Sustained secretion of human growth hormone from TheraCyte devices encapsulated with PiggyBac-engineered retinal pigment epithelium cells

Claudia R. Cecchi [a,b], Sidsel Alsing [a], Gustavo P.P. Jesus [b,c], Enio A. Zacarias [b], Lisbeth Kjaer [a], Michelle S. Clement [a], Makiko Kumagai-Braesch [d], Thomas J. Corydon [a,e], Paolo Bartolini [b], Cibele N. Peroni [b], Lars Aagaard [a,*]

[a] Department of Biomedicine, Aarhus University, DK-8000 Aarhus C, Denmark
[b] Biotechnology Center, Instituto de Pesquisas Energéticas e Nucleares (IPEN-CNEN), Cidade Universitária, São Paulo, SP, Brazil
[c] Centro Universitario Lusiada, Santos, SP, Brazil
[d] Division of Transplantation Surgery, CLINTEC, Karolinska Institutet, Stockholm, Sweden
[e] Department of Ophthalmology, Aarhus University Hospital, 8200 Aarhus N, Denmark

A B S T R A C T

Growth hormone (GH) deficiency is characterized by impaired growth and development, and is currently treated by repeated administration of recombinant human GH (hGH). Encapsulated cell therapy (ECT) may offer a less demanding treatment-strategy for long-term production and release of GH into circulation. We used PiggyBac-based (PB) transposon delivery for engineering retinal pigment epithelial cells (ARPE-19), and tested a series of viral and non-viral promoters as well as codon-optimization to enhance transgene expression. Engineered cells were loaded into TheraCyte macrocapsules and secretion was followed *in vitro* and *in vivo*. The cytomegalovirus (CMV) promoter supports strong and persistent transgene expression, and we achieved clonal cell lines secreting over 6 μg hGH/10^6 cells/day. Codon-optimization of the hGH gene did not improve secretion. ARPE-19 cells endured encapsulation in TheraCyte devices, and resulted in steady hormone release for at least 60 days *in vitro*. A short-term pilot experiment in immunodeficient SCID mice demonstrated low systemic levels of hGH from a single 40 μL capsule implanted subcutaneously. No significant increase in weight increase or systemic hGH was detected after 23 days in the GH-deficient lit/SCID mouse model using 4.5 μL capsules loaded with the highest secreting clone of ARPE-19 cells. Our results demonstrate that PB-mediated engineering of ARPE-19 is an efficient way to generate hormone secreting cell lines compatible with macroencapsulation, and our CMV-driven expression cassette allows for identification of clones with high level and long-term secretory activity without addition of insulator elements. Our results pave the way for further *in vivo* studies of encapsulated cell therapy.

1. Introduction

Growth hormone (GH) is known for its important role in mammalian growth and development. Biosynthetic GH is being used as an effective means for treating GH deficiency (GHD) and short stature in humans by promoting structural growth (Zhang et al., 2010; He and Barkan, 2020). GHD is a syndrome of impairment in GH secretion which requires daily administration of human recombinant GH to improve growth and normalization of other aspects including body composition, metabolism, and quality of life (Ranke and Wit, 2018).

Encapsulated cell therapy (ECT) is a potential alternative treatment strategy which offers an opportunity to continuously synthetize and release therapeutic factors in a sustained way, making it possible to avoid the daily injections of GH in GHD patients. The ECT strategy is based on transplantation of therapeutic-secreting cells within a semipermeable device. Genetically engineered cells, islets, or human embryonic stem cells (hESC) differentiated into pancreatic epithelium secreting therapeutic proteins have been loaded into semipermeable polymeric devices that allow supply of oxygen and nutrients as well as release of therapeutic protein, while protecting the transplanted cells from host immune response, thus creating a long-term release system (Fjord-Larsen et al., 2010; Kirk et al., 2014; Lathuiliere et al., 2014). The *in vivo* efficacy of ECT has been tested in several clinical studies related to both diabetes type 1, chronic retinal degenerative diseases, and

---

Alzheimer's Disease (ClinicalTrials.gov Identifier NCT0293911, NCT04678557) (Eyjolfsdottir et al., 2016); Kauper et al., 2012). Viacyte has optimized a planar macroencapsulation device (Encaptra Drug Delivery System) to implant allogeneic hESC derived pancreatic cells and perform the first ever clinical trial for a stem cell-derived cell replacement therapy for diabetes (Viacyte.com). Two phase I/II clinical trial studies (ClinicalTrials.gov Identifier NCT02239354, NCT02939118) with cell device VC-01 are ongoing or enrolling patients. Multiple research groups have described the use of a macro semipermeable device (capsule) from TheraCyte (Kirk et al., 2014; Josephs et al., 1999; Kompa et al., 2021; Kumagai-Braesch et al., 2013; Malavasi et al., 2010; Sorenby et al., 2008; Rafael et al., 1999), which is developed to encapsulate tissue, secrete therapeutic proteins, and protect allografts from immune destruction. The TheraCyte capsule is composed of a two-layer polytetrafluoroethylene (PTFE) membrane that exhibits extreme durability and maintains its integrity after freezing (Geller et al., 1997; Yakhnenko et al., 2012). The outermost PTFE membrane consists of polymer with a pore size of 5 μm that supports neo-vascularization, while the inner PTFE membrane is a hydrophilized membrane with 0.4 μm pore size that isolates and shields the inner cells from the contact of the host tissue (Geller et al., 1997). Cells located inside the device are supplied with nutrients and oxygen by inwards diffusion, while the secreted protein of interest and waste diffuse outwards. This device has been shown to present a physiologically relevant secretion profile in a study where pancreatic cells were able to produce insulin and maintain glycemic control in a mouse model of immune-mediated diabetes (Boettler et al., 2016). Encapsulated xenografts from TheraCyte are protected from immune cells, but not from the factors produced by the host immune system, as the inner membrane is permeable to antibodies and cytokines. Xenoantigens produced inside the capsule, leaking into the surrounding environment, may provoke host production of such immune factors. Therefore, xenograft survival required an immunologically deficient animal model (Geller et al., 1997; Brauker et al., 1996; McKenzie et al., 2001). Immunoreactivity against xenotransplants has been a problem for many years. Nevertheless, the use of xenograft transplantation of rat islets in diabetic pigs using a multilayer immunprotective membrane of alginate and PTFE, demonstrated potential benefits that does not require the use of immunosuppression therapy and may be an attractive strategy for cell transplantation (Neufeld et al., 2013).

Josephs et al (Josephs et al., 1999). carried out experiments indicating the potential of utilizing the TheraCyte system with human fibroblast cells (MSU 1.2) for delivery of human GH (hGH) in nude rats. Based on this original study, we aim to investigate if the expression levels of hGH can be improved using ECT with PiggyBac-engineered ARPE-19 cells. The ARPE-19 cell line was established by selective trypsinization of human primary retinal pigment epithelium (RPE) cells, and spontaneous immortalization (Dunn et al., 1996). PiggyBac-based vector technology mediates stable transgene integration through a 'cut and paste' transposition mechanism. It is a non-viral system that offers advantages over viral delivery in terms of increased safety, ease of use, and low genotoxicity (Wilson et al., 2007; Woodard and Wilson, 2015). In particular, the integration profile of transposon-based vectors is favorable as compared with retro- or lentiviral vectors (LVs) (Hudecek et al., 2017), reducing the risk of cell transformation and altered growth behavior. In this work we identify CMV as a strong promoter for use in PiggyBac-engineered ARPE-19 cells, with the aim of reaching sustained high-level expression of hGH. Furthermore, we demonstrate that modified ARPE-19 cells are useful for long-term encapsulation and maintain high hormone secretion. Pre-clinical studies using plasmid-based gene therapy for treatment of isolated GHD in lit/lit and lit/SCID mice have only obtained partial reversal of the GHD phenotype (Cecchi et al., 2014; Higuti et al., 2016; Oliveira et al., 2010; Rosa Lima et al., 2020). The present study provides evidence that our encapsulated genetically manipulated ARPE-19 cells efficiently and potently express hGH *in vitro*, and lays the foundation for future *in vivo* studies which may result in rescue of the GHD phenotype.
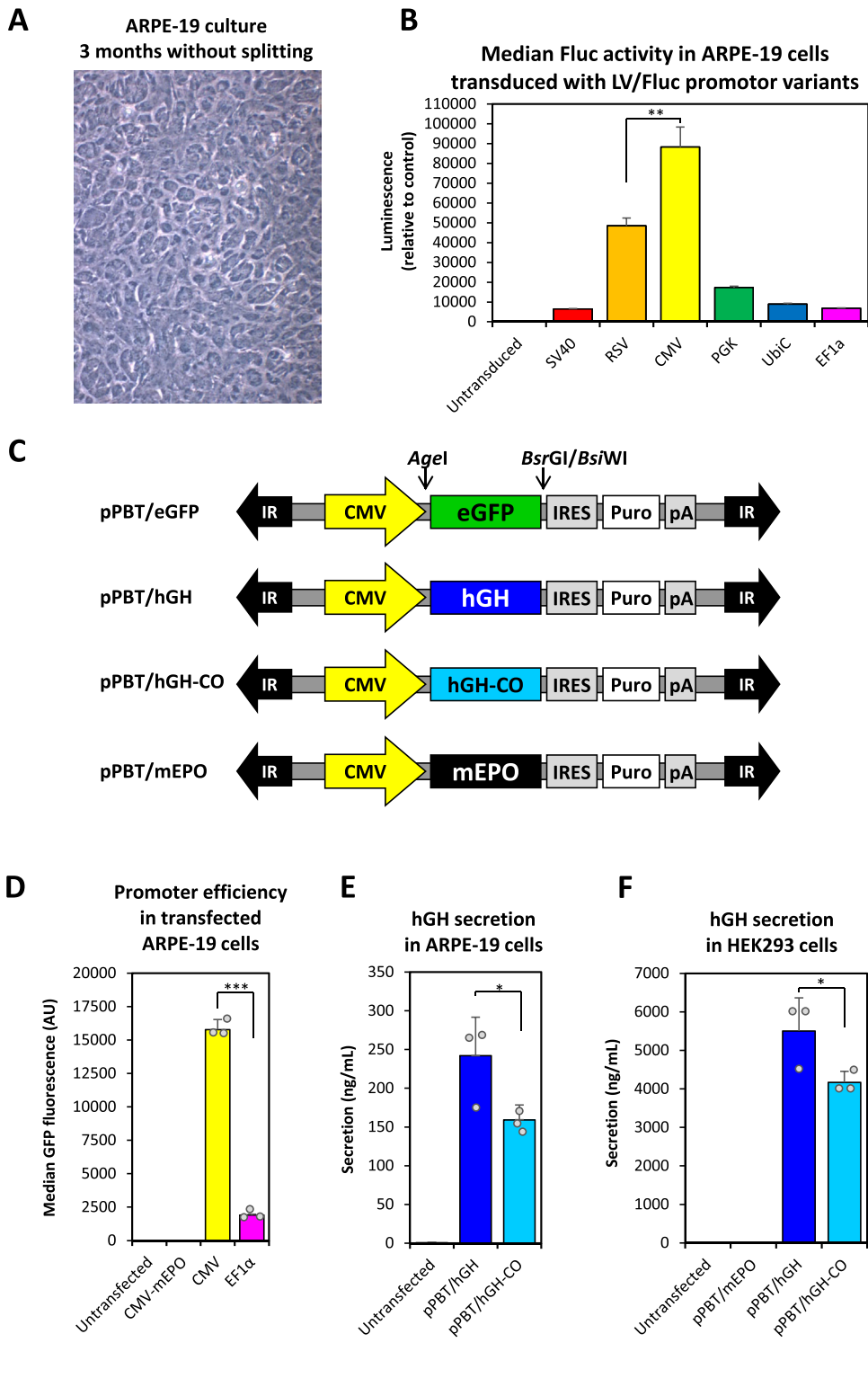
2. Results

