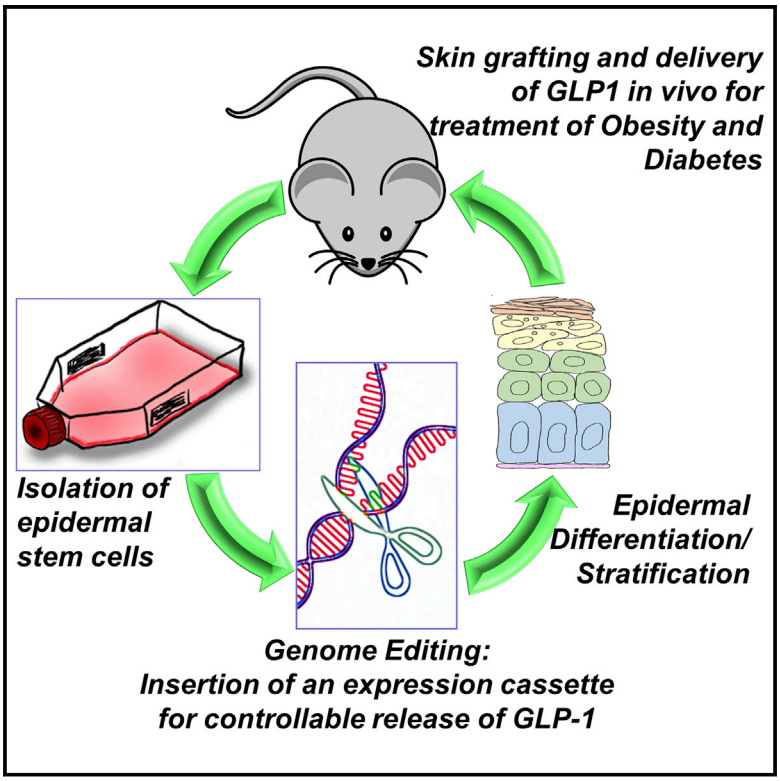
Short Article

Cell Stem Cell

Engineered Epidermal Progenitor Cells Can Correct Diet-Induced Obesity and Diabetes

Graphical Abstract



Highlights

- CRISPR-based editing of epidermal stem cells provides inducible GLP-1 expression
- An immunocompetent transplantation assay allows evaluation of engineered skin
- Graft-derived GLP-1 normalizes blood glucose homeostasis in vivo

Authors

Jiping Yue, Xuewen Gou, Yuanyuan Li, Barton Wicksteed, Xiaoyang Wu

Correspondence

xiaoyangwu@uchicago.edu

In Brief

Wu and colleagues show that engineered epidermal progenitor cells can function as a gene therapy vehicle. Using an immunocompetent skin transplantation model, they show skin grafts that secrete GLP-1 in a controllable manner can normalize body weight and blood glucose levels in diet-induced obese and diabetic mice.

Yue et al., 2017, Cell Stem Cell *21*, 256–263
August 3, 2017
http://dx.doi.org/10.1016/j.stem.2017.06.016

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Engineered Epidermal Progenitor Cells Can Correct Diet-Induced Obesity and Diabetes

Jiping Yue,[1] Xuewen Gou,[1] Yuanyuan Li,[1] Barton Wicksteed,[2] and Xiaoyang Wu[1,3,*]
[1]Ben May Department for Cancer Research, The University of Chicago, Chicago, IL 60637, USA
[2]Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA
[3]Lead Contact
*Correspondence: xiaoyangwu@uchicago.edu
http://dx.doi.org/10.1016/j.stem.2017.06.016

SUMMARY

Somatic gene therapy is a promising approach for treating otherwise terminal or debilitating diseases. The human skin is a promising conduit for genetic engineering, as it is the largest and most accessible organ, epidermal autografts and tissue-engineered skin equivalents have been successfully deployed in clinical applications, and skin epidermal stem/progenitor cells for generating such grafts are easy to obtain and expand in vitro. Here, we develop skin grafts from mouse and human epidermal progenitors that were engineered by CRISPR-mediated genome editing to controllably release GLP-1 (glucagon-like peptide 1), a critical incretin that regulates blood glucose homeostasis. GLP-1 induction from engineered mouse cells grafted onto immunocompetent hosts increased insulin secretion and reversed high-fat-diet-induced weight gain and insulin resistance. Taken together, these results highlight the clinical potential of developing long-lasting, safe, and versatile gene therapy approaches based on engineering epidermal progenitor cells.

INTRODUCTION

Obesity and diabetes have become a major healthcare issue worldwide (Ahima, 2011; Ashcroft and Rorsman, 2012). Diabetes is characterized by increased blood glucose levels as a consequence of insufficient insulin to maintain homeostatic levels of glucose. The hormone GLP1 is a major physiological incretin that controls homeostasis of blood glucose by stimulation of glucose-dependent insulin secretion, inhibition of glucagon secretion, delay of gastric emptying, and protection of islet beta cell mass (Sandoval and D'Alessio, 2015). However, native GLP1 must be delivered through a parenteral route to achieve its effect, and it has an extremely short circulating half-life. Thus, somatic gene transfer may provide a more effective way for long-term and stable delivery of GLP1 (Prud'homme et al., 2007; Rowzee et al., 2011).

The recent development of genome editing technology, including the CRISPR system, has made it possible to perform precise genetic engineering, providing an ideal tool for somatic gene therapy (Wright et al., 2016). However, clinical application of CRISPR technology has been challenging due to inadequate efficacy in vivo using conventional delivery approach. Thus, the development of an ex vivo platform that can combine both precise genome editing in vitro with effective application of engineered cells in vivo will provide significant benefits for the treatment of many human diseases.

The epidermal progenitor cells of skin (Blanpain and Fuchs, 2006; Watt, 2014) have several unique advantages, making them particularly suited for somatic gene therapy ex vivo: (1) Human skin is the largest and most accessible organ in the body, offering availability for collection of epidermal progenitor cells by well-established procedures (Rheinwald and Green, 1975). Moreover, it is easy to monitor the skin for potential off-target effects of gene targeting and, if necessary, to remove it in case of an adverse consequence. (2) Cultured epidermal progenitor cells have tremendous proliferative capacity (Blanpain and Fuchs, 2006; Watt, 2014). With support of fibroblast feeder cells, we can culture mouse or human primary basal cells for more than 150 doublings in vitro. These cells can also be readily induced to differentiate and the resultant stratified skin tissue can be transplanted to donor patients with well-established protocols (Blanpain and Fuchs, 2006; Watt, 2014). Compared with other somatic gene therapy approach, autologous skin grafts are relatively inexpensive, and the procedure is minimally invasive, safe, and has been clinically used for treating burn wounds for decades (Carsin et al., 2000). (3) Somatic gene therapy with epidermal progenitor cells is tissue specific. Anatomically, skin epidermis is not directly vascularized but receives nutrients from blood vessels located in the underlying dermal tissue. The physical separation by the basement membrane precludes potential dissemination of genetically modified cells in vivo, making it extremely tissue specific and safe for the cutaneous gene therapy. (4) Epidermal progenitor cells can withstand long-term culture in vitro without losing stemness (Rheinwald and Green, 1975), making it possible to perform precise genome editing with non-viral approaches. Potential genotoxicity, particularly from viral vectors, has been a significant hurdle for somatic gene therapy (Kotterman et al., 2015). (5) Epidermal progenitor cells have low immunogenicity. Gene-therapy-derived products can be recognized as foreign antigens by the host immune system, which may mount an immune response leading to clearance of genetically modified cells. However, skin autograft or allograft developed from cultured epidermal progenitor cells can achieve long-term and stable transplantation in human patients without eliciting significant immune reaction (Centanni et al., 2011; Zaulyanov and Kirsner,

