-AAACGTTTTTCGAA GCATGCGCG-3′. The lenti EF1α Ins (F) IRES GFP vector was constructed by ligation of the elongation factor (EF)1α promoter DNA insert into the *Bcl*I, *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*I site and a reverse primer incorporating a *Bsi*WI site. The EF1α promoter was subsequently ligated into the prepared promoter-less lenti 'X' Ins(F) IRES GFP plasmid vector to generate lenti EF1α Ins(F) IRES GFP vector. The primers used were: forward-SV40 5′-GCATGATCAGGCAATTGAACCGG-3′; reverse-SV40 5′-ATCGCAAA TTTGAAGCATGCCGACGA-3′.The mock control plasmid, lenti SFFV GFP, was a kind gift from Professor John Sinclair (Department of Medicine, University of Cambridge, Cambridge, UK). All the plasmid vectors generated were sequenced by GATC Biotech Ltd.

2.3 | Virus production

Replication deficient HIV-1 based lentiviruses were produced by transient cotransfection involving a three plasmid system.[27] 293 T 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 x 10^6 293 T cells were seeded in a T75 flask in complete DMEM medium 24 h prior to transfection with plasmid DNA. A lipid based transfection reagent (Effectine; Qiagen, Manchester, UK) was used in accordance with the manufacturer's instructions to produce lentivirus particles. Viral harvest was carried out at 48 and 72 h post transfection. The harvested medium was subsequently filtered to remove cell debris using a 0.45-μm Millex-HA filter (Millipore, Watford, UK). Virus was concentrated by centrifugation at 64 000 ***g*** for 2 h at 4 °C in a Optima L-100 XP ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). Lastly, the virus pellet was re-suspended in 300–500 μl of DMEM media with no additives and stored at −80 °C in 70-μl aliquots. The multiplicity of infection (MOI) of lentiviruses produced from each of the vector was determined by titrating the viruses on 293 T cells.

2.4 | Lentiviral transduction

Twenty-four hours prior to lentiviral transduction, Hep3B hepatoma cells and primary cMSCs were each seeded at a density of 2 x 10^5 cells per well in 24-well tissue culture plates in 150 μl of complete DMEM media (Hep3B) or custom MSC media (cMSCs). Viral transduction was carried out by adding concentrated lentivirus particles dropwise to each well. Twenty-four hours post transduction, the culture media was topped up to 250 μl. The cells were then incubated at 37° C in 5% CO_2 for 5–30 days depending on the experiment. For the time course and the promoter comparison experiments, 50 μl of supernatant was collected and 50 μl of fresh media was added every 24 h post transduction. At the end of the experiment, 120 h post transduction for cMSCs and 72 h for Hep3B cells, the cells were harvested and GFP expression was analysed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). For the long-term cell culture assay, the first sample (250 μl supernatant) and cells were collected at 72 h post transduction, and then at days 14 and 21. The cells were passaged (1:2) and cultured in 250 μl of fresh media every 7 days.

2.5 | Flow cytometry

A FACSCalibur flow cytometer (BD Biosciences) fitted with an argon-ion (488 nm) and red diode laser (635 nm) capable of four channels of fluorescent detection was used to carry out flow cytometry analysis in the present study. Acquired data were analysed using FlowJo software (Tree Star Inc, Ashland, OR, USA). Where GFP was the fluorochrome to be detected, cells were washed twice with PBS and re-suspended in either 300 μl of PBS for immediate analysis or 300 μl 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 h post transduction. The cMSCs were first blocked in 1% bovine serum albumin/PBS and then stained for CD14 RPE:CY5 (mouse anti-human; MCA2804C; Bio-Rad, Hemel Hempstead, UK), CD29 PE (mouse anti-human; ab64629; Abcam, Cambridge, UK), CD34 Alexa Fluor®647 (mouse anti-dog; MCA2411A647; Bio-Rad), CD45 APC (rat anti-dog; MCA1042APC; Bio-Rad), CD90 PE (rat anti-dog; 12–5900-42; eBioscience, Hatfield, UK), CD44 FITC (rat anti-dog; 11–5440-42; eBioscience) 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.

2.6 | C-peptide measurements

C-peptide concentration in supernatant of transduced cMSCs or Hep3B cells was assayed using Liaison clinical diagnostic C-peptide enzyme-linked immunosorbent assay (ELISA) assay kits (product code: 316171; DiaSorin SpA, Saluggia, Italy). The C-peptide assays were performed by clinical diagnostic laboratories at Addenbrooke's Hospital (Cambridge, UK).