2.1. Optimizing transgene expression and delivery to ARPE-19 cells

With the aim of engineering an hGH-secreting cell line suitable for ECT-based macroencapsulation for the potential treatment of GHDs, we first sought a cell type that was sturdy enough and compatible with encapsulation and confinement of space. ARPE-19 cells, a cell line derived from human retinal pigment epithelium, exhibited sufficient contact inhibition, decreased mitotic activity, and prolonged longevity, to survive and express therapeutic genes inside a capsule for an extended period of time. In order to observe cell expansion pattern, ARPE-19 cells were cultured in a tissue flask without splitting, and the monolayer growth was monitored over time. As shown in Fig. 1a, ARPE-19 formed a confluent monolayer of viable cells and stayed intact for months.

Next, we sought to optimize our transgene expression cassette for high activity. This was done in two ways; 1) by screening several viral and non-viral promoters for their intrinsic activity in ARPE-19 cells and 2) by testing a codon-optimized version of the gene encoding hGH. The promoter strength in ARPE-19 cells was assessed using lentiviral transduction to ensure efficient and even delivery across constructs. A series of pCCL-based LVs established in our laboratory, encoding the reporter gene Firefly Luciferase (Fluc) driven by various promoters, were used to transduce ARPE-19 cells. Luminescence data clearly demonstrated that the viral CMV promoter provides the strongest activity in ARPE-19 cells among the six promoters tested (Fig. 1b). The LV dose was adjusted by p24/capsid levels, but importantly, the actual vector copy number (VCN) that integrated into the ARPE-19 cells post transduction was measured by qPCR and used to adjust expression level data as measured by luminescence to correct for minor titer differences among the LVs. Supplementary Information (SI) Fig. 1a-c provides an overview of LVs and the experimental protocol.

To engineer ARPE-19 cells to stably secrete hGH we turned to transposon-based delivery, more specifically the PiggyBac-based vector technology. We built a series of vectors (Fig. 1c), that encode either a hormone or a reporter gene (eGFP) in conjunction with the Puromycin resistance gene as a bicistronic transcript driven by the CMV promoter. We included the mEPO gene to have an independent validation of our engineering strategy for secretory proteins. Using the X-tremeGENE 9 transfection reagent we obtained high transfection efficiency of ARPE-19 cells (>50%, see SI Fig. 2). Furthermore, we confirmed the potency of the CMV promoter in the context of our PB-based vectors. As seen in Fig. 1d the strong activity of the CMV promoter observed in the transduced ARPE-19 cells was replicated in transiently transfected ARPE-19 cells, where a ~10 fold higher activity of the CMV promoter relative to EF1α was observed. We also constructed two additional variants in which we included the 1.5 kb insulator fragments ubiquitous chromatin opening element (UCOE) (Skipper et al., 2019) in either 5′ or 3′ orientation upstream of the CMV promoter (SI Fig. 3a). The UCOE elements may potentially prolong transgene expression by protecting the promoter from epigenetic silencing and shielding the integrated vector from heterochromatization (Williams et al., 2005). We established that the intrinsic promotor activity was not compromised by the inclusion of the isolator elements (SI Figs. 3b and 3c).

Codon-optimization (CO) may in some cases offer enhanced transgene expression levels by matching tRNA profiles and/or altering primary sequences or secondary structures to improve transcriptional or translational efficacy without changing the amino acid sequence. Using the proprietary algorithm OptimumGene offered at Genscript (Piscataway, NJ, USA) we tested a CO version of the gene encoding hGH with 134 silent substitutions (see alignment in SI Fig. 4). However, in this case the CO gene did not improve hGH secretion from ARPE-19 cells to the growth media as deemed from transient transfection experiments (Fig. 1e). Rather, we observed a slight decrease in hGH levels from the
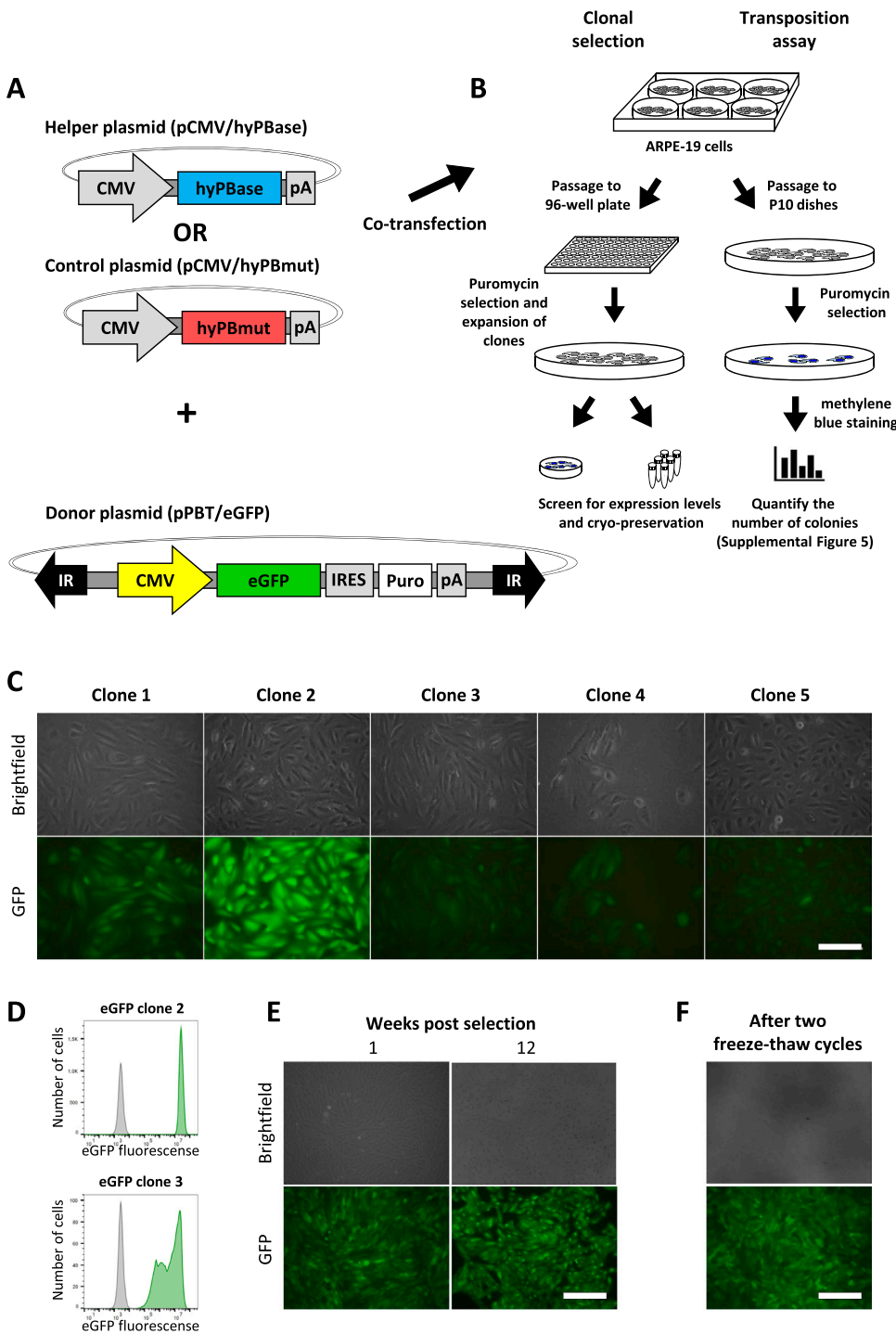
Fig. 2. High and robust transgene expression PiggyBac-engineered ARPE-19 cells. a) Diagram of the helper plasmid encoding a hyperactive version of the PiggyBac transposase (hyPBase) and the inactivated transposase control (hyPBmut) used in the transposition assay, as well as a diagram of the bicistronic eGFP-IRES-Puro reporter-encoding donor vector (pPBT/eGFP) used to validate our strategy. **b)** Outline of the experimental procedure, in which helper (or control) and donor plasmid DNA were co-transfected into target ARPE-19 cells before selection and isolation of Puromycin resistant clones. Briefly, ARPE-19 cells were seeded in a 6-well dish and co-transfected with pPBT/eGFP and the hyPBase. For isolation and screening of individual PB-engineered clones (clonal selection, diagram on left hand side), the cells were diluted and transferred to 96-well plate the following day, and selected for Puromycin resistance for two weeks after which surviving clones were isolated and expanded. Next, individual clones were screened for expression levels and cryopreserved. For validation of transfer efficacy, we performed a transposition assay (diagram on right hand side). Here, cells were transferred to P10 dishes with different dilutions the next day and selected for Puromycin resistance for two weeks before surviving clones were isolated and expanded. The clones were submitted to methylene blue staining for quantification of number of colonies (SI Fig 5). **c)** Images of five PiggyBac-engineered ARPE-19 clonal cell lines stably expressing eGFP. Pictures were taken 8 weeks after transfection, corresponding to 6 weeks after the initial Puromycin selection. Top row: Bright field, bottom row: eGFP. Scale bar: 250 μm. **d)** Flow cytometry analysis of eGFP expression in eGFP clone 2 and eGFP clone 3 cells. Upper panel (clone 2) shows a pure population with extremely high eGFP expression, while the lower panel (clone 3) reveals a bimodal pattern indicating a mixed population of cells with intermediate and high eGFP expression. The x-axis shows the relative eGFP expression levels, and the Y-axis cell count. Grey peaks: control (untransfected) ARPE-19 cells. Green peaks: Isolated ARPE-19 clones (2 and 3) after hyPB transposon-mediated integration of the CMV-eGFP-IRES-Puro expression cassette. **e)** Stable expression of eGFP (lower panel) from ARPE-19 clone 2 following one or twelve weeks of growth after the initial Puromycin selection and clone expansion. The upper panel shows the corresponding bright field images. Scale bar: 1000 μm. **f)** Stable expression of eGFP from ARPE-19 clone 2 after two freeze-thaw cycles, as compared to the reference images in panel E. Scale bar: 1000 μm. Abbreviations: CMV, Cytomegalovirus promoter; eGFP, enhanced Green Fluorescent Protein gene (cDNA); hyPBase, hyperactive PiggyBac transposase; hyPBmut, mutated (non-functional) PiggyBac transposase; IR, inverted repeat; IRES, Internal Ribosomal Entry Site; pA, polyadenylation signal; Puro, Puromycin resistance gene.