2007). (6) It has been well documented that proteins secreted by skin epidermal cells, such as ApoE (apolipoprotein E) and large blood clotting proteins Factor VIII and Factor IX, can cross the epidermal/dermal barrier and reach circulation to achieve therapeutic effect in a systematic manner (Christensen et al., 2002; Del Rio et al., 2004; Fakharzadeh et al., 2000; Fenjves et al., 1989; Gerrard et al., 1993; Morgan et al., 1987). Thus, the potential applicability of skin stem cell therapy is broad and beyond the skin diseases.

Despite the potential clinical relevance, research in cutaneous gene therapy has been greatly hampered by the lack of an appropriate mouse model. Although it has been shown that mouse skin or human skin can be transplanted to immunodeficient mice (Christensen et al., 2002; Del Rio et al., 2004; Fakharzadeh et al., 2000; Fenjves et al., 1989; Gerrard et al., 1993; Morgan et al., 1987; Sebastiano et al., 2014), lack of an intact immune system in the host animals makes it impossible to determine the potential outcomes that the therapy may elicit in vivo. Immune clearance of engineered cells has been one of the major complications for somatic gene therapy (Collins and Thrasher, 2015). Additionally, it remains technically challenging to perform skin organoid culture with mouse epidermal progenitor cells and generate mouse skin substitute for transplantation. In this report, we resolved the technical hurdles and develop a mouse-to-mouse skin transplantation model with immunocompetent host animals. With this platform, we present the key evidence that genome-edited epidermal progenitor cells can be exploited for robust delivery of GLP1 and effective treatment of diabetes and obesity.

RESULTS

Ectopic Expression of *GLP1* in Epidermal Progenitor Cells via CRISPR-Mediated Genome Editing

By genetic engineering of skin epidermal progenitor cells, we can potentially transform skin into an in vivo reactor that produces GLP1 in a controllable manner (Figure S1A). To test CRISPR-mediated genome editing in mouse epidermal progenitor cells, we developed DNA vectors encoding the D10A mutant of *Cas9* (CRISPR-associated protein 9) (Ran et al., 2013), two gRNAs (guide RNAs) targeting the mouse *Rosa26* locus, and a *Rosa26*-targeting vector. The targeting vector contains two homology arms for the *Rosa26* locus, flanking an expression cassette that encodes a *GLP-1* and mouse *IgG-Fc* fragment fusion protein (Figures 1A and S1B). Fusion with IgG-Fc enhances the stability and secretion of GLP-1 when ectopically expressed (Kumar et al., 2007). To control the level of GLP-1 release, we further modified the targeting vector so the expression of *GLP-1* fusion protein is driven by a tetracycline-dependent promoter (Figure 1A).

Primary epidermal keratinocytes were isolated from CD1 newborn mice and electroporated with the *Rosa26* targeting vector together with plasmids encoding *Cas9* and *Rosa26*-specific gRNAs. Clones were isolated upon selection and the correct integration to the *Rosa26* locus was confirmed by both PCR screening and Southern blotting analysis (Figure 1B). Engineered epidermal cells exhibited robust GLP1 production upon stimulation with doxycycline in a dose-dependent manner (Figure 1C). The secreted GLP1 fusion protein was functional as the conditioned medium significantly induced secretion of insulin when added to insulinoma cells cultured in vitro (Figure 1D). Expression of *GLP1* fusion protein in epidermal cells did not significantly change cell proliferation (Figures 1E and S1C) or differentiation (Figures 1F and 1G) in vitro. To confirm that modified epidermal cells are not tumorigenic, we examined the potential anchorage-independent growth of cells. Our results indicated that epidermal progenitor cells with *GLP1* targeting cannot grow in suspension with or without doxycycline stimulation (Figure 1H). As a positive control, cancer initiating cells (Schober and Fuchs, 2011) isolated from mouse SCC (squamous cell carcinoma) exhibited robust colony formation in soft agar medium (Figure 1H). Expression of *GLP1* did not affect the ability of epidermal progenitor cells to stratify. When subjected to skin organoid culture, the targeted cells readily produced stratified epithelial tissue (Figure S1D).

Stable Delivery of GLP1 In Vivo through Skin Transplantation

To efficiently transplant mouse epidermal progenitor cells, we developed an organotypic culture model with mouse epidermal progenitor cells in vitro by culturing the cells on top of acellularized mouse dermis (Liu et al., 2015; Yue et al., 2016). Exposure to the air/liquid interface can induce stratification of cultured cells to generate a skin-like organoid in vitro (Pruniéras et al., 1983). Previously, we have demonstrated that transplantation of this cultured skin organoid to *nude* hosts led to efficient skin engraftments (Liu et al., 2015; Yue et al., 2016). Using a modified surgical procedure and skin graft maintenance protocol, we have now fully developed the technology to engraft the isogenic mouse skin substitute onto an immunocompetent host.

To investigate the potential therapeutic effect of GLP1 in vivo, we prepared skin organoid culture with epidermal keratinocytes targeted with a *GLP1*-expression vector or a control vector and transplant the organoids to CD1 host animals (Figures 2A and 2B). No significant rejection of the skin grafts was observed after transplantation, suggesting that the targeted epidermal progenitor cells are well tolerated immunologically in vivo. Grafted skin exhibited normal epidermal stratification, proliferation, and cell death regardless of doxycycline treatment (Figures 2C–2E, S2A, and S2B). When fed with food containing doxycycline, the mice that were grafted with *GLP1*-expressing cells displayed significantly enhanced levels of GLP1 in the blood (Figure 2F). Expression of *GLP1* in grafted animals was stable for more than 3 months (Figure 2G). Consistent with previous observations (Christensen et al., 2002; Del Rio et al., 2004; Fakharzadeh et al., 2000; Fenjves et al., 1989; Gerrard et al., 1993; Morgan et al., 1987; Sebastiano et al., 2014), our results confirm that a skin-derived therapeutic protein can cross the basement membrane barrier and achieve a systematic effect in vivo. To test the controllability of *GLP1* expression in vivo, we measured plasma GLP1 level after administration of different amount of Doxycycline through intraperitoneal injection. Our results showed a dose-dependent release of GLP1 in plasma (Figure S2C). Significant level of GLP1 can be detected with the doxycycline dosage as low as 10 μg/kg body weight. The expression of *GLP1* reached saturation with doxycycline dosage between 250 and 500 μg/kg (Figure S2C). GLP1 is a potent incretin hormone. Our results indicated that induction of *GLP1* expression by doxycycline can significantly elevate fasting blood insulin level in vivo (Figure S2D).