2.7 | Statistical analysis

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

3 | RESULTS

3.1 | 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 (Figure 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

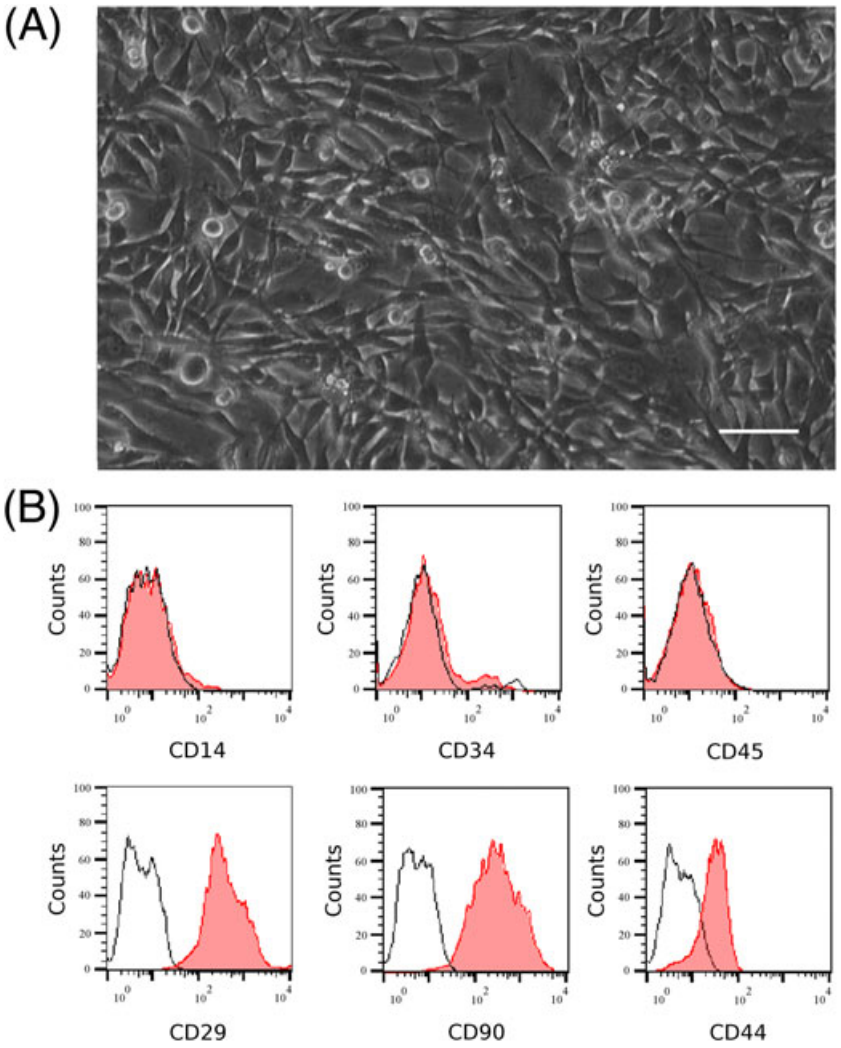

FIGURE 1 Characterisation of cMSCs. (a) Microscopy image of cMSCs at passage 0. Cells were derived from canine bone marrow aspirate and have a fibroblast-like morphology. Scale bar =80 µm. (b) Expression of mesenchymal cell specific surface markers by cMSCs CD29, CD90 and CD44 but not cell markers CD34, CD14 and CD45. Black lines indicate the isotype control

CD29, CD90 and CD44 but not the haematopoietic markers CD34, CD14 and CD45 (Figure 1b).[28]

3.2 | 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 (Figure 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 as 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, 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] Because 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 the expression of GFP, to measure the transduction efficiency.[30]

Fluorescence microscopy of cMSCs confirmed successful GFP reporter gene expression in lenti-SFFV Ins (F) IRES GFP transduced cells (Figure 2b). As the 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 (from 1700 to 4000 pmol/l), which was statistically significant at MOI 10 and 20 in the medium of the transduced cMSCs and consistent with the published literature (Figure 2c).[30,31]