transposition assay replicas, our experimental parameters and a dilution factor of 10 yields approximately 100 Puromycin resistant colonies per p10 dish (SI Figure 5a). Increasing the dilution factor results in fewer colonies, which is favorable in terms of minimizing the risk of picking non-clonal colonies. Comparison of hyPBase and the inactivated hyPBmut at the same dilution factor clearly demonstrates that the vast majority of emerging colonies results from PB-mediated transposition events and only a few percent (1–3 colonies out of >100) are likely the result of aberrant insertions.

Using this experimental procedure, we next isolated and expanded a

handful of eGFP-positive clones for further analysis and validation of transgene activity. As seen by fluorescence microscopy the isolated colonies all express eGFP at clearly detectable levels, while clone 2 produced a particularly high level of expression (Fig. 2c). We quantified the eGFP fluorescence by flow cytometry and indeed found extremely

high eGFP activity in clone 2 with over 5 logs increase in median fluorescent intensity relative to naïve ARPE-19 cells (Fig. 2d, upper panel). Moreover, the well-defined narrow peak in the histogram analysis of clone 2 compared to clone 3 (Fig. 2d, compare upper and lower panel), suggests that the homogenous clone 2 cells are in fact clonal. In contrast,
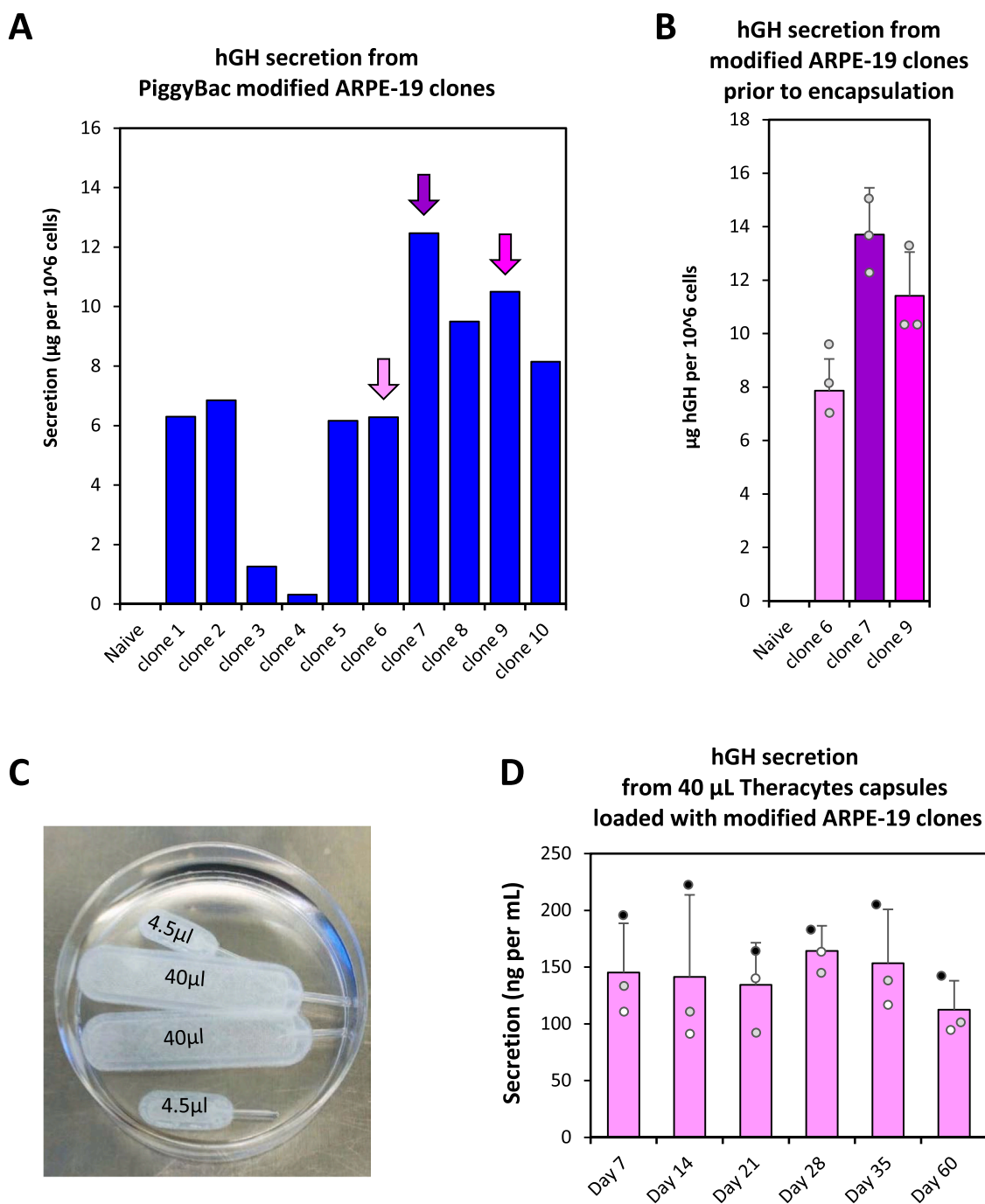
Fig. 3. Sustained hGH expression from TheraCyte-encapsulated ARPE-19 cells. a) Secretion of hGH (measured by ELISA) to media in 10 randomly selected PB-engineered colonies to identify clones with a high expression level. Samples were harvested after 24 h exposure of 10 mL media to a confluent P10 dish, and hGH values were normalized to the number of cells at harvest (single experiment). Naïve ARPE-19 cells served as negative controls. The three clones chosen for further studies are highlighted with color coded arrows. **b)** hGH secretion from the three clonal lines, clone 6, 7 and 9, selected in panel A was quantified once more to establish pre-encapsulation values. Samples were media from confluent P10 dishes harvested after 24 h, and the number cells per dish was counted at sample harvest to normalize expression levels per cell. Values (μg per 10^6 cells per day) are plotted as the mean of three biological replicates plus standard deviations. Individual data points are shown as grey dots. **c)** Picture showing the two sizes of the TheraCyte capsules used in this study, the 4.5 μL and 40 μL type, placed in a 60 mm wide P5 culture dish for illustrative purposes. **d)** hGH secretion from TheraCyte devices as measured by media samples at day 7, 14, 21, 28, 35, and 60 post encapsulations. Three 40 μL TheraCyte capsules were loaded with 3×10^6 clone 6 cells at day zero, and placed in T25 flasks containing 10 mL medium. The media was replaced 24 h prior to harvest. Values are reported as ng/mL per capsule (mean of three biological replicates plus standard deviations) as the actual number of encapsulated and live cells are unknown. Individual data points are shown as dots (capsule 1, white; capsule 2, grey; capsule 3, black).

the clear bimodal histogram pattern in clone 3 clearly indicates a mixed population of cells, most likely consisting of two distinct clones (Fig. 2d, lower panel).

Using the highly eGFP positive clone 2 cells as a model for transgene expression in PB-engineered ARPE-19 cells, we followed the persistence of expression over time. First, we compared clone 2 cells expanded from the initial cryopreserved stock after growth for either 1 or 12 weeks. As evident from fluorescent microscopy analysis eGFP expression appears persistent following 12 weeks of culture growth (Fig. 2e); a result confirmed by flow cytometry analysis (data not shown). Two additional freeze thaw cycles did also not affect transgene expression notably (Fig. 2f). In conclusion, it is possible to derive clones with a high expression level using PB-mediated transposition and puromycin selection, and the PB-embedded CMV-driven bicistronic expression cassette works very well in the sturdy ARPE-19 cells, and may importantly support long-term and robust transgene expression. While the viral CMV promoter is prone to epigenetic silencing and reduced transgene expression in some cell types such as muscle and Chinese hamster ovary (CHO) cells (Brooks et al., 2004; Osterlehner et al., 2011), our data indicate that is possible to find integration events in ARPE-19 in which epigenetic silencing is not a major concern. In light of these findings, we decided to proceed with the simple 'CMV only' promoter configuration, and not the 3′ or 5′ UCOE-modified versions, even though the intrinsic CMV promotor activity was not reduced by the addition of the 1.5 kb UCOE insulator element (SI Fig. 3a-c).

2.3. Sustained secretion of human growth hormone from TheraCyte devices

Confident that PB-engineered ARPE-19 cells supported high and persistent transgene expression, we next isolated and screened hGH secreting cells using the pPBT/hGH donor vector (Fig. 1c). Eight out of ten clones secreted over 6 µg hGH/10^6 cells/day (Fig. 3a). We achieved over 10-fold higher expression with PB-mediated delivery compared to a similar experiment in which we delivered a CMV-driven hGH gene in a Sleeping Beauty-based transposon vector (SI Figure 6a-c). We confirmed the potential of our expression cassette and PB-mediated delivery for efficient and robust engineering ARPE-19 cells with high transgene expression, by screening a handful of clones after pPBT/mEPO transposition for mEPO secretion, which, despite clonal variation, yielded with a high level of expression (SI Figure 5c). We selected three hGH clones with a high expression level for further analysis and encapsulation experiments, as marked by arrows in Fig. 3a. First, we repeated the expression profiles for each of the selected clones after expanding the clones from a cryopreserved sample (Fig. 3b). For the top-three clones, clone 6, 7, and 9, the assay was repeated three times to ensure reliable and highly robust hGH expression. Repeatedly, we observed over 10 µg hGH/10^6 cells/day for hGH-clone 7 and 9 (Fig. 3b). Next, we examined the vector copy number (VCN) by Southern blot analysis (SI Figure 7a). Based on the presence of unique bands in *Sac*I-digested gDNA we estimate that clone 6, 7, and 9 carry 15, 3, or 6 vector integration events, respectively. In the eGFP-clone 2 and 5 we found 21 and 1 vector integration events, respectively, the former being an example of so-called hyper-transposition.