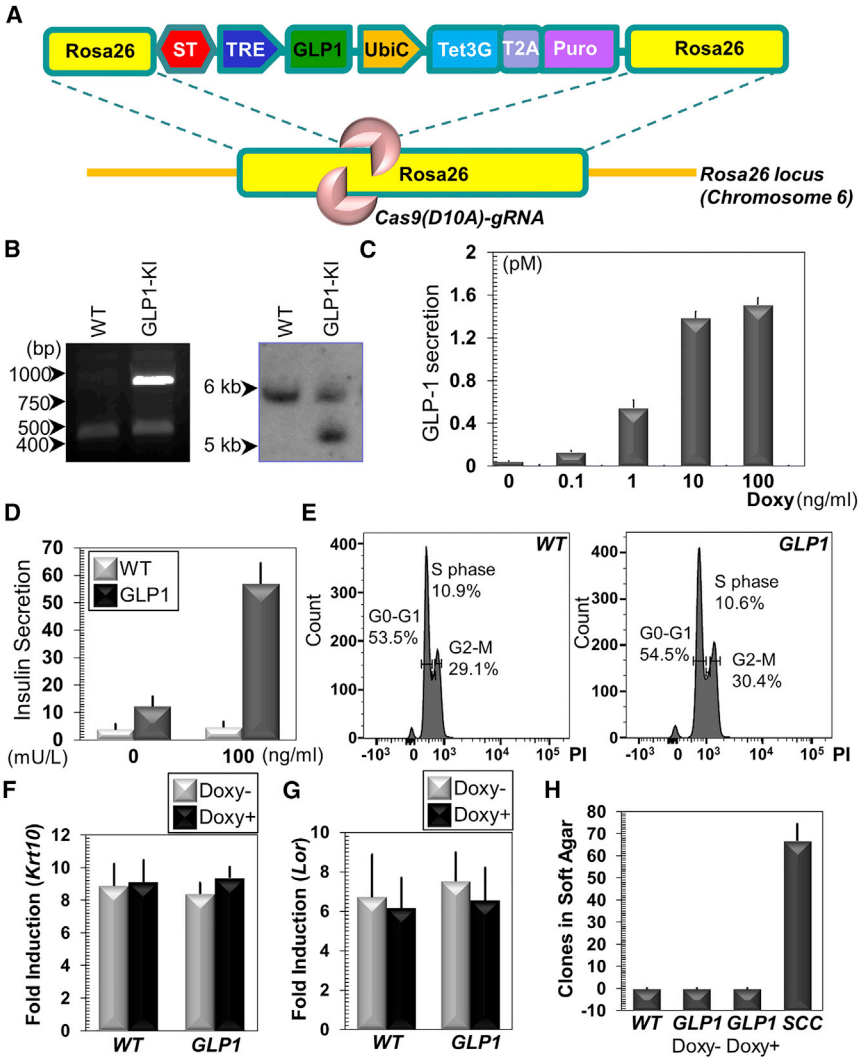

Figure 1. Engineering *GLP1*-Producing Skin Epidermal Progenitor Cells with CRISPR

(A) The targeting vector contains two *Rosa26* homology arms, flanking the expression cassette for *GLP1* (driven by TRE/tet-on promoter). Tet3G (tetracycline transactivator) protein and a selection marker (Puro) are separated by a self-cleavable peptide *T2A* and driven by a constitutive promoter UbiC (Ubiquitin C promoter). ST, transcriptional stop signal (ST).

(B) Integration of the targeting vector into *Rosa26* locus is verified by PCR (left panel) and Southern blotting (right panel).

(C) Secretion of *GLP1* in cell culture medium is determined by ELISA (enzyme-linked immunosorbent assay) upon stimulation with doxycycline (Doxy). All error bars represent SD unless otherwise specified. Sample size n = 3 independent experiments.

(D) Conditioned medium is collected and used to treat starved insulinoma cells. Secretion of insulin in vitro is determined by ELISA. n = 4 independent experiments.

(E) FACS (fluorescence-activated cell sorting) demonstrates similar cell cycle profiles for WT (wild-type) and *GLP1*-expressing epidermal progenitor cells after doxycycline treatment. PI, propidium iodine.

(F and G) Western blotting analysis of early (F) and late (G) differentiation marker expression in WT and *GLP1*-expressing cells upon calcium shift. Band intensity was determined by densitometry and fold of induction is quantified. Krt10, keratin 10; Lor, loricrin. n = 4 independent experiments.

(H) WT cells or *GLP1* cells with or without doxycycline treatment are tested for anchorage independent growth in soft agar. SCC, mouse squamous cell carcinoma cancer initiating cells. n = 3 independent experiments.

Cutaneous Gene Transfer with GLP1 Can Achieve Therapeutic Effects In Vivo

To examine the potential effect in diet-induced obesity and diabetes, we transplanted *GLP1*-expressing cells and control cells to two cohorts of CD1 adult mice, and employed high-fat diet (HFD) to induce obesity in grafted animals. Doxycycline was applied in drinking water to half of the animals to induce expression of *GLP1*. To minimize gender difference, only male animals were used in our study. Compared with animals on regular chow diet, the HFD greatly accelerated body-weight gain in mice grafted with control cells or *GLP1* cells without doxycycline treatment. Induction of *GLP1* expression with doxycycline led to a significant decrease in body weight in mice grafted with *GLP1* cells but not control cells (Figure 3A and quantification in Figure 3C). Consistently, histological examination of white fat tissue demonstrated that HFD can progressively induce adipocyte hypertrophy in the control groups (Figure 3B). By contrast, induction of *GLP1* expression dramatically suppressed this effect (Figure 3B). Skin transplantation and exogenous expression of *GLP1* did not significantly affect animal behavior or locomotor activity, as determined by analysis of mouse energy metabolism (Figure S3A). DEXA (dual-energy X-ray absorptiometry) scan revealed increased fat mass upon HFD feeding, and decreased fat mass with expression of *GLP1* (Figure S3B). By contrast, BMD (bone mineral density) was not significantly altered by HFD or *GLP1* (Figure S3B).