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 h

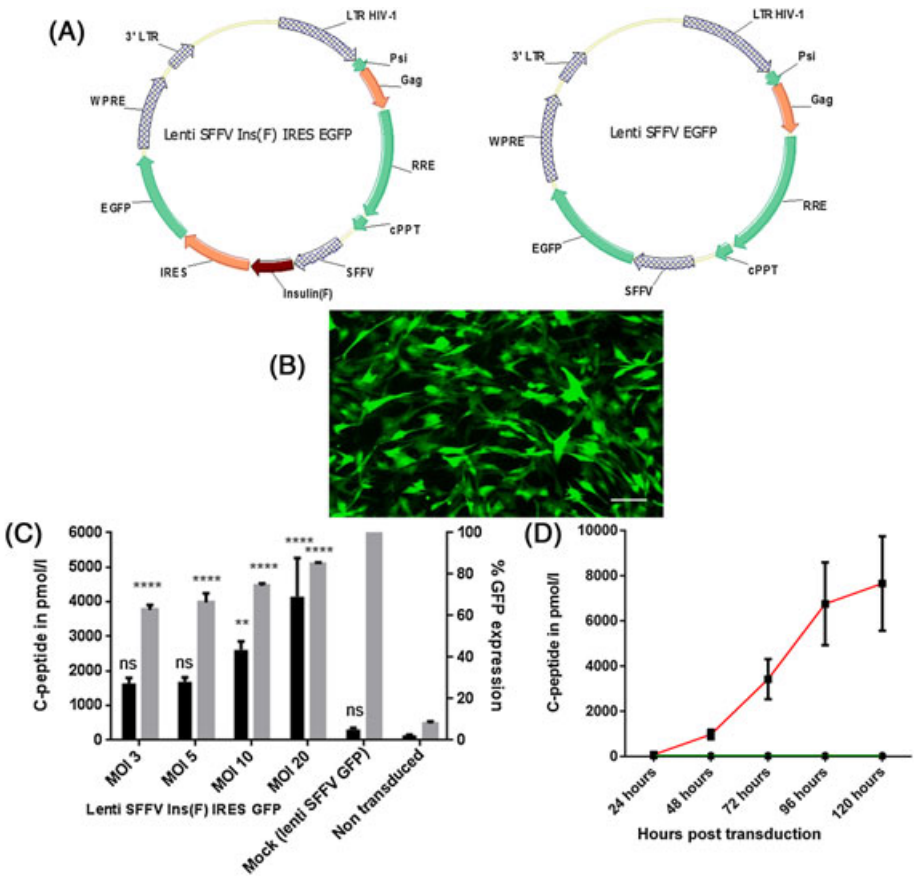

FIGURE 2 Lentiviral transduction of cMSCs. (a) Schematic representation of the lentivirus vectors encoding either the furin cleavable human proinsulin gene and an enhanced GFP reporter gene under the control of SFFV (top left) or the mock vector encoding enhanced GFP gene under the control of SFFV promoter (top right). (b) Expression of GFP reporter gene by the lenti SFFV Ins (F) IRES GFP transduced cMSCs. The GFP expression (green) indicated the successful transduction of the cMSCs by lenti SFFV Ins (F) IRES GFP viruses. (c) Graph illustrating the concentration of human C-peptide in the medium of transduced cMSCs (black bar; left axis) and the percentage of GFP expressing transduced cMSCs (grey bar, right axis) 72 h after transduction. Showing 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 =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 were untreated (green). Results are from three separate experiments, each performed in triplicate. The data are presented as the mean ± SEM. NS, nonsignificant. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$ by one-way analysis of variance compared to nontransduced cMSCs

post transduction for 5 days (Figure 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 the published data.[32]

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

Next, we evaluated whether the SFFV promoter used was the optimal promoter for lentiviral transduction of primary cMSCs. We generated lentiviral vectors that were isogenic other than being driven by different constitutive promoters and compared these with the original vector driven by the SFFV promoter (Figure 3a). Two of the three were virus-derived constitutive promoters (CMV, SV40); the third was a mammalian constitutive promoter (EF-1α). 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 with 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 a 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 h 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 (Figure 3b). The SFFV promotor was also the only promotor 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, although there was a large difference in the amount of C-peptide detected in the medium (Figure 3c). This may be a result of the difference in stability between the two proteins (the half-lives of GFP and C-peptide are 24 h and 20–30 min, respectively).[33,34] Therefore, the difference in the percentage of cells expressing GFP between the different promoters may be masked by the relative stability of expressed GFP protein. Nevertheless, the SFFV promoter within the lentiviral vector conferred the strongest transcriptional activity in cMSCs at 72 h post transduction.