Using the top-three hGH-secreting ARPE-19 clones (clone 6, 7 and 9), we then moved on to encapsulation experiments. We utilized the large 40 µL TheraCyte devices as shown in Fig. 3c. We injected approx. 3×10^6 clone 6 cells per device and submerged the capsule in a T25 flask containing 10 mL medium. Secretion of hGH was monitored by ELISA assays of the media surrounding capsule and followed over time. As evident from the continuous hGH secretion plotted in Fig. 3d, engineered ARPE-19 cells endured encapsulation in TheraCyte 40 µL devices and maintained a high and steady hormone production, amounting up to ~150 ng/mL, for up to 2 months *in vitro*. Importantly, we did not observe leakage of cells from the capsules during the two months in culture as demonstrated by a sensitive colony stain protocol (data not shown). Moreover, as evident by the continuous hGH release, the ARPE-19 cells were metabolically active. However, we did observe a decrease in viability following the two months of encapsulation, as measured by ATP level per cell in clone 6 cells retrieved by opening and trypsinization of the device (SI Figure 8c). We also tested bioactivity of the hGH produced and secreted to the media by our engineered ARPE-19 clones in culture, and hGH released from loaded Theracyte devices. As shown for clone 6, we see very potent bioactivity of hGH in media samples from capsules as measured by the Ba/F3-hGHR bioassay (Ikeda et al., 2000) (SI Fig. 7c).

Using the top-two hGH-secreting ARPE-19 clones (clone 7 and 9), we did a second long term *in vitro* encapsulation experiments with two 40 µL TheraCyte devises (before using the same capsules for *in vivo* studies). We injected approx. 1.5×10^7 cells per device and followed secretion of hGH over time with ELISA assays. Once more we found that our engineered ARPE-19 clones endured encapsulation and released high hGH amounts for 2 months, amounting up to 800 ng/mL (SI Figure 8a). For the smaller device (4.5 µL capsule), *in vitro* hGH secretion was up to approximately 100 ng/mL, and hGH secretion levels were measured weekly until day 35 (SI Figure 8b).

### 2.4. Limited in vivo secretion of hGH from implanted capsules

Encouraged by the high and continuous secretion levels measured from both 40 µL TheraCyte devices used in the second *in vitro* culture experiments (SI Figure 8a), we decided to proceed to an *in vivo* pilot test. We surgically implanted the capsules subcutaneously on the back of adult SCID mice as described previously (Kumagai-Braesch et al., 2013) and included a control mouse that did not have any capsule implanted. We monitored secretion of hGH in the animals by drawing blood samples at day 3 and 10 post surgery and followed their weight and behavior. As seen in Table 1, we confirmed the presence of exogenous hGH in the blood of both treated animals. However, the levels were low, in the range of 0.1–0.2 ng/mL, which is far from endogenous production of 8.3 ± 6.5 ng/mL GH in normal C57BL/6 J mice (Cecchi et al., 2014). The feeding behavior and growth parameters of the capsule-implanted mice and the control did not appear different (see Fig. 4a for body-weight), albeit the small number of animals used in this pilot test does not allow statistical assessment. Importantly, the treated mice seemed to thrive and maintain a normal 'curious' behavior (Fig. 4b). However, due to the limited hGH secretion, we terminated the experiment after 13 days. Having obtained proof that TheraCyte devices loaded with two distinct hGH-producing ARPE-19 clones at least to some extent result in systemic delivery of hGH when implanted in the back of mice, we next sought to test the applicability of our strategy in a more sensitive mouse model. For this purpose, we selected the GH-deficient lit mouse model which we have previously used at IPEN-CNEN (Higuti et al., 2016; Oliveira et al., 2010). As shown in Fig. 4c the lit/SCID mice are considerably smaller than the normal SCID animals, and adults reach a bodyweight of ~9 g, which is approx. 2.8-fold less than normal mice (Higuti et al., 2016). For these animals we chose the smaller 4.5 µL

Table 1
Human growth hormone (hGH) measurements (ng/mL) after capsule implant in SCID mice (day 3 and day10) and lit/SCID (end of the experiment - day 23).

Murine model and treatment	Number of mice (n)	Day 3	Day 10	End of experiment
SCID mice				Day 13
Control	1	< 0.001	< 0.001	NA
hGH-clone 7	1	0.21	0.23	NA
hGH-clone 9	1	0.15	0.06	NA
lit/SCID mice				Day 23
Control	4	NA	NA	< 0.001
hGH-clone 7	3	NA	NA	< 0.001

NA, represents not measured samples. < 0.001 values represent undetectable levels of hGH in the sample.

A

40 µl capsules in SCID mice

B

C

lit/SCID mice   SCID mice

D

4.5 µl capsules in lit/SCID mice
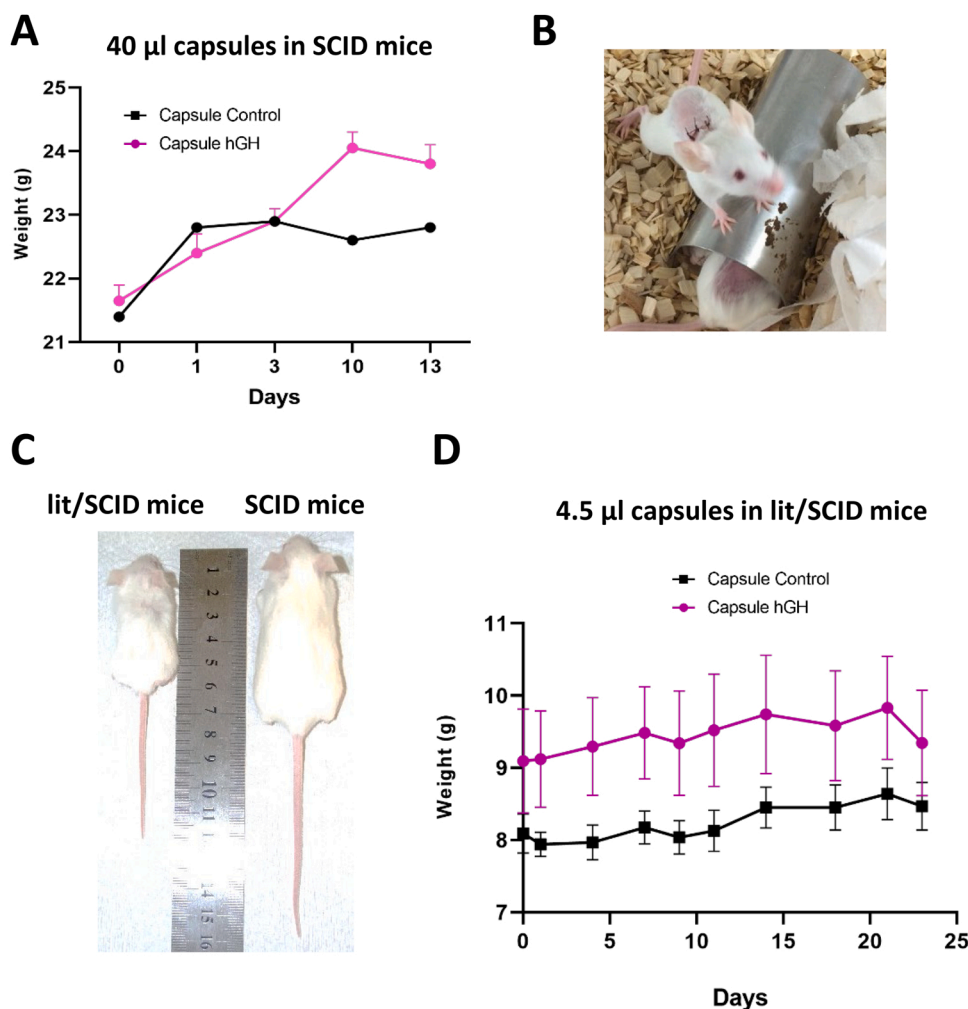
Fig. 4. Limited weight gain in mice implanted with hGH-secreting capsules. a) Weight on day 0, 1, 3, 10, and 13 of SCID mice implanted with a single 40 µL capsule loaded with PiggyBac-engineered ARPE-19 hGH cells (n = 2, shown in pink). The capsules are the same as the ones used *in vitro* in SI Figure 8a, and each of the 40 µL TheraCyte devices were surgically implanted immediately after the day 60 sample time point. The control mouse (n = 1, shown in black) did not receive any capsule. b) Picture of the SCID mouse implanted with a single 40 µL TheraCyte device on its back. c) Picture of the two mouse models used in this study, placed on either side of a ruler (cm). On the left hand side is an adult lit/SCID mouse, a growth hormone deficient mouse model (Deitel et al., 2002), and on the right hand side is a normal adult SCID mouse, for comparison. d) The weight of lit/SCID mice implanted with a single 4.5 µL capsule loaded with ARPE-19 cells (n = 3, shown in purple) at day zero and followed for 23 days. The control group (n = 4, shown in black) were implanted with capsules loaded with naïve ARPE-19 cells.