To examine glucose homeostasis, we conducted IPGTT (intraperitoneal glucose tolerance test) and ITT (insulin tolerance test). HFD resulted in decreased glucose tolerance in control mice or *GLP1*-grafted mice without doxycycline treatment (Figures 3D and 3E). By contrast, expression of *GLP1* significantly reduced glycemic excursion in vivo (Figures 3D and 3E). Expression of *GLP1* in grafted skin also significantly reduced insulin resistance compared with control mice or GLP1 mice without doxycycline treatment (Figures 3F and 3G). Together, our data strongly suggest that cutaneous gene therapy with inducible expression of *GLP1* can be used for the treatment and prevention of diet-induced obesity and pathologies. Expression of *GLP1* via skin grafts increased insulin secretion (Figure S2D). However, area of insulin-positive islets was not significantly altered in vivo (Figures S3C and S3D, $p > 0.05$), suggesting that the observed effects of GLP1 are likely due to its role in enhancing insulin secretion, instead of increasing proliferation or expansion of β cells.

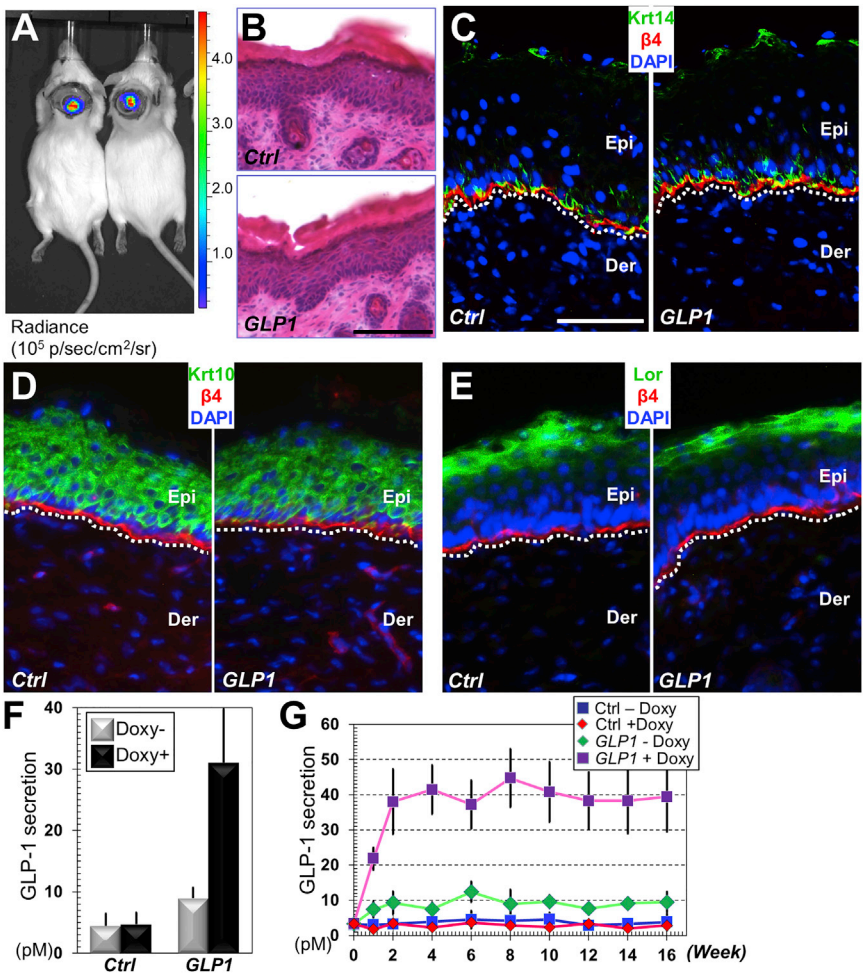

Figure 2. Stable Delivery of GLP1 In Vivo through Mouse-to-Mouse Skin Transplantation

(A) Images of immunocompetent mice (CD1) grafted with isogenic skin organoids generated from *GLP1*-expressing cells. Cells were infected with lentivirus encoding *Luciferase* before grafting.

(B) Histological examination of grafted *GLP1* skin and adjacent host skin as control (Ctrl). Scale bar, 50 μm.

(C–E) Sections of grafted skin and adjacent host skin control (ctrl) were immunostained with different antibodies as indicated (Krt14, keratin 14, β4, β4-integrin, CD104) to examine basal (C), early differentiation (D), and late differentiation (E) layers. Scale bar, 50 μm. Epi, epidermis; Der, dermis; HF, hair follicle.

(F) The level of *GLP1* in blood is determined by ELISA from mice engrafted with control or GLP1 cells. n = 4 different animals.

(G) Level of GLP1 in blood is determined by ELISA for 16 weeks after skin engraftment. n = 3 different animals.

Cutaneous Delivery of GLP1 with Human Epidermal Progenitor Cells

To test the feasibility of cutaneous gene therapy with human epidermal progenitor cells, we cultured human skin organoids from primary epidermal keratinocytes isolated from human newborn foreskin. The human epidermal keratinocytes can readily produce organoids in vitro, which can be transplanted to *nude* mice. When infected with lentivirus, the grafted human cells exhibited robust expression of the exogenous *Luciferase* gene (Figure 4A). The grafted tissue showed normal skin stratification when stained for early or late epidermal differentiation markers (Figure 4B).

To examine CRISPR-mediated genome editing in human epidermal cells, we developed vectors encoding two gRNAs targeting human *AAVS1* (adeno-associated virus integration site 1) locus, and an *AAVS1*-targeting vector (Figure S4A) that harbors a tetracycline-inducible expression cassette encoding the *GLP-1* and *IgG-Fc* fragment fusion protein. Human epidermal keratinocytes were transfected with the targeting vector together with plasmids encoding *Cas9* and the gRNAs. Clones were isolated and correct integration confirmed by Southern blotting analysis (Figure 4C). Like mouse cells, engineered human epidermal cells exhibited strong dose-dependent GLP1 production upon stimulation with doxycycline (Figure 4D). The GLP1 fusion protein secreted from these cells can induce insulin secretion in vitro (Figure 4E).

Expression of the *GLP1* fusion protein in human cells did not significantly change cell proliferation (Figure S4B) or differentiation (Figures S4C and S4D) in vitro. The engineered cells stratified and formed skin organoids in vitro, which were transplanted to *nude* host (Figure 4F). Grafted skin exhibited normal epidermal stratification and proliferation regardless of doxycycline treatment (Figures S4E–S4G). Skin grafts derived from control or *GLP1*- expressing human epidermal cells also displayed comparable apoptosis rate compared with adjacent host skin (Figure S4H). Together, this suggests that CRISPR editing of human epidermal progenitor cells does not significantly alter cellular dynamics or persistence in vivo. When fed with food containing doxycycline, significant secretion of GLP1 was detected in the blood from the *nude* mice that are grafted with *GLP1*-expressing cells (Figure 4G). Expression of *GLP1* also increased insulin secretion in vivo (Figure S4I), suggesting the potential clinical relevance of cutaneous gene delivery for treatment of obesity and diabetes in the future.