3.3 | 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] Figure 4b shows that the 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

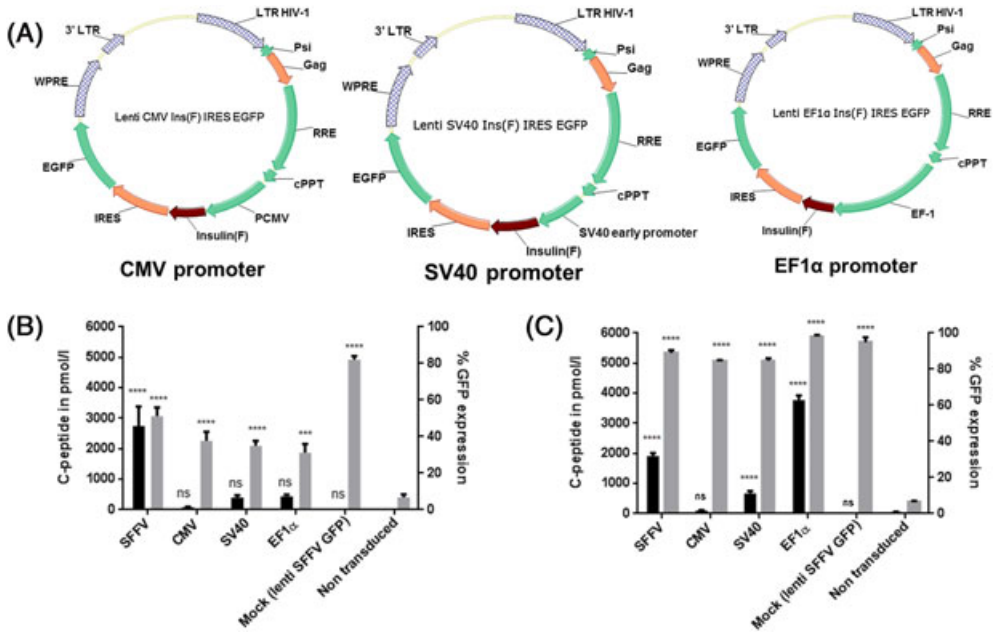

FIGURE 3 Comparison of promoters. (a) Schematic representation of lentivirus vectors encoding CMV, SV40 or EF1α internal promoters (left to right) driving the transcription of furin cleavable proinsulin and GFP transgenes. The lentivirus vectors were identical to the lenti SFFV Ins (F) IRES GFP vector, except for the promoters driving transgene expression. (b) 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 h after transduction. Canine MSCs were transduced with lentiviruses with four different internal promoters driving transgene expression. (c) 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 h after transduction with lentivirus vector encoding four different promoters. Showing the results of three (Hep3B) and four (cMSCs) separate experiments. The data are presented as the mean ± SEM. NS, nonsignificant. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$ by one-way analysis of variance compared to nontransduced cMSCs