TheraCyte devices. Each capsule was loaded with 1×10^6 cells, either a PB-engineered hGH-secreting clone or unmodified ARPE-19 control cells. Three to four 90 days-old mice of mixed gender were included in each group, and a single 4.5 µL capsule was surgically implanted on the back of each mouse. The body weight was measured every week. As seen in Fig. 4d most of the treated and control mice gained weight for the first ~21 days following capsule implantation. Encouragingly, we note a tendency for the hGH-treated mice to gain weight slightly faster than controls during the first three weeks. This difference was translated into a weight variation correlation curve until day 23, and the following values were found: hGH: $Y = 0.031X + 0.063$ (r = 0.914; n = 8; P < 0.01) and Control: $Y = 0.035X - 0.245$ (r = 0.941; n = 8; P < 0.001). No significant difference between hGH and control (unmodified cells) curves were found. A 2-way ANOVA followed by Dunnett's multiple comparison test days x treatment showed a significant difference in weight increase - day 1 *vs.* day 21 (P < 0.0022) for the hGH capsules group. No difference was observed within the control group during the entire experiment. However, on day 23, two mice deviated negatively in the group implanted with the hGH capsules and a significant decrease in body weight was seen compared to first day of the experiment (P < 0.0491). We noticed that the cannula of the devices was exiting the skin of the mice in the hGH group, causing severe tissue inflammation and discomfort. Due to the weight loss of the animals and skin wound, we decided to terminate the experiment. The animals in the control group did not present any problem, showing the capsules and the suture were good. However, the limited number of mice in this study does not allow any solid conclusions to be drawn. Due to the small size of

lit/SCID mice it was not possible to draw blood samples at multiple time points during the experiment. Hence, we were able to assess hGH levels in the blood only after terminating the experiment where we did not detect any systemic delivery of hGH in either of the three animals treated with hGH-secreting capsules, translating into a level of exogenous hGH below 0.001 ng/mL, which is the detection limit of the assay. Based on the pilot test in SCID mice, we suspect that the capsules likely released hGH to the lit/SCID mice early in the experiment, but based on our experience these values never exceeded ~0.25 ng/mL for more than 10 days, as we did not observe any alleviation of GH deficiencies in terms of growth.

3. Discussion

Here we report that engineering ARPE-19 cells using PiggyBac-mediated transposition is a simple, safe, and efficient way to establish a hormone secreting cell line compatible with encapsulation. The CMV promoter has the capacity of promoting strong and robust transgene expression, and PB-engineered ARPE-19 cells sustain high levels of hGH secretion encapsulated within TheraCyte devices *in vitro*. We obtained hundreds of individual clones from a single transfection experiment, of which the vast majority stem from transposase-dependent integrations. The expression levels of hGH and mEPO varied from clone to clone, but was generally very high. This was also evident from the fluorescence activity in clones carrying the eGFP reporter.

For hGH we achieved secretion levels of 6–12 µg hGH/10^6 cells/day for 8 out of 10 clones. This result was similar to the 7–11 µg mGH/10^6

cells/day achieved with a retroviral vector (pLXSN) for transduced human keratinocytes with murine growth hormone (mGH) gene using a LTR promoter (Bellini et al., 2003; Peroni et al., 2006). However, the safety of using a non-viral system makes the use of a PB vector an advantage compared to retroviral vectors. To our knowledge, we are the first group to describe the use of a hyPBase for delivering hGH secreted by ARPE-19 cells. Our data also indicates that PB-engineered ARPE-19 clones seem to express higher transgene levels as compared to SB-generated clones (10 of 10 randomly selected SB clones were less than 0.4 μg hGH/10^6 cells/day). While this observation does not warrant any firm conclusion to be drawn, we believe that PB vectors are a solid choice for engineering of ARPE-19 clones with a high level of expression.

We observed a very robust and persistent expression by the CMV-driven cassette in our PB vectors. Neither freeze-thaw cycles nor prolonged *in vitro* growth seemed to affect expression from our eGFP reporter clone, and we also observed steady hGH secretion from TheraCyte capsules 60 days after loading high-expressing clones. This also suggest that ARPE-19 cells are not particularly sensitive to promoter silencing, at least for the CMV promoter, although this will depend on the actual site (s) of vector integration. Our data suggest that the 1.5 kb UCOE element may be placed upstream of the CMV promoter without compromising transcriptional activity in ARPE-19 cells. This epigenetic insulator element (Skipper et al., 2019) may thus be included to shield the CMV promoter from silencing. In the case of ARPE-19 cells we did not deem this to be the needed. However, for other cells lines or applications it may be beneficial to include this insulator element to prevent or reduce epigenetic silencing.

Our results emphasize that transgene expression, as expected, varies substantially between clones, and importantly, it is possible to identify and pick 'super expressors' if a sufficient number of clones are screened. This is clearly demonstrated by the extreme fluorescence seen in eGFP clone 2. Our Southern blot data demonstrate the occurrence of so-called hyper-transpositions events, in which multiple vector copies are inserted by the hyPBase (Skipper et al., 2018). In particular in eGFP-clone 2 we find an example of hyper-transposition, which may explain the extremely high eGFP expression displayed by this particular clone. However, transgene expression does not necessarily correlate with VCN, as evident from our hGH-secreting clones. PB-mediated transposition follows a more or less random integration profile into chromosomal TTAA-site. This contrasts retro- or lentiviral transduction which favors insertion into regulatory elements of active genes and transcription units, respectively (Hudecek et al., 2017; Cavazza et al., 2013). Random integration into transcriptionally favorable chromosomal regions is clearly a major determinant of transgene activity. It appears that the expression level depends on both the specific integration site and the VCN, with the former playing the most significant role. We further highlight that selected clones should be carefully selected and characterized by Southern blot analysis or a similar method to establish clonality, as outgrowth of less abundant clones in mixed cultures may compromise transgene expression levels over time. The flow cytometry analysis of eGFP-clone 3 exemplifies expression heterogeneity and likely represents a non-clonal population.

Importantly, our study demonstrates that ARPE-19 cells are sturdy and compatible with encapsulations. Naïve ARPE-19 cells can be maintained and survive in culture for more than 12 weeks without splitting, showing a superconfluent, adherent state. ARPE-19 cells have been proven to have the ability to form tight junctions and also demonstrate a high level of resistance in monolayer culture and do not present signals for senescence for several passages (Dunn et al., 1996). We provide evidence that PB-engineered ARPE-19 cells are excellent for encapsulation, and show that clones are viable and survive at least 60 days when loaded into a 40 μL TheraCyte device and maintain high secretion of bioactive hormone. This is to our knowledge the first time ARPE-19 cells have been reported use in TheraCyte device. The use of the TheraCyte demonstrated that this capsule is suitable for loading

ARPE-19 cells, and allowing the devices to be filled at high cellular density without leakage. This is important to obtain therapeutic levels of the recombinant protein (Lathuiliere et al., 2014). In the short-term pilot trial, we demonstrated secretion of hGH into circulation of adult SCID mice. The detected secretion levels were modest, in the range of 0.06–0.23 ng/mL, despite using the large 40 μL TheraCyte device. The experiment was terminated after 10 days to avoid complications and discomfort, but clearly for at least 10 days oxygen and nutrition was supplied to the implant to maintain viable cells. In contrast, we could not detect hGH circulatory levels *in vivo* at the end of the experiment in lit/SCID mice. We were not able to collect blood during the experiment, to assess the secretion of hGH before the termination of the experiment, since the lit mice are too small to withdraw blood and need a long recovery time. A possible explanation for the absence of circulatory hGH levels could be the low viability of cells inside the capsule leading to low perfusion after 23 days. Non-circulating hGH levels could also be due to a lack of vascularization of the implanted device. Some studies have reported on pre-implanting empty TheraCyte capsules up to three months prior to loading the device *in situ*. This procedure may help to induce neovascularization of the capsules prior the cell transplantation, improve endocrine tissue, and reduce the formation of necrotic tissue or cores (Kumagai-Braesch et al., 2013; Sorenby et al., 2008). Formation of necrotic cores is the result of dense clusters of cells, which makes diffusion of oxygen and nutrients to the center of the clusters so difficult that they eventually die (Lathuiliere et al., 2015). Other possible causes could be the inflammatory reaction in the skin caused by the device, that would release cytokines as interleukin-1β, interferon-γ, and tumor necrosis factor, and even without leakage cytokines might penetrate the membrane and be harmful to the ARPE-19 cells inside the device (Barkai et al., 2016). Our study reports high *in vitro* expression levels (6 – 12 μg hGH/10^6 cells/day) using PiggyBac engineered ARPE-19 to secrete hGH, which is approximately 8 times higher than the levels reached by Josephs et al. using a human fibroblasts cell line MSU 1.2 (Josephs et al., 1999). Using MSU 1.2 cells Josephs et al. demonstrated *in vivo* secretion of hGH for six months in nude rats at therapeutically relevant levels, of up to 2.5 ng/mL in plasma. In that study however, four 40 μL TheraCyte devices loaded with 2×10^7 cells were implanted per animal. More studies are needed to improve this gene therapy technique to promote *in vivo* expression levels of hGH and growth of lit/SCID mice implanted with TheraCyte device or alternative macroencapsulation technologies. The main obstacles for the use of TheraCyte devices include the limitation in use at immunoprivileged sites (or in immunodeficient models) and the leakage of yet unknown antigens that can initiate an immune attack (Boettler et al., 2016). This seems in particular to be mediated by CD4^{+} T cells (Geller et al., 1997). The ability to retrieve the implanted device and halt or adjust the treatment, is a major advantage of the use of ECT, and provides a strong motivation for pursuing more pre-clinical experiments. Potential clinical applications evidently warrant close examination of durability of the ECT device and safety in form of containing allograft or immortalized cells within the capsule. This is also important when considering engineered cell lines and potential genotoxicity as a result hereof (Micklethwaite et al., 2021). However, studies from animal models suggest that TheraCyte devices are well tolerated and not leaking (Kirk et al., 2014; Lee et al., 2009).

In this study, we report that vectors encompassing the CMV promoter yields high and persistent transgene expression ARPE-19, and clonal cells lines appear resilient to epigenetic silencing. We find that PiggyBac-based transposition is an efficient way to engineer clonal ARPE-19 cells lines with high and long-term secretory levels of human growth hormone. Importantly, we demonstrate that ARPE-19 cells are sturdy and compatible with long-term encapsulation in TheraCyte devices, and that capsules release stable, high levels of hGH for over eight weeks *in vitro*. Pilot experiment of hGH secreting capsules in immunodeficient SCID mice revealed low systemic release, and warrant further investigations for successful application of ECT *in vivo*.