DISCUSSION

Skin epidermal progenitor cells are an ideal platform for ex vivo gene therapy, allowing efficient genetic manipulation with minimal risk of tumorigenesis or other detrimental complications in vivo (Del Rio et al., 2004). In this study, we resolved the technical hurdles for mouse-to-mouse skin transplantation and provide proof-of-concept evidence supporting the efficacy of cutaneous gene therapy for systematic disorders in an immunocompetent mouse model. In addition to expression of a therapeutic hormone or protein factors, such as *GLP-1*, ectopic

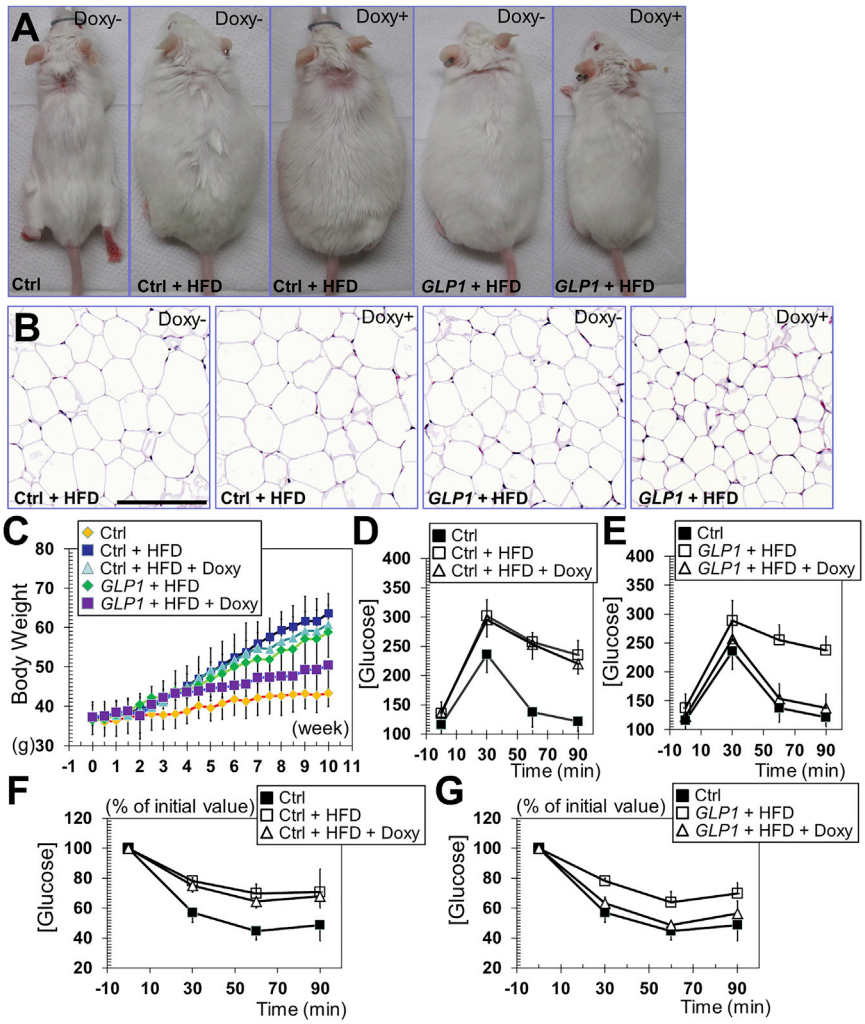

Figure 3. Expression of *GLP1* in Epidermal Progenitor Cells Improves Body Weight and Glucose Homeostasis In Vivo

(A) Images of control and grafted animals fed a regular diet or a HFD (high-fat diet).

(B) Representative histological staining's of white fat tissue. Scale bar, 100 μm.

(C) Body-weight change of different cohorts of mice measured from ~10 weeks of age. n = 5 different animals for each group.

(D and E) IPGTT (intraperitoneal glucose tolerance test) for control (D) and *GLP1* grafted (E) animals. Blood glucose concentrations as a function of time following intraperitoneal injection of glucose showed improved glucose tolerance in *GLP1*-expressing mice. n = 5 different animals.

(F and G) ITT (insulin tolerance test). Profile of glucose concentrations (percentage of initial value) as a function of time following intraperitoneal injection of insulin shows reduced insulin resistance in *GLP1*-expressing mice (G), but not control mice (F), after HFD treatment. n = 5 different animals.

expression of metabolic enzymes in skin epidermal cells can also transform the engineered skin into a potential "metabolic sink" for correction of various other metabolic disorders (Christensen et al., 2002). Thus, the applicability of cutaneous gene therapy is very broad.

Cultured human epidermal progenitor cells have been used to generate CEA (cultured epidermal autograft), which has been clinically used for treatment of massive burn wounds for decades (Carsin et al., 2000). It has been well documented that CEA can be well taken by the patients and contribute significantly to wound coverage and survival. CEA has also been used for treatment of other skin diseases, including vitiligo and epidermolysis bullosa. The regenerated skin is stable in vivo and can last for many years in the clinical follow-up studies (Guerra et al., 2000; Shinkuma et al., 2014). Thus, we expect that the cutaneous gene delivery will be long term if not permanent. The engineered epidermal cells can also be cryopreserved and used for generation of new skin grafts if needed.

The peptide hormone GLP1 has the essential requisite properties to maintain homeostatic levels of glucose in order to effectively treat diabetes. Compounds that elongate the half-life of endogenous GLP1 or synthetic GLP1 receptor agonists have already been clinically used for adjunctive antidiabetic treatments (Sandoval and D'Alessio, 2015). Skin is a tempting target organ, providing a long-lasting, safe, and affordable way to deliver GLP1 through somatic gene transfer. In this report, by CRISPR-mediated genome editing, we have achieved controllable release of GLP1 in skin with doxycycline. Doxycycline is a well-developed antibiotic, which has been widely used for treatment of bacterial infections. Because of the enhanced sensitivity of the tetracycline-dependent promoter, a very small amount of doxycycline is sufficient to activate the promoter and induce *GLP1* expression in vivo. We expect that administration of doxycycline at this dosage (less than 1/100 of the dosage used for bacterial infection) would be very unlikely to cause significant side effect (Sloan and Scheinfeld, 2008). Interestingly, it has been shown that *GLP1* expression can be driven by a synthetic signaling circuit that couples glycolysis-mediated calcium entry to an excitation-transcription system (Xie et al., 2016). Alternatively, expression of *GLP1* can also be controlled by non-invasive signals, such as a light-inducible transgene expression system (Ye et al., 2011). Cultured skin epidermal progenitor cells can be very easily engineered. It will be interesting to test and compare these synthetic pathways for control of *GLP1* expression in the future.