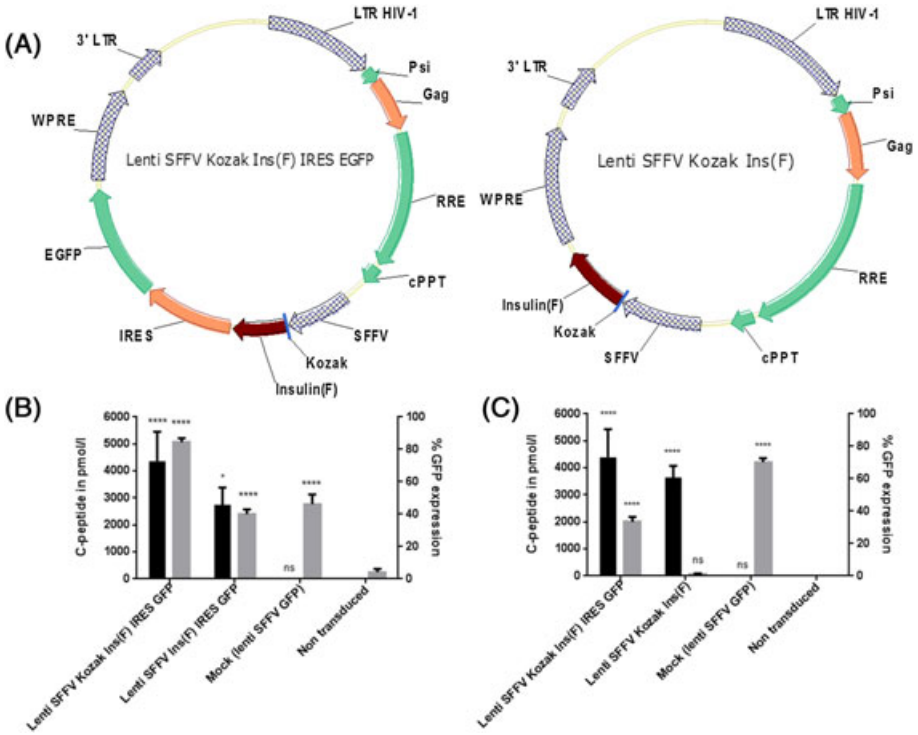

FIGURE 4 Optimisation of insulin production. (a) Schematic representation of lentivirus vectors generated to further optimise insulin production by transduced cMSCs. Showing lenti Kozak Ins (F) IRES GFP with an additional Kozak sequence added upstream of the proinsulin gene (left) and lenti Kozak Ins(F) 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) 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. Showing the results of three separate experiments, each performed in triplicate. The data are presented as the mean ± SEM. NS, nonsignificant. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$ by one-way analysis of variance compared to nontransduced cMSCs

vector, although this difference was not statistical significant (Figure 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 a significant alteration in transduction efficiency, we generated a lentiviral vector without the IRES GFP gene (Figure 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 (Figure 4c).

Transcriptional activities of promoters have been shown to decrease with time in permanently transduced cells.[32,36] Additionally, we have previously reported that early GFP protein 'expression' seen in transduced cells can be a result of 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 EF1α promoters ($p < 0.0001$) (Figures 5a and 5b). The percentage of GFP expression in cMSCs transduced with lentivirus containing the viral constitutive promoter CMV and the mammalian constitutive promoter EF1α were slightly lower at day 21 than that seen at day 3 post transduction (Figures 2b and 5b). Analysis of MSC surface markers showed that the cells were positive for the MSC marker CD29 and CD90 and negative for the haematopoietic markers CD34, CD14 and CD45 despite long-term culture (Figure 5c). In addition, the expression levels of the surface markers from these cells were similar to that observed in untreated cells (Figures 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.

4 | 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] In the present study, we show that the expression of