4. Materials and methods

4.1. Construction of PiggyBac-based transposon vectors

The PiggyBac (PB) transposon-based vector containing the multiple cloning site (MCS), pPBT/CMV-MCS-IRES-Puro (Sharma et al., 2012), was used as recipient vector for expression of eGFP, hGH, hGH-CO (codon optimized), and mEPO (murine erythropoietin) genes. The pPBT/CMV-MCS-IRES-Puro vector was kindly donated by Professor Jacob Giehm Mikkelsen (Department of Biomedicine, Aarhus University). Forward and reverse primers tagged with restriction enzyme sites were used to amplify the specific genes by PCR. PCR templates were obtained from pT2/CMV-eGFP.SV40neo plasmids for eGFP (Sharma et al., 2012), pT2/CMV-hGH.SV40neo for hGH, and pT2/CMV-mEPO. SV40neo for mEPO genes (Aagaard, L, unpublished), and pUC57-hGH-CO (purchased at GenScript, Piscataway, NJ, USA) served as template for amplification of the codon optimized version of hGH. The codon-optimized version of hGH presented an improvement of the codon adaptation index from 0.81 to 0.94 and the percentage of CG from 55.69 to 56.30. The Sleeping Beauty (SB) transposon-based vector pT2/CMV/eGFP.SV40neo was also kindly donated by Professor Jacob Giehm Mikkelsen. PCR was performed using the high fidelity polymerase Phusion (ThermoFisher Scientific, Roskilde, Denmark), and isolated amplicons were digested with restriction enzymes *Age*I and *Bsr*GI or *Bsi*WI (ThermoFisher Scientific) before cloning into the multiple cloning site (MCS) of pPBT/CMV-MCS-IRES-Puro using the T4 DNA ligase (ThermoFisher Scientific) as indicated in Fig. 1c. Vector DNA was purified from transformed bacteria clones using Maxi-prep kit (Qiagen, Copenhagen, Denmark), followed by restriction analysis with *Apa*LI (ThermoFisher Scientific) and 1% agarose gel electrophoresis to confirm overall plasmid integrity, and all PCR inserts were verified by Sanger sequencing at GATC Biotech (MWG/Eurofins, Ebersberg, Germany). A list of primers are found in Table 2.

4.2. Construction of lentiviral vectors

A series of pCCL-based Firefly luciferase (Fluc) vectors with different promoters was prepared as follows: pCCL-PGK-Fluc (Dull et al., 1998) was digested with *Cla*I and *Bam*HI (ThermoFisher Scientific) to remove the PGK promoter. Inserts holding the viral promoters SV40 and RSV were PCR-amplified using *Cla*I- and *Bam*HI-tagged primers and psi-Check2.2 (Promega, Madison, WI, USA) and pRSV-Rev (Dull et al., 1998) template DNA, respectively. Similarly, the EF1α and UbiC promoters were cloned into *Cla*I/*Bam*HI of pCCL-PGK-Fluc as PCR amplicons prepared from pUC57-miRvector (purchased at GenScript, Piscataway, NJ, USA) and pUC-Ubi-hGH (Cecchi et al., 2014) template DNA, respectively. Construction of pCCL-CMV-Fluc has been described earlier (Jakobsen et al., 2015).

4.3. Cell culturing and Puromycin kill-curve experiment

All cell lines were kept at 37 °C and 5 V/V% CO_2. HEK293 (catalog no. CRL-1573; American Type Culture Collection (ATCC), Manassas, VA, USA), and HEK293T (catalog no. CRL-11268, ATCC), were kept in Dulbecco's Modified Eagles Medium (DMEM). ARPE19 cells (catalog no. 2302; ATCC) were kept in 50/50% DMEM/ F12 media (RPE medium). All media were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. DMEM media, F12 media, and supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA).

To establish a lethal dose of Puromycin in ARPE-19 cells, cells were seeded in a 6 well dish (1.5×10^5 cells/well). A stock concentration of 10 mg/mL of Puromycin (Sigma-Aldrich, Milwaukee, WI, USA) was prepared. RPE media containing the different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 µg/mL) of Puromycin were added to the ARPE-19 cells. Doses from 0.1 to 0.5 µg/mL were tested in comparison with a negative

Table 2
List of primers used in this study. All primers were purchased from MWG/Eurofins (Ebersberg, Germany) or TAG Copenhagen (Frederiksberg, Denmark).

Name	Sequence (5′–3′)	Purpose (flanking restriction site)
mEPO-F	GCGCGACCGGTGCCACCATTGGGGTGCCCGAACGTCCC	Cloning (*Age*I)
mEPO-R	GCGCGCGTACGTCACCTGTCCCCTCTCCTG	Cloning (*Bsi*WI)
eGFP-F	GCGCGACCGGTGCCACCATGGTGAGCAAGGGCGAGG	Cloning (*Age*I)
eGFP-R	GCGCGCGTACGTTACTTGTACAGCTCGTCCATGCC	Cloning (*Bsi*WI)
hGH-F	GCGCGACCGGTGCCACCATGGCTACAGGCTCCCGGACG	Cloning (*Age*I)
hGH-R	CGGCGTGTACACTAGAAGCCACAGCTGCCCTCC	Cloning (BsrGI)
EF1a-F	GAGGGAATCGATTAGAGCCTCCCCGTCACCAC	Cloning (*Cla*I)
EF1a-R	GCGCGCTCGAGGAACGTTCACGGCGACTACTGC	Cloning (*Xho*I)
UCOE-F	CGCGCATCGATTCGTCTCCGGCCCTCCGCGCCTAC	Cloning (*Cla*I)
UCOE-R	CGCGCATCGATTGAGACGCCGTGGCCCCCGAAGC	Cloning (*Cla*I)
pPBT-F-seq	TATTGTTATTTCATGTTCTAC	Sequencing
CAG-F-seq	CTCCGAAAGTTTCCTTTTATG	Sequencing
eGFP-R-seq	CGTCCAGCTCGACCAGGATGG	Sequencing
IRES-R-seq	CCTAGGAATGCTCGTCAAGA	Sequencing
CMV-F-seq	GGAGGTCTATATAAGCAGAG	Sequencing
CMV-F-seq	CGTCAATGACGGTAAATGGC	Sequencing
CMV-F-seq	GCAAATGGGCGGTAGGCGTGTAC	Sequencing
KS55	CCCTAGAAAGATAGTCTGCGT	Sequencing
KS56	TACTATGGGAACATACGTC	Sequencing
KS175	TCTGGAGGATTCCACACC	Sequencing

control without Puromycin in the media. The RPE medium containing the Puromycin was replaced every 2–3 days. After 2 weeks the cells were visualized in a brightfield microscope and pictures were taken to analyze the cell death (SI Figure 5c). A dose of 0.3 µg/mL was chosen as selection scheme for isolation of Puromycin resistant ARPE-19 cells.

4.4. Assaying promoter strength in LV transduced ARPE-19 cells

HEK293T cells were seeded in 15-cm dishes (1×10^7 cells/dish) with 20 mL DMEM medium for transient lentivirus (LV) production. Twenty-four hours later, the cells were transfected with 7.3 mg pRSV-Rev, 9 mg pMD 0.2 G, 31.5 mg pMDGP-Lg/RRE, and 31.5 mg of the lentiviral transfer plasmid (encoding Fluc driven by the different viral and non-viral promoter, see SI Figs. 1a and 1b) using the calcium phosphate co-precipitation method as previously described (Chen and Okayama, 1987). In brief, the DNA was diluted with double distilled water (ddH2O) to a final volume of 1.089 mL. 121 µL 2.5 M $CaCl_2$ was added to the DNA solution. 1.212 mL 2X HEPES buffer was pipetted into a 15-mL tube, and the DNA-$CaCl_2$ solution was quickly added and vortexed thoroughly. Following incubation for 15 min at room temperature, the solution was added to the cells in a drop-wise manner and distributed by gently swirling. The medium was replaced the day after. The

virion-containing media was harvested 48 hrs post-transfection and filtered through a 0.45-μm filter (Sarstedt, Nümbrecht, Germany) to remove cell debris. The vector concentration was estimated in the resulting virus preparation by HIV-1 gag p24 antigen ELISA kit (ZeptoMetrix, Buffalo, NY, USA) according to manufacturer's protocol. The virus was stored at $-80°C$ in aliquots.

Vector titers were determined by quantitative polymerase chain reaction (qPCR) essentially as previously described (Barde et al., 2010). In short, 5×10^4 cells (HEK293) were seeded in 12-well dishes (Sarstedt, Nürnbrecht, Germany), and the next day, cells were transduced with 160 ng p24 units of the viral vectors per well using media supplemented with 8 μg/mL polybrene (Sigma-Aldrich). Twenty-four hours post transduction, the cultivation medium was replaced. Three days post transduction, cells were washed in phosphate-buffered saline (PBS) and harvested by trypsinization, while 1/10 were reseeded for evaluation of Fluc activity (see below). Purification of genomic DNA (gDNA) was performed using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. qPCR was carried out on the gDNA samples using two sets of primers and probes: one set for a LV sequence (WPRE) and another set for a single-copy human reference gene (Albumin). Estimation of the infectious titer was performed using serial dilutions of a plasmid containing the human albumin amplicon as well as the WPRE sequence (pCCL+Alb, Aagaard, L, unpublished) for generation of a standard curve. The first point of the standard curve contained 1×10^7 molecules in 8 μL. The standard curve was generated by serial 10-fold dilutions until 100 molecules in 8 μL (in total six dilutions). PCR conditions and primer sequences are available upon request. For evaluation of Fluc expression, 1/10 of the transduced HEK293 cells were reseeded in a white 96 well plate, and the Fluc activity was measured four days post transduction using the Fluc reagent from the Dual-Glo Luciferase Assay System (Promega Madison, WI, USA) according to the manufacturer's instructions.

ARPE-19 cells were seeded directly in the white 96 well plate at a density of 2000 cells/well and transduced the following day using a dose of 6.5 ng p24 of viral vectors. Media was replaced the next day and Fluc activity was measured four days post transduction, and the luminescence values were adjusted to the HEK293 estimated titers to normalize the promotor activity to the vector copy number. Fluc activity was measured on a plate reading luminometer (MicroLuminat plus LB 96 V, Berthold Technologies, Bad Wild-bad, Germany).

4.5. Assaying promoter strength in transiently transfected ARPE-19 cells

ARPE-19 cells were seeded in a 24 well dish (3.2×10^4 cells/well) and transfected with pPBT/CMV-eGFP-IRES-Puro, pPBT/3'UCOE-CMV-eGFP-IRES-Puro, pPBT/5'UCOE-CMV-eGFP-IRES-Puro, or pPBT/EF1α-eGFP-IRES-Puro using 1 μg DNA and a ratio of 1:3 (DNA:transfection reagent) X-tremeGENE 9 (Sigma-Aldrich, Milwaukee, WI, USA). The cells were analyzed two days post transfection using flow cytometry (see below) and promoter efficiency was estimated by measuring the median fluorescence intensity.

4.6. Transfection assay with a codon optimized version of hGH

HEK293 or ARPE-19 cells were seeded in triplicates in a 24 well dish (3.2×10^4 cells/well) and next day transfected using X-tremeGENE 9 reagent (Sigma-Aldrich, Milwaukee, WI, USA). A mixture of 1 μg DNA, transfection reagent and serum-free media (SFM) were added to each well in a 1:3 ratio (DNA:transfection reagent). After one day the media was replaced with fresh 500 μL media. After three days, 300 μL media was collected for ELISA assay.