Cultured epidermal progenitor cells have tremendous proliferative potential in vitro (Blanpain and Fuchs, 2006). However, if the culture of epidermal progenitor cells imposes a limit on the application of cutaneous gene therapy, we could take advantage of the iPSC (induced pluripotent stem cell) technology, which has been successfully used to generate skin grafts (Sebastiano et al.,

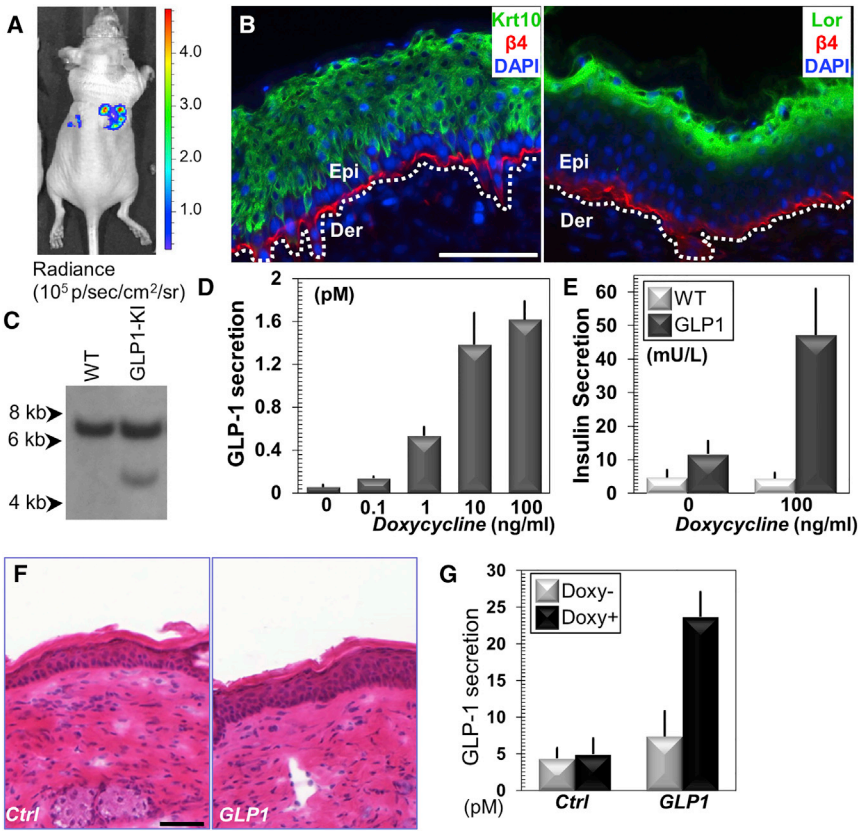

Figure 4. Expression of *GLP1* in Human Epidermal Progenitor Cells with CRISPR

(A) Image of nude mouse grafted with organotypic human skin culture.

(B) Sections of grafted skin were immunostained with different antibodies as indicated. Scale bar, 50 μm.

(C) Integration of the targeting vector into *AAVS1* locus is verified by Southern blotting.

(D) Secretion of *GLP1* into the culture medium was determined by the ELISA upon stimulation. n = 3 independent experiments.

(E) Secretion of insulin upon treatment with conditioned medium was determined by ELISA. n = 4 independent experiments.

(F) H&E staining of skin organoids developed from control or GLP1-producing human cells.

(G) Level of *GLP1* was determined by ELISA in blood from control or grafted *nude* mice. n = 4 different animals.

2014). Although humans have a relatively smaller body surface to weight ratio compared with rodents, adult human skin epidermis (average thickness ~100 μm) (Sandby-Møller et al., 2003) is significantly thicker than mouse epidermis (~10 μm) (Hansen et al., 1984), providing more metabolically active cells for synthesis and secretion of therapeutic proteins. Calculating on a per cell basis (based on GLP1 synthesis in vitro, Figure 1C), a graft of 20 × 20 cm (containing ~2 × 10^9 epidermal cells) (Weinstein et al., 1984) will deliver ~150 pmol of *GLP1* per day, which can lead to ~27 pM of plasma GLP1 for an adult human, suggesting that cutaneous gene therapy for GLP1 secretion could be practical and clinically relevant.

Within normal skin epidermis, the Langerhans cells function as the sole cell type that expresses *major histocompatibility complex* (*MHC*) class II and presents antigen (Haniffa et al., 2015). Epidermal keratinocytes only express *MHC* class I molecules on their cell surface and are considered as "non-professional" antigen-presenting cells. Thus, in skin substitute generated from epidermal progenitor cells, without the presence of Langerhans cells or leukocytes as antigen-presenting cells, potential antigenicity and immunogenicity are significantly reduced. It has been shown that skin allografts developed from cultured human keratinocytes can been clinically used for the treatment of wounds, without eliciting significant immune reaction (Centanni et al., 2011; Zaulyanov and Kirsner, 2007). Our study demonstrates that grafted skin stem cells expressing therapeutic proteins can be efficiently and stably grafted to host mice with intact immune systems. Together, our study demonstrates the tempting potential of cutaneous gene therapy for the treatment of various human diseases in the future.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2017.06.016.

AUTHOR CONTRIBUTIONS

X.W. and J.Y. designed the experiments. J.Y., X.G., Y.L., and B.W. performed the experiments. J.Y., B.W., and X.W. analyzed the data. X.W. wrote the manuscript. All authors edited the manuscript.

ACKNOWLEDGMENTS

We are very grateful to Dr. Lev Becker at the University of Chicago, Dr. Markus Schober at New York University School of Medicine, and Dr. Elaine Fuchs at the Rockefeller University for sharing reagents and technical assistance. We thank Linda Degenstein at the transgenic core facility and Drs. Honggang Ye and Graeme Bell at the Diabetes Research Laboratory at University of Chicago for excellent technical assistance. The animal studies were carried out in the ALAAC-accredited animal research facility at the University of Chicago. This work was supported by grants R01-AR063630 and R01OD023700 from the National Institutes of Health, the Research Scholar Grant (RSG-13-198-01) from the American Cancer Society, the V scholar award from V foundation to X.W., and National Institute of Health grant DK-020595 to B.W.