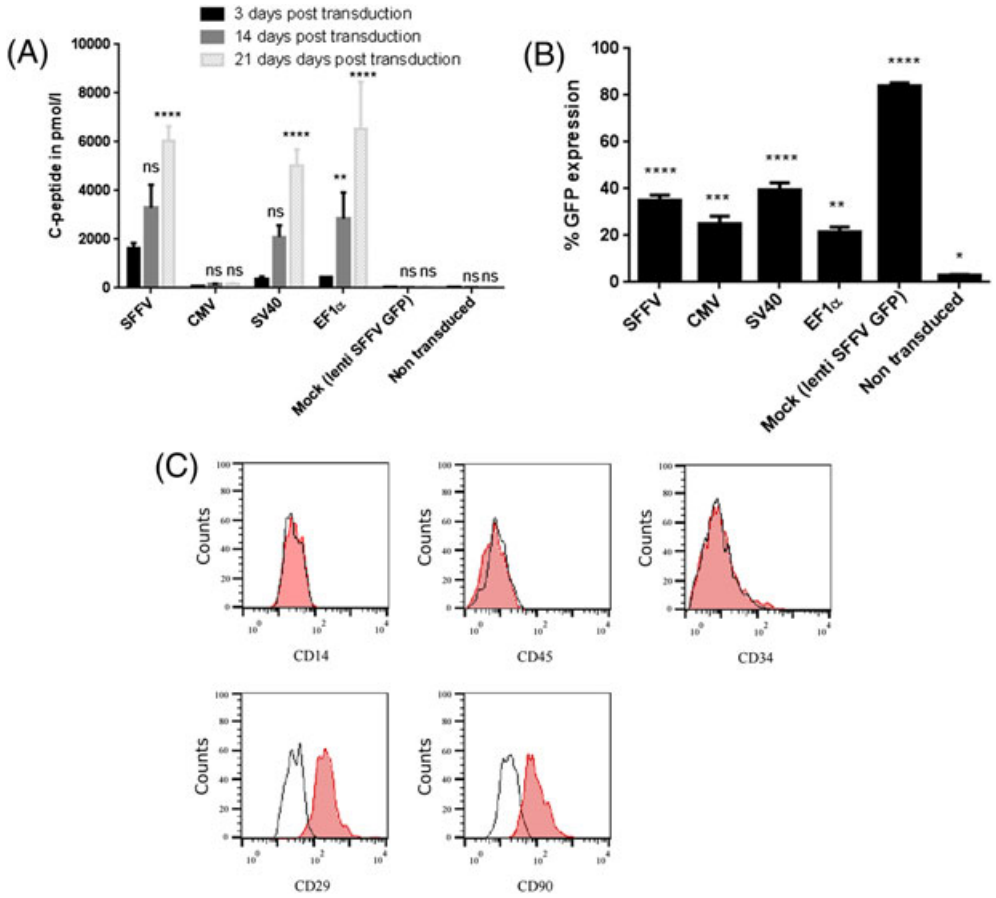

FIGURE 5 Long-term transcriptional activities of constitutive promoters in the lentivirus vector. (a) Comparison of the amount of C-peptide produced at day 21 post transduction by cMSCs transduced with lentiviruses driven by the SFFV, CMV, SV40 or the EF1α promoters. (b) Comparison of the percentage of cells with GFP expression at day 21 post transduction by cMSCs transduced with lentiviruses driven by the four different promoters. Showing the results of three separate experiments, each performed in triplicate. The data are presented as the mean ± SEM. NS, nonsignificant. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$ by two-way analysis of variance 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

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[33] 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 min, whereas that of C-peptide is generally considered to be at least five times as long. Thus, the time window for accurate measurement of C-peptide makes it a far better reflection of secreted insulin than any attempt 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 h post transduction (as judged by C-peptide production and GFP expression). Expression from CMV, SV40 and EF1α promoters at an identical MOI was less efficient. The CMV promoter conferred the weakest transcriptional ability in primary cMSCs. This is a somewhat surprising finding because 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 confirms that the observed difference in concentration of C-peptide produced by cells transduced with lentiviral vectors with four different internal promoters is not a result of differences in transduction efficiency. We observed a more 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 the 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 (Figure 2) demonstrates that the secretion of insulin increases steadily to a plateau over the first 5 days. The rise in the concentration of supernatant C-peptide does not reflect a simple accumulation of peptide because the half-life is too short. This typical depiction of expression

rising to a peak and then plateauing is an encouraging indicator showing that stable expression can be expected using 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 the silencing of any promoter in cMSCs when tested at day 21. This is consistent with our previous findings indicating that, once a lentiviral vector has integrated and is expressing a transgene, it is rarely switched off.[43] Additionally, there was no difference in the expression of the MSC marker CD29 and stem cell marker CD34 before transduction, at 72 h 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 is currently provided via the exogenous administration of long-acting insulin. Continuous low-level insulin secretion by *ex vivo* modified MSCs, as reported in the present study, 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 in 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 (i) optimise the viral dose; (ii) investigate the *in vivo* retention, mobilisation and stability of *ex vivo* modified insulin secreting MSCs; and (iii) 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 way of regulating constitutive insulin production. Ideally a glucose-regulated insulin secretion system would be utilised but, to date, the regulated promoters that have been tested have been either too slow or not reliable in their responsiveness to glucose.[4,47,48]

In conclusion, the data obtained in the present study demonstrate that lentiviral transduction of the insulin genes into primary cMSCs derived from the bone marrow is a promising way forward for establishing the secretion of insulin, both over the short- and long-term.

ACKNOWLEDGMENTS

Thanks to Professor John Sinclair (Department of Medicine, University of Cambridge) for the lenti SFFV GFP vectors and for assistance with MSC phenotyping. We would like to thank Professor K.O. Lee for helpful discussions. The study was funded by the Lollipop Trust. Work in the laboratory is supported by the Biomedical Research Centre. The authors declare that they have no conflicts of interest. The study was funded by the Lollipop Trust. Work in the laboratory is supported by the Biomedical Research Centre.