4.7. Isolation of PiggyBac-engineered ARPE-19 clones and transposition assay

ARPE-19 cells were seeded in 6-well plates (1.5×10^5 cells/well),

and the day after co-transfected with the bicistronic vector (like pPBT/CMV-eGFP-IRES-Puro) using 2 μg DNA and the transposase vectors pCMVhypPBase or pCMVhypPBmut at an established ratio of 1:10 (vector:transposase). The cells were transfected using a ratio of 1:3 of DNA:X-tremeGENE 9 transfection reagent (Sigma-Aldrich, Milwaukee, WI, USA) in duplicates according to the manufacturer's instructions. On day three, the cells were trypsinized and re-seeded into either 96 well plates for isolation of individual clones or in 10-cm dishes at different dilutions (1:10, 1:50, 1:100) for the transposition assay. One day later, the cells were subjected to selection with 0.3 μg/mL Puromycin added to the RPE media for 8 days. To quantify the number of colonies in the transposition assay the plates were stained with methylene blue (Sigma-Aldrich, Milwaukee, WI, USA). The transposition efficiency for the hyperactive transposon hyPBase was calculated according to the total number of colonies stained multiplied by the dilution factor, and compared to inactive hyPBmut transposon. For isolation of clones, Puromycin resistant colonies were trypsinized from confluent 96 well plates and transferred to 24 well plates and finally expanded in 10 cm dishes.

4.8. eGFP fluorescence microscopy and flow cytometry

ARPE-19 clones engineered with the PB-vector encoding eGFP or transiently transfected ARPE-19 cells (see promoter assay above) were visualized and images captured using a Fluorescent Inverted Microscopy Leica DM-IRB/E (Leica Microsystem AS, Brønshøj, Denmark) equipped with a Leica camera (DFC 360 FX). To quantify fluorescence, cells were washed with PBS before being harvested by trypsinization, then washed twice with PBS, fixed with 4% formaldehyde (BHD Prolabo - VWR, Søborg, Denmark) and finally resuspended in PBS. Fixed cells were analyzed for eGFP expression using a NovoCyte 3000 flow cytometer equipped with three lasers (405 nm, 488 nm and 640 nm) and 13 detectors (ACEA Biosciences Inc, Agilent) at the FACS CORE facility (Department of Biomedicine, Aarhus University) and data was acquired and analyzed using NovoExpress software (v. 1.2.1, ACEA Biosciences).

4.9. Quantification of hGH and mEPO secretion by ELISA

1 mL of growth medium (cell-free samples) from ARPE-19 cells secreting hGH or mEPO was collected after 24 hrs of conditioning. The cells were washed with 10 mL PBS and trypsinized, resuspended and homogenized in 10 mL of fresh media. A Neubauer counting chamber (Marienfeld, Germany) was used to count the cells and the collected media was submitted to ELISA to quantify our protein of interest. The hGH ELISA Kit (Life Technologies Europe, Naerum, Denmark) or the mouse Erythropoietin (mEPO) Quantikine ELISA Kit (Biotechne R&D Systems Europe, Abingdom, OX, UK) was utilized according to the manufacturer's specification. The optical density was measured using a BioTek ELX808 microplate reader (Agilent, Vermont, USA) at 450 nm and the results were calculated using a four parameter logistic curve fit (https://www.myassays.com/).

4.10. Southern blot analysis to determine vector copy number

Isolation of genomic DNA (gDNA) from individual ARPE-19 clones was carried out using standard salt-precipitation method. Briefly, gDNA was extracted from a confluent P10 dish with approximately $2.5–3.5 \times 10^6$ cells (harvested by trypsinization, suspended in 1 mL PBS⁻/⁻). 5 μL proteinase K (18 mg/mL) and 500 μL Chorion Villis buffer were added to the cell pellet to lyse the cells. This suspension was incubated for 2 hrs at 55 °C and a solution of 170 μL 6 M NaCl was added to the lysate and centrifuged for 5 min at 13,000 rpm at 4 °C. The supernatant was transferred to a 2 mL tube and placed at $-20°C$ while glass capillary tubes were prepared to capture the DNA strands. The DNA was precipitated with isopropanol (1:1). At the end of the procedure the gDNA was transferred to a new tube containing 50 μL TE

buffer (10 mM, pH 8.5) and stored at 4 °C. For Southern blot analysis 15 µg of gDNA from each clone was digested (3 U/µg DNA) with the FastDigest *Sac*I restriction enzyme (ThermoFisher Scientific, Roskilde, Denmark) for 5 h, before gel electrophoresis (using 0.8% agarose), and transferred to a 15 × 20 cm membrane by vacuum blotting (50 mBar). The membrane was probed with a Puromycin-specific probe after performing three washes using 4 different buffers (Nick's buffer 0.25 M HCl; denaturing buffer 1.5 M NaCl, 0.5 M NaOH; neutralizing buffer 1.5 M NaCl, 1.0 M Tris-HCl, pH 7.6; and buffer SSC 20X). Random labeling of the Puromycin probe was carried out by using the Prime-It-II random primer labeling kit (Agilent Technologies) and [α-32 P] dCTP-labelling (3.000 Ci/mmol) according to the manufacturer's instructions. After pre-hybridization and hybridization overnight at 42 °C, the membrane was washed with SSC buffer. The membrane was exposed to an X-ray film (Konica Minolta, Ballerup, Denmark) in a dark room. After 2 days at − 70°C the film was developed and the copy numbers of each clone was then visualized by the autoradiography.

4.11. In vitro cell encapsulation experiments

The TheraCyte macrocapsules utilized in this work were provided by Baxter Healthcare, Round Lake, Ill., USA (Geller et al., 1997). We have used capsules of volumes of 4.5 µL, or 40 µL, ARPE-19 clones engineered to express high levels of hGH were encapsulated as follows: ARPE-19 clone 7 and 9 were cultivated in T125 flasks and at confluence, the media (conditioned for approx. 24 hrs) was collected for ELISA to measure the *in vitro* productivity. The cells were then trypsinized from the dishes, counted, washed with 15 mL RPE medium and centrifuged for 5 min at 1500 rpm. The media was discarded and 20 µL of medium was added to the cell pellet. A total of approximately 1.5×10^7 cells were injected into the TheraCyte devices of 40 µL, using a Hamilton syringe (Sigma-Aldrich, Milwaukee, WI, USA). The port of the capsule was closed with a Silastic medical adhesive (Downcorning-Biesterfield, Hamburg, Germany) for surgical procedures. The capsule was placed in a T25 flask containing 10 mL RPE medium. The cells were monitored while in cell culture for up to 60 days. During the entire assay the conditioned medium from the T25 flasks (1 out of 10 mL) was collected for measurement of *in vitro* capsule secretion rate and the productivity of the clones was calculated.

4.12. Viability assay

ARPE-19 cells were seeded in 100 µL media in an opaque-walled 96-well plates (1×10^4 cells/well) and cultured for 24 h. CellTiter-Glo® Assay (catalog no.G7570, Promega, Madison, WI, USA) was then perfomed according to the manufactures description. Brifly, 100 µL Cell-Titer-Glo® Reagent was added to each well, and after shaking the plate for 2 min and incubating at room temperature for an additional 10 min, the luminescence was recorded on a Varioskan LUX Multimode Microplate Reader (ThermoFisher Scientific, Roskilde, Denmark).

4.13. Ba/F3-hGHR bioassay

The BAF-hGHR (BAF 3. I. g, Novo Nordisk) cells are an immortalized murine bone marrow-derived pro-B cell line. The BAF-cells were grown in culture medium for 10–11 passages before transfer to starvation medium (medium without GH) at a density of 0.111×10^6 cells/mL, in which it is grown for 24 h at 37 °C, 5% CO2. After 24 h, the cells were centrifuged, the medium removed, and the cells were resuspended in starvation medium to 0.222×10^6cells/mL. 90 µL of the cell supernatant were seeded into microtiter plates (96 well NUNC-clone; catalog no. 156545). Ten µL of hGH-standard in known concentrations or serum samples were added to the cells, and the plates were incubated for 72 h. The proliferation of the BAF-cells were quantified by addition of the redox indicator alamarBlue (4 h incubation), and the number of viable cells were measured in a fluorescence plate reader using an excitation

filter of 544 nM and an emission filter of 590 nM.

4.14. In vivo cell encapsulation experiments

SCID mice were purchased from Taconic Bioscience (Laven, Denmark) and the original mutant strain of CB17-Ghrhr lit/+ Prkdc SCID/Bm (lit/SCID) mice was obtained from The Jackson Laboratory (Bar Harbor, ME, USA) (Deitel et al., 2002) and bred in house in the vivarium at Biotechnology Center – IPEN (University of Sao Paulo, SP, Brazil). These animals were maintained in a specific pathogen free (SPF) room on a vented shelf and used to breed colonies of lit/SCID mice. Males and females of approximately 80–90 days of age were used in the experiments. All experiments were performed in accordance with relevant guidelines and regulations (ARRIVE). The protocol was approved by the Danish Animal Experiments Inspectorate (license number: 2016–15–0201–00947) and in Brazil with the approval of the Ethical Committee for the Use of Animals (CEUA – IPEN) following the standard rules approved by the Brazilian National Council for Control of Animal Experimentation (CONCEA). Two 90-days SCID mice were anesthetized with isoflurane (Forene, Abbott Scandinavia AB, Solna, Sweden), induction at 4% isoflurane in oxygen and maintained at 1.5–2% isoflurane. The hair at the dorsum was removed using a pet shaver machine and afterwards, depilatory cream (Matas, Denmark) was applied to remove the excessive hair. The depilatory cream was removed using a wet cotton tissue. Using a surgical scissor a cut was made in the dorsum of the mice to allow access to the subcutaneous area. The 40 µL capsules were implanted subcutaneously and the skin was sutured. A 90-days old mouse with no capsule implant was included as a non-treated control. After the implant Carprofen (Rimadyl) was administered intraperitoneally (0.05 mg/10 g animal) to reduce postoperative pain and minimize animal suffering (Refinement). In the following 3 days, the analgesic was added to the water bottle in a concentration of 0.1 mL of carprofen in 150 mL water. The mice were maintained for 10 days. The animals were weighed, and at the end of the experiment tail length was measured and the blood was collected for hGH measurements using ELISA (see above). At the end of the experiment, the mice were killed by cervical dislocation and one of the capsules was retrieved in order to assess the viability and hGH secretion of the ARPE-19 cells. The capsule was carefully detached from the subcutaneous tissue and washed in PBS to remove cells and cell debris. The capsule was treated with trypsin 0.25%, and placed in the CO_2 incubator (37 °C) for 3–5 min. Capsule fluids were pipetted into a 10 mL tube with RPE media, centrifuged 5 min at 1500 rpm. The cell pellet was resuspended in a P10 dish containing fresh RPE media. After 1 week in cell culture, the 24 hrs conditioned media was collected for ELISA.