Received: August 5, 2016
Revised: February 15, 2017
Accepted: June 27, 2017
Published: August 3, 2017

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea pig anti-Keratin 5 (K5)	from Elaine Fuchs lab	gift
Guinea pig anti-Keratin 14 (K14)	from Elaine Fuchs lab	gift
Rabbit anti-Keratin 10 (K10)	from Elaine Fuchs lab	gift
Rabbit anti-Loricin (Lor)	from Elaine Fuchs lab	gift
Rat monoclonal β4-integrin	BD PharMingen	553745; RRID: AB_395027
Ser10 pho-histone antibody	EMD Millipore	06-570; RRID: AB_310177
Cleaved caspase-3 antibody	Cell Signaling	9661; RRID: AB_2341188
Anti-insulin	Cell signaling	3014; RRID: AB_2126503
HRP-conjugated goat anti-rabbit serum	Jackson Lab	111-036-046; RRID: AB_2337944
Chemicals, Peptides, and Recombinant Proteins		
Keratinocyte E base medium (DMEM:F12 = 3:1, without calcium)	Invitrogen/GIBCO	custom order
FBS	Omega Scientific	FB-11
OCT	Tissue-Tek	4583
paraformaldehyde	EMS	15710
Ethanol	Fischer	64-17-5
Xylene	Fischer	UN1307
Hematoxylin/Eosin stain	Cancer Diagnostics	CM3951/EM000G-P
Propidium Iodide	Fischer	25535-16-4
PBS	GIBCO	14190
SDS-polyacrylamide gel	National Diagnostics	EC-890
Odyssey blocking buffer	Li-Cor	921-40000
doxycycline	Sigma	D9891
glucose	Sigma	D9434
insulin	Sigma	91077C
Ingenio electroporation solution	Mirus Bio	MIR 50111
Mitomycin C	Santa Cruz	Sc-3514
Critical Commercial Assays		
Insulin ELISA kit	EMD Millipore	EZRMI-13K
GLP-1 ELISA kit	Sigma	RAB0201-1KT
SignalStain DAB substrate kit	Cell signaling	8059
Experimental Models: Cell Lines		
Mouse: primary epidermal basal cells	this paper	N/A
Human: primary epidermal basal cells	Invitrogen	C0015C
Experimental Models: Organisms/Strains		
Mouse: CD-1	Transgenic Core Facility/University of Chicago	N/A
Mouse: Nude	Jackson Laboratories	Stock 007850
Software and Algorithms		
Pannoramic Viewer 1.15	3DHISTECH	http://www.3dhistech.com/pannoramic_viewer
ImageJ	NIH	https://imagej.nih.gov/ij/
FlowJo	FlowJo	https://www.flowjo.com/
Excel	Microsoft	https://products.office.com/en-us/excel
OriginLab	OriginLab	http://www.originlab.com/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Xiaoyang Wu (xiaoyangwu@uchicago.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

WT CD1 mice were obtained from the Transgenic Core Facility at University of Chicago. Athymic nude mice were purchased from Jackson Laboratories. All the mice were housed under pathogen-free conditions in ARC (animal resource center) of University of Chicago under a 12 hr light-dark cycle. All the subjects were not involved in any previous procedures. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of University of Chicago.

Primary Epidermal Cell Culture

Primary mouse epidermal basal keratinocytes were isolated from the epidermis of newborn male mice using trypsin, after prior separation of the epidermis from the dermis by an overnight dispase treatment. Keratinocytes were plated on mitomycin C–treated 3T3 fibroblast feeder cells until passage 5-8. Cells were cultured in E-media supplemented with 15% serum and a final concentration of 0.05 mM Ca^{2+} (Wu et al., 2008).

Primary human epidermal basal keratinocytes (male foreskin) were purchased from Invitrogen. Cells were cultured on mitomycin C–treated 3T3 fibroblast feeder cells with E-media supplemented with 15% serum and a final concentration of 0.05 mM Ca^{2+} (Rasmussen et al., 2013; Rheinwald and Green, 1975).

METHOD DETAILS

Plasmid DNA Constructions

Lentiviral vector encoding *Luciferase* and *H2B-RFP* has been described before (Liu et al., 2015; Yue et al., 2016). Plasmid encoding *hCas9*-D10A mutant was a gift from George Church, obtained from Addgene (plasmid #41816). Plasmid encoding gRNA expression cassette was constructed with primers: AAG GAA AAA AGC GGC CGC TGT ACA AAA AAG CAG G; and gGA ATT CTA ATG CCA ACT TTG TAC, using gBlock as a template. *Rosa26* –targeting gRNA is constructed with primers: ACA CCG GCA GGC TTA AAG GCT AAC CG, AAA ACG GTT AGC CTT TAA GCC TGC CG, ACA CCG AGG ACA ACG CCC ACA CAC Cg, AAA ACG GTG TGT GGG CGT TGT CCT CG. *AAVS1*–targeting gRNA is constructed with primers: ACA CCG TCA CCA ATC CTG TCC CTA GG, AAA ACC TAG GGA CAG GAT TGG TGA CG, ACA CCG CCC CAC AGT GGG GCC ACT AG, AAA ACT AGT GGC CCC ACT GTG GGG CG. *Rosa26* targeting vector is constructed with pRosa26-GT as template (a gift from Liqun Luo, addgene plasmid 40025) using primers: GAC TAG TGA ATT CGG ATC CTT AAT TAA GGC CTC CGC GCC GGG TTT TGG CG, GAC TAG TCC CGG GGG ATC CAC CGG TCA GGA ACA GGT GGT GGC GGC CC, CGG GAT CCA CCG GTG AGG GCA GAG GAA GCC TTC TAA C, TCC CCC GGG TAC AAA ATC AGA AGG ACA GGG AAG, GGA ATT CAA TAA AAT ATC TTT ATT TTC ATT ACA TC, CCT TAA TTA AGG ATC CAC GCG TGT TTA AAC ACC GGT TTT ACG AGG GTA GGA AGT GGT AC. *AAVS1* targeting vector was constructed with AAVS1 hPGK-PuroR-pA donor (a gift from Rudolf Jaenisch, addgene plasmid 22072) as template using primers: CCC AAG CTT CTC GAG TTG GGG TTG CGC CTT TTC CAA G, CCC AAG CTT CCA TAG AGC CCA CCG CAT CCC C, CAG GGT CTA GAC GCC GGA TCC GGT ACC CTG TGC CTT CTA GTT GC, GGA TCC GGC GTC TAG ACC CTG GGG AGA GAG GTC GGT G, CCG CTC GAG AAT AAA ATA TCT TTA TTT TCA TTA CAT C, GCT CTA GAC CAA GTG ACG ATC ACA GCG ATC. Genotyping primers for CIRPSR mediated knockin: GAG CTG GGA CCA CCT TAT ATT C, GGT GCA TGA CCC GCA AG, GAG AGA TGG CTC CAG GAA ATG.

Genome editing of epidermal cells

Human or mouse primary epidermal basal cells were electroporated with a mixture of plasmid DNA containing plasmid encoding *hCas9*-D10A, plasmids encoding the gRNAs targeting *Rosa26* locus for mouse cells or *AAVS1* locus for human cells, and the *GLP1* targeting construct. Electroporation was carried out with BioRad gene pulser using Ingenio electroporation solution. Cells were electroporated using exponential decay mode at 250 Volts and 950 μF. Electroporated cells were immediately suspended in culture medium and plated. Cells were selected with puromycin (2.5μg/ml) 2-3 days post electroporation.

Mouse and human cells were electroporated at passage 8-10. Mouse epidermal cells at this stage can grow in feeder-free condition, whereas human cells are continuously cultured with feeder cells. Limited trypsinization or brief EDTA (20 mM in PBS) treatment was used to remove the feeder cells before suspension of human epidermal cells.