REFERENCES

1. World Health Organisation and International Diabetes Foundation. *Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycaemia*. WHO Document Production Services: Geneva, Switzerland.
2. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343:230–238.
3. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet*. 2001;358:221–229.
4. Chen NK, Tan SY, Udolph G, Kon OL. Insulin expressed from endogenously active glucose-responsive EGR1 promoter in bone marrow mesenchymal stromal cells as diabetes therapy. *Gene Ther*. 2010;17:592–605.
5. Callejas D, Mann CJ, Ayuso E, et al. Treatment of diabetes and long-term survival after insulin and glucokinase gene therapy. *Diabetes*. 2013;62:1718–1729.
6. Fodor A, Harel C, Fodor L, et al. Adult rat liver cells transdifferentiated with lentiviral IPF1 vectors reverse diabetes in mice: an ex vivo gene therapy approach. *Diabetologia*. 2007;50:121–130.
7. Han J, Lee HH, Kwon H, Shin S, Yoon JW, Jun HS. Engineered enteroendocrine cells secrete insulin in response to glucose and reverse hyperglycemia in diabetic mice. *Mol Ther*. 2007;15:1195–1202.
8. Chen NK, Wong JS, Kee IH, et al. Nonvirally modified autologous primary hepatocytes correct diabetes and prevent target organ injury in a large preclinical model. *PLoS One*. 2008;3:e1734.
9. Aiuti A, Cassani B, Andolfi G, et al. Multilineage hematopoietic reconstitution without clonal selection in ADA-SCID patients treated with stem cell gene therapy. *J Clin Investig*. 2007;117:2233–2240.
10. Nathwani AC, Gray JT, McIntosh J, et al. Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood*. 2007;109:1414–1421.
11. Aiuti A, Bachoud-Levi AC, Blesch A, et al. Progress and prospects: gene therapy clinical trials (part 2). *Gene Ther*. 2007;14:1555–1563.
12. Muul LM, Tuschong LM, Soenen SL, et al. Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. *Blood*. 2003;101:2563–2569.
13. Alexander BL, Ali RR, Alton EW, et al. Progress and prospects: gene therapy clinical trials (part 1). *Gene Ther*. 2007;14:1439–1447.
14. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell*. 2013;13:392–402.
15. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143–147.
16. Centeno CJ, Schultz JR, Cheever M, et al. Safety and complications reporting update on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique. *Curr Stem Cell Res Ther*. 2011;6:368–378.
17. Amara I, Touati W, Beaune P, de Waziers I. Mesenchymal stem cells as cellular vehicles for prodrug gene therapy against tumors. *Biochimie*. 2014;105:4–11.
18. Li C, Ding J, Jiang L, et al. Potential of mesenchymal stem cells by adenovirus-mediated erythropoietin gene therapy approaches for bone defect. *Cell Biochem Biophys*. 2014;70:1199–1204.