The *in vivo* cell encapsulation procedure was also performed in lit/SCID mice in the laboratory in Brazil. Due to the small size of the mice, 4.5 µL capsules loaded with 1×10^6 ARPE-19 cells were implanted. Three mice were implanted with a device containing an hGH-expressing ARPE-19 clone and four mice were implanted with a device containing untransfected ARPE-19 control cells. After the implant Carprofen (Rimadyl) was administered intraperitoneally and added in the water for analgesia for 3 days. The mice were maintained and weighed at least once a week for up to 23 days. At the end of the experiment, blood was collected for hGH measurement and the mice were killed by cervical dislocation.

4.15. Statistical analyses of the in vivo data

Data are presented as the mean ± SEM. Statistical differences between the weight increase of each group (implanted with capsule containing hGH-expressing ARPE-19 cells or non-transfected ARPE-19 cells) in comparison to the weight at the initial day were analyzed using two-way ANOVA followed by Dunnett's multiple comparison test applying the case "One between-subject variable and one within subject variable", using GraphPad Prism (version 9). In all analyses, a P value

< 0.05 was considered statistically significant.

Ethics approval

The experiments were performed in the animal facility at Department of Biomedicine (Aarhus University, Denmark) for the SCID mice and in the vivarium at Biotechnology Center – IPEN (University of Sao Paulo, SP, Brazil) for the lit/SCID mice, with the approval from the Danish Animal Experiments Inspectorate and the ethical committee for the use of animals (CEUA – IPEN), respectively.

Funding

CRediT authorship contribution statement

C.R.C., L.K. and M.S.C. cloned the vectors, performed cell transfections, colony forming assay, fluorescence microscopy analysis and flow cytometry and isolated individual transposon-modified cell clones. S.A. performed promoter assay studies using lentiviral vectors. C.R.C. performed the promoter efficiency assay in ARPE-19 cells, Southern blot analysis, the ELISA assays, viability assays, and encapsulated and tested the TheraCyte devices *in vitro* and *in vivo*. M.K.B. provided the TheraCyte devices and provided technical support on the encapsulation and engraftment techniques. C.R.C. carried out the murine experiments in Denmark, and C.R.C, G.P.P.J. and E.A.Z. performed the animal experimentation, and the ELISA assays in Brazil. The study was conceived by C.R.C. and L.A. and supervised C.N.P., T.J.C., and L.A. The manuscript was drafted by C.R.C. and L.A. and revised by T.J.C., P.B, S.A., C.N.P. and L.A. All authors read and approved the revised manuscript.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

Data will be made available on request.

Acknowlegdements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tice.2023.102095.

References

Barde, I., Salmon, P., Trono, D., 2010. *Curr. Protoc. Neurosci.* 4, 21.
Barkai, U., Rotem, A., de Vos, P., 2016. World J. Transpl. 6, 69–90.
Bellini, M.H., Peroni, C.N., Bartolini, P., 2003. FASEB J. 17, 2322–2324.
Boettler, T., Schneider, D., Cheng, Y., Kadoya, K., Brandon, E.P., et al., 2016. Cell Transpl. 25, 609–614.
Brauker, J., Martinson, L.A., Young, S.K., Johnson, R.C., 1996. Transplantation 61, 1671–1677.
Brooks, A.R., Harkins, R.N., Wang, P., Qian, H.S., Liu, P., Rubanyi, G.M., 2004. J. Gene Med 6, 395–404.
Cavazza, A., Moiani, A., Mavilio, F., 2013. Hum. Gene Ther. 24, 119–131.
Cecchi, C.R., Higuti, E., Oliveira, N.A., Lima, E.R., Jakobsen, M., et al., 2014. Curr. Gene Ther. 14, 44–51.
Chen, C., Okayama, H., 1987. Mol. Cell Biol. 7, 2745–2752.
Deitel, K., Dantzer, D., Ferguson, P., Pollak, M., Beamer, W., et al., 2002. J. Surg. Oncol. 81, 75–79.
Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., et al., 1998. J. Virol. 72, 8463–8471.
Dunn, K.C., Aotaki-Keen, A.E., Putkey, F.R., Hjelmeland, L.M., 1996. Exp. Eye Res 62, 155–169.
Eyjolfsdottir, H., Eriksdotter, M., Linderoth, B., Lind, G., Juliusson, B., et al., 2016. Alzheimers Res Ther. 8, 30.
Fjord-Larsen, L., Kusk, P., Tornoe, J., Juliusson, B., Torp, M., et al., 2010. Mol. Ther. 18, 2164–2172.
Geller, R.L., Loudovaris, T., Neuenfeldt, S., Johnson, R.C., Brauker, J.H., 1997. Ann. N. Y Acad. Sci. 831, 438–451.
He, X., Barkan, A.L., 2020. Pituitary 23, 294–306.
Higuti, E., Cecchi, C.R., Oliveira, N.A., Lima, E.R., Vieira, D.P., et al., 2016. Growth Horm. IGF Res 26, 1–7.
Hudecek, M., Izsvak, Z., Johnen, S., Renner, M., Thumann, G., Ivics, Z., 2017. Crit. Rev. Biochem Mol. Biol. 52, 355–380.
Ikeda, M., Wada, M., Fujita, Y., Takahashi, S., Maekawa, K., Honjo, M., 2000. Growth Horm. IGF Res. 10, 248–255.
Jakobsen, M., Askou, A.L., Stenderup, K., Rosada, C., Dagnaes-Hansen, F., et al., 2015. Hum. Gene Ther. Methods 26, 123–133.
Josephs, S.F., Loudovaris, T., Dixit, A., Young, S.K., Johnson, R.C., 1999. J. Mol. Med. 77, 211–214.
Kauper, K., McGovern, C., Sherman, S., Heatherton, P., Rapoza, R., et al., 2012. Invest Ophthalmol. Vis. Sci. 53, 7484–7491.
Kirk, K., Hao, E., Lahmy, R., Itkin-Ansari, P., 2014. Stem Cell Res. 12, 807–814.
Kompa, A.R., Greening, D.W., Kong, A.M., McMillan, P.J., Fang, H., et al., 2021. Cardiovasc Res. 117, 918–929.
Kumagai-Braesch, M., Jacobson, S., Mori, H., Jia, X., Takahashi, T., et al., 2013. Cell Transpl. 22, 1137–1146.
Lathuiliere, A., Cosson, S., Lutolf, M.P., Schneider, B.L., Aebischer, P., 2014. Biomaterials 35, 779–791.
Lathuiliere, A., Mach, N., Schneider, B.L., 2015. Int J. Mol. Sci. 16, 10578–10600.
Lee, S.H., Hao, E., Savinov, A.Y., Geron, I., Strongin, A.Y., Itkin-Ansari, P., 2009. Transplantation 87, 983–991.
Malavasi, N.V., Rodrigues, D.B., Chammas, R., Chura-Chambi, R.M., Barbuto, J.A., et al., 2010. Cell Transpl. 19, 269–277.
McKenzie, A.W., Georgiou, H.M., Zhan, Y., Brady, J.L., Lew, A.M., 2001. Cell Transpl. 10, 183–193.
Micklethwaite, K.P., Gowrishankar, K., Gloss, B.S., Li, Z., Street, J.A., et al., 2021. Blood 138, 1391–1405.
Neufeld, T., Ludwig, B., Barkai, U., Weir, G.C., Colton, C.K., et al., 2013. PLoS One 8, e70150.
Oliveira, N.A., Cecchi, C.R., Higuti, E., Oliveira, J.E., Jensen, T.G., et al., 2010. J. Gene Med. 12, 580–585.
Osterlehner, A., Simmeth, S., Gopfert, U., 2011. Biotechnol. Bioeng. 108, 2670–2681.
Peroni, C.N., Cecchi, C.R., Damiani, R., Soares, C.R., Ribela, M.T., et al., 2006. Mol. Biotechnol. 34, 239–245.
Rafael, E., Wernerson, A., Arner, P., Wu, G.S., Tibell, A., 1999. Cell Transpl. 8, 317–326.
Ranke, M.B., Wit, J.M., 2018. Nat. Rev. Endocrinol. 14, 285–300.
Rosa Lima, E., Regina Cecchi, C., Higuti, E., Protasio Pacheco de Jesus, G., Moura Gomes, A., et al., 2020. Molecules 25.
Sharma, N., Hollensen, A.K., Bak, R.O., Staunstrup, N.H., Schroder, L.D., Mikkelsen, J.G., 2012. PLoS One 7, e48421.
Skipper, K.A., Nielsen, M.G., Andersen, S., Ryo, L.B., Bak, R.O., Mikkelsen, J.G., 2018. Mol. Ther. Nucleic Acids 11, 253–262.
Skipper, K.A., Hollensen, A.K., Antoniou, M.N., Mikkelsen, J.G., 2019. BMC Biotechnol. 19, 75.
Sorenby, A.K., Kumagai-Braesch, M., Sharma, A., Hultenby, K.R., Wernerson, A.M., Tibell, A.B., 2008. Transplantation 86, 364–366.
Williams, S., Mustoe, T., Mulcahy, T., Griffiths, M., Simpson, D., et al., 2005. BMC Biotechnol. 5, 17.
Wilson, M.H., Coates, C.J., George Jr., A.L., 2007. Mol. Ther. 15, 139–145.
Woodard, L.E., Wilson, M.H., 2015. Trends Biotechnol. 33, 525–533.

Yakhnenko, I., Wong, W.K., Katkov, I.I., Itkin-Ansari, P., 2012. Cryo Lett. 33, 518–531.

Yusa, K., Zhou, L., Li, M.A., Bradley, A., Craig, N.L., 2011. Proc. Natl. Acad. Sci. USA 108, 1531–1536.

Zhang, T., Qu, H., Li, X., Zhao, B., Zhou, J., et al., 2010. J. Pharm. Sci. 99, 4880–4891.