Skin organoid culture and transplantation

Decelluralized dermis (circular shape with 1cm diameter) was prepared by EDTA treatment of newborn mouse skin (Pruniéras et al., 1983). 1.5×10^6 cultured keratinocytes were seeded onto the dermis in cell culture insert. After overnight attachment, the skin culture was exposed to air/liquid interface.

For grafting with skin organoids, CD1 males with the ages of 6-8 weeks were anesthetized. A silicone chamber bottom with the interior diameter of 0.8cm and the exterior diameter of 1.5cm was implanted on its shaved dorsal mid-line skin, which was used

to hold the skin graft. A chamber cap was installed to seal the chamber right after a piece of graft was implanted. About one week later, the chamber cap was removed to expose the graft to air. A single dose of 0.2mg α-CD4 (GK1.5) and 0.2mg α-CD8 (2.43.1) antibodies was administered intraperitoneally for skin grafting.

Histology and Immunofluorescence

Skin or wound samples were embedded in OCT, frozen, sectioned, and fixed in 4% formaldehyde. For paraffin sections, samples were incubated in 4% formaldehyde at 4°C overnight, dehydrated with a series of increasing concentrations of ethanol and xylene, and then embedded in paraffin. Paraffin sections were rehydrated in decreasing concentrations of ethanol and subjected to antigen unmasking in 10 mM Citrate, pH 6.0. Sections were subjected to hematoxylin and eosin staining or immunofluorescence staining as described (Guasch et al., 2007). Antibodies were diluted according to manufacturer's instruction, unless indicated.

Pancreata were fixed for 4 hr in 4% paraformaldehyde were embedded in paraffin, and 5-μm sections stained for insulin (Cell Signaling Technology, cat # 3014; 1:1000) and the HRP-conjugated goat anti-rabbit antiserum (Jackson Laboratories cat # (111-036- 046; 1:1000) using the SignalStain DAB Substrate kit (Cell Signaling Technology, cat # 8059) and counterstained with hematoxylin (Dako). Images were collected on a Cri Pannoramic whole slide scanner and analyzed using Pannoramic Viewer 1.15 software and ImageJ. Sections were used to quantify total pancreas area on each slide and to measure the area of each islet. Data were analyzed for each slide to calculate islet area (insulin+ area/total pancreas area), islet number per pancreas area (number of islets/area of pancreas in mm^2) and average islet size (sum of the area of all islets in a section/number of islets in the section). 3 mice and 3 slides (9 sections) were analyzed per group, with slides collected at 25 μm intervals. Data were compared between the control, HFD and HFD+GLP1 groups using student's t test.

Cell Cycle Analysis

Propidium Iodide (PI) staining followed by Flow Cytometry Assay were used to determine the effect of cell cycle profiles. Mouse and human epidermal cells were cultured in two 6cm cell culture dish for 24 hr, respectively. Cells were trypsinized and 1×10^5 cells from each dish were collected, followed by one PBS wash. Fixation of cells was carried out using 70% (v/v) ice cold ethanol for 1 hr. Then, the fixed cells were centrifuged at 500 g at 4°C for 10 min, followed by PBS wash for two times. The cells were then treated with 75μg RNase A in 100μl PBS and incubated at 37°C for 1 hr. After incubation, the cells were collected by centrifuging at 500 g at 4°C for 10 min, followed by another PBS wash. The cell pellet was re-suspended in 200μl PBS, in addition of PI solution at a final concentration of 25ng/μl. After staining, the cells were analyzed immediately using flow cytometer BD FACSCanto™ II (BD Biosciences, San Jose, CA) with an excitation wavelength at 488 nm and emission at 585 nm. DNA content and histograms of cell cycle distribution were analyzed using FlowJo software, version 10 (FLOWJO LLC, OR).

Protein biochemical analysis

Western blotting was performed as described previously (Wu et al., 2004). Briefly, equal amounts of the cell lysates were separated on a SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a NC membrane. The immunoblot was incubated with Odyssey blocking buffer (Li-Cor) at room temperature for 1 hr, followed by an overnight incubation with primary antibody. Blots were washed three times with Tween 20/Tris-buffered saline (TBST) and incubated with a 1:10000 dilution of secondary antibody for 1 hr at room temperature. Blots were washed three times with TBST again. Visualization and quantification was carried out with the LI-COR Odyssey scanner and software (LI-COR Biosciences).

Mouse obesity model

Male CD-1 mice were housed (5 per cage, ~8 weeks old) in a central-controlled animal facility for air, humidity and temperature. These mice were fed either a regular chow or an HFD (60% kcal from fats, 20% from carbohydrates, and 20% from proteins) purchased from Bio-Serv (Frenchtown, NJ). Body weight and food intake were measure biweekly. To induce transgene expression, drinking water containing doxycycline (0.5 mg/ml) was provided to the animals.

For glucose tolerance and insulin tolerance test, an intraperitoneal glucose tolerance test (IPGTT) was performed on mice fed an HFD for 10 weeks. Mice were fasted for 6 hr before the test. Animals were injected (1 g/kg glucose/body weight, i.p.) with glucose dissolved in saline, and blood glucose was measured at 0, 30, 60 and 90 min using glucose test strips and glucose meters. An intraperitoneal insulin tolerance test was carried out 1 week after the IPGTT. Mice were fasted for 4 hr and injected (2 U/kg, i.p.) with insulin purchased from Sigma (St. Louis, MO). Blood glucose levels were determined thereafter at 0, 30, 60 and 90 min.

Metabolic analysis

Evaluation of energy homeostasis: Energy expenditure (metabolic rate) was determined by indirect calorimetry using a TSE LabMaster metabolic cage system (TSE Systems, Chesterfield, MO). Mice were individually housed in metabolic cages and allowed 2 days of acclimation. They were then monitored for 4 more consecutive days to measure metabolic rate, mouse movement, as well as food and water intake by periodic weighing (Savic et al., 2011). Metabolic rate, [expressed as oxygen consumption VO2 (ml/hr/kg)] or [expressed as kcal/hr/kg], was normalized to body weight.

Body Composition: Mice were anesthetized prior to DEXA imaging. Mice were injected with a mixture of Ketamine (80 mg/kg body weight) and Xylazine (5 mg/kg body weight) solution. Body Composition was measured by DEXA (Lunar PIXImus densitometer

system, GE Healthcare) using PIXImus 2 software. The system was calibrated according to manufacturer's instructions prior to the start of the experiment. Mean values of the both femur of each animal were reported.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using Excel or OriginLab software. Boxplots are used to describe the entire population without assumptions on the statistical distribution. A Student's t test or ANOVA was used to assess the statistical significance (P value) of differences between two experimental conditions. Sample size (n) for each analysis can be found in the figure legends.

DATA AND SOFTWARE AVAILABILITY

A complete list of software for data analysis and processing can be found in the Key Resources Table.