19. Durinikova E, Kucerova L, Matuskova M. Mesenchymal stromal cells retrovirally transduced with prodrug-converting genes are suitable vehicles for cancer gene therapy. *Acta Virol*. 2014;58:1–13.
20. Hajizadeh-Sikaroodi S, Hosseini A, Fallah A, et al. Lentiviral mediating genetic engineered mesenchymal stem cell for releasing IL-27 as a gene therapy approach for autoimmune diseases. *Cell J*. 2013;16.
21. Gabr MM, Zakaria MM, Refaie AF, et al. Insulin-producing cells from adult human bone marrow mesenchymal stem cells control streptozotocin-induced diabetes in nude mice. *Cell Transplant*. 2013;22:133–145.
22. Lever A, Gottlinger H, Haseltine W, Sodroski J. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J Virol*. 1989;63:4085–4087.
23. Zhao J, Pettigrew GJ, Bolton EM, et al. Lentivirus-mediated gene transfer of viral interleukin-10 delays but does not prevent cardiac allograft rejection. *Gene Ther*. 2005;12:1509–1516.
24. Zhao J, Lever AM. Lentivirus-mediated gene expression. *Methods Mol Biol*. 2007;366:343–355.
25. Kaiser J. Gene therapy. Beta-thalassemia treatment succeeds, with a caveat. *Science*. 2009;326:1468–1469.
26. Groskreutz DJ, Sliwkowski MX, Gorman CM. Genetically engineered proinsulin constitutively processed and secreted as mature, active insulin. *J Biol Chem*. 1994;269:6241–6245.
27. Buchschacher GL Jr, Wong-Staal F. Development of lentiviral vectors for gene therapy for human diseases. *Blood*. 2000;95:2499–2504.
28. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315–317.
29. Wilcox G. Insulin and insulin resistance. *Clin Biochem Rev*. 2005;26:19–39.
30. Wahlers A, Schwieger M, Li Z, et al. Influence of multiplicity of infection and protein stability on retroviral vector-mediated gene expression in hematopoietic cells. *Gene Ther*. 2001;8:477–486.
31. Fleury S, Simeoni E, Zuppinger C, et al. Multiply attenuated, self-inactivating lentiviral vectors efficiently deliver and express genes for extended periods of time in adult rat cardiomyocytes in vivo. *Circulation*. 2003;107:2375–2382.
32. Gerolami R, Uch R, Jordier F, et al. Gene transfer to hepatocellular carcinoma: transduction efficacy and transgene expression kinetics by using retroviral and lentiviral vectors. *Cancer Gene Ther*. 2000;7:1286–1292.
33. Jones AG, Hattersley AT. The clinical utility of C-peptide measurement in the care of patients with diabetes. *Diabet Med*. 2013;30:803–817.
34. Li X, Zhao X, Fang Y, et al. Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem*. 1998;273:34970–34975.
35. Kozak M. Evaluation of the fidelity of initiation of translation in reticulocyte lysates from commercial sources. *Nucleic Acids Res*. 1990;18:2828
36. Norrman K, Fischer Y, Bonnamy B, Wolfhagen Sand F, Ravassard P, Semb H. Quantitative comparison of constitutive promoters in human ES cells. *PLoS One*. 2010;5:e12413.
37. Nash KL, Lever AM. Green fluorescent protein: green cells do not always indicate gene expression. *Gene Ther*. 2004;11:882–883.
38. McDonald TJ, Perry MH, Peake RW, et al. EDTA improves stability of whole blood C-peptide and insulin to over 24 hours at room temperature. *PLoS One*. 2012;7:e42084.
39. Teschendorf C, Warrington KH Jr, Siemann DW, Muzyczka N. Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. *Anticancer Res*. 2002;22:3325–3330.
40. Damdindorj L, Karnan S, Ota A, et al. A comparative analysis of constitutive promoters located in adeno-associated viral vectors. *PLoS One*. 2014;9:e106472.
41. Qin JY, Zhang L, Clift KL, et al. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One*. 2010;5:e10611.
42. Kozak M. The scanning model for translation: an update. *J Cell Biol*. 1989;108:229–241.
43. Mok HP, Javed S, Lever A. Stable gene expression occurs from a minority of integrated HIV-1-based vectors: transcriptional silencing is present in the majority. *Gene Ther*. 2007;14:741–751.
44. Battezzati A, Zerbi A, Perseghin G, et al. Effect of hemipancreatectomy and of pancreatic diversion on the tolerance to a glucose load in humans. *Eur J Clin Investig*. 2000;30:397–410.
45. Battelino T. Risk and benefits of continuous subcutaneous insulin infusion (CSII) treatment in school children and adolescents. *Pediatr Diabetes*. 2006;7(Suppl 4):20–24.
46. Weinzimer SA, Ahern JH, Doyle EA, et al. Persistence of benefits of continuous subcutaneous insulin infusion in very young children with type 1 diabetes: a follow-up report. *Pediatrics*. 2004;114:1601–1605.
47. Burkhardt BR, Parker MJ, Zhang YC, Song S, Wasserfall CH, Atkinson MA. Glucose transporter-2 (GLUT2) promoter mediated transgenic insulin production reduces hyperglycemia in diabetic mice. *FEBS Lett*. 2005;579:5759–5764.
48. Hsu PY, Kotin RM, Yang YW. Glucose- and metabolically regulated hepatic insulin gene therapy for diabetes. *Pharm Res*. 2008;25:1460–1468.

How to cite this article: P. Gautam, A. Recino, R. D. Foale, J. Zhao, S. U. Gan, M. Wallberg, R. Calne, and A. M. L. Lever (2016), Promoter optimisation of lentiviral vectors for efficient insulin gene expression in canine mesenchymal stromal cells: potential surrogate beta cells, *J Gene Med*, 18, 312–321. doi: 10.1002/jgm.2900