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WU et al.

(54) **TISSUE ORGANOIDS**

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(57) **ABSTRACT**

Physiologically-tailored tissue organoids are disclosed for monitoring and treating diseases and improving an individual's health.

Specification includes a Sequence Listing.

1A

Isolation of epidermal stem cells

Differentiation engraftment

Genome Editing

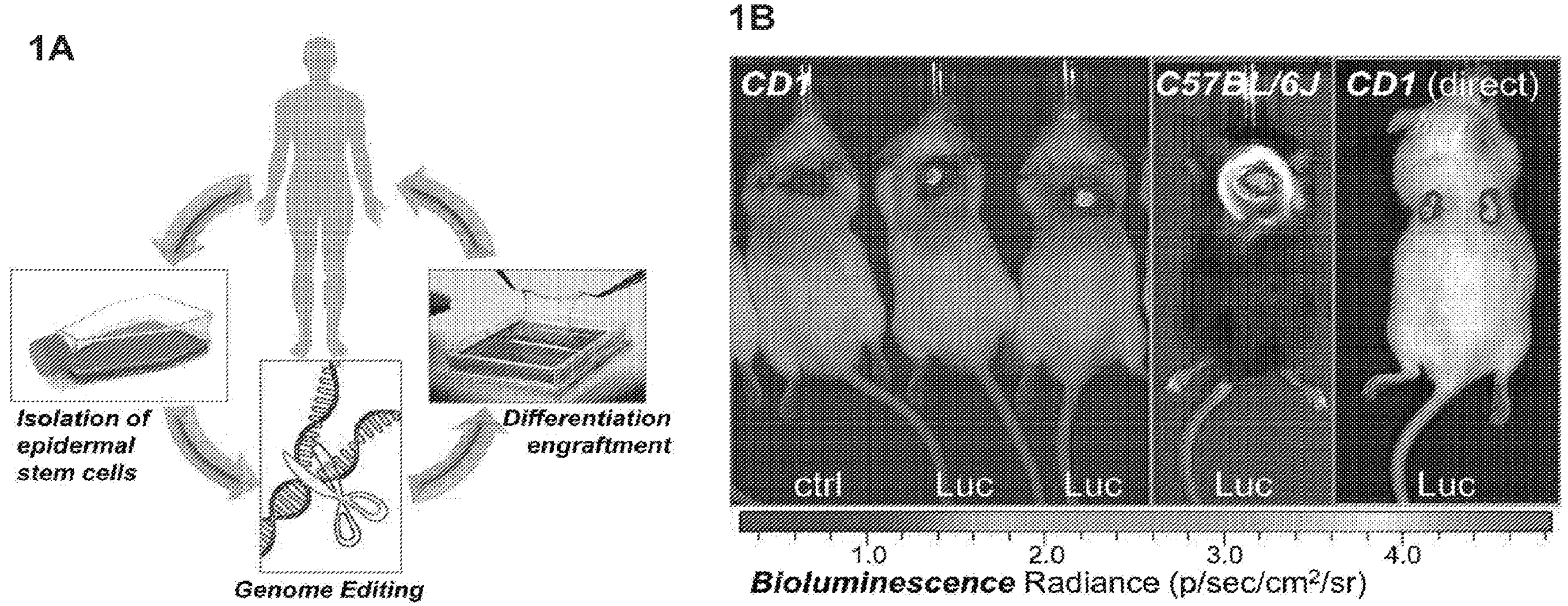


Figure 1

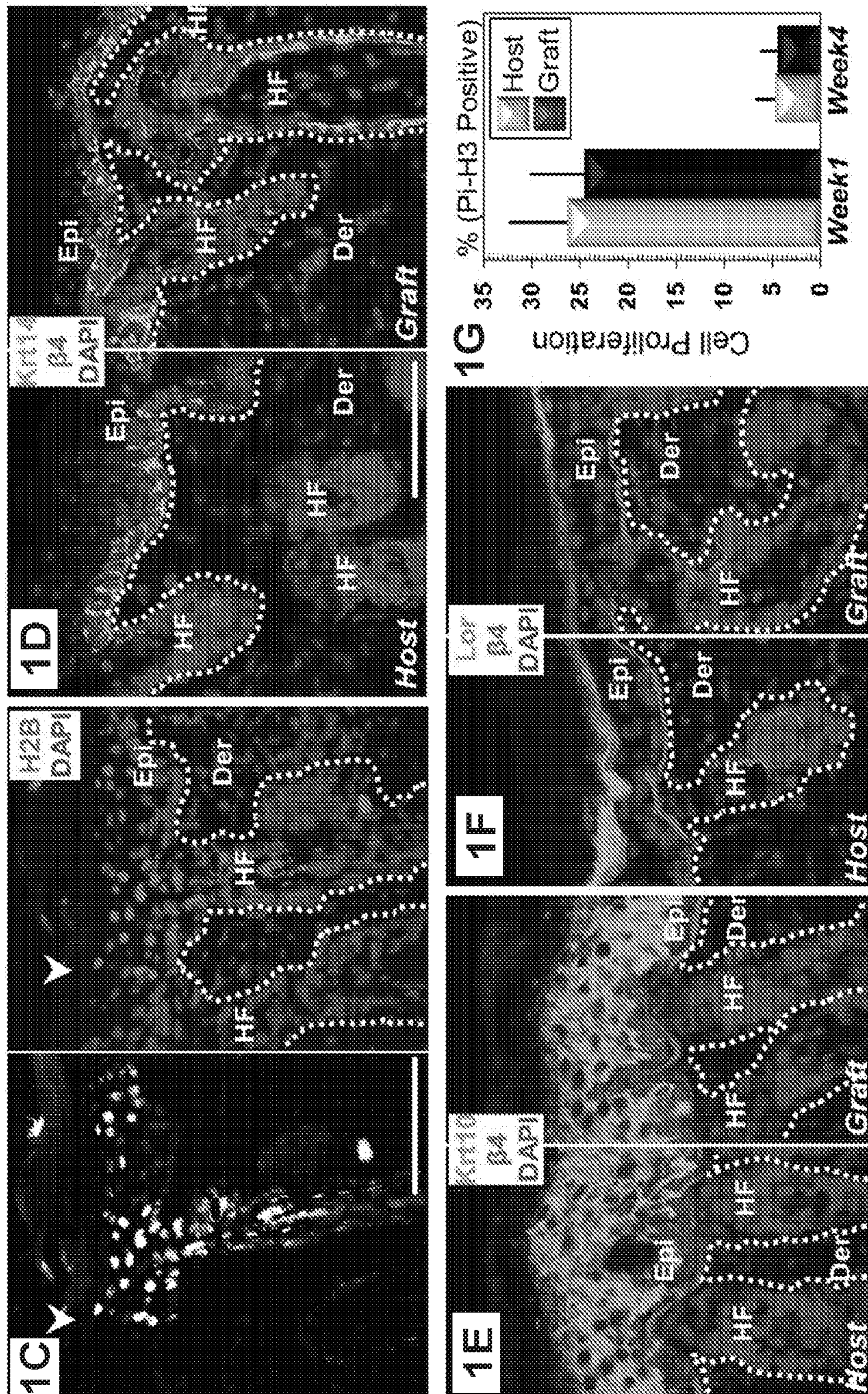


Figure 1 (cont.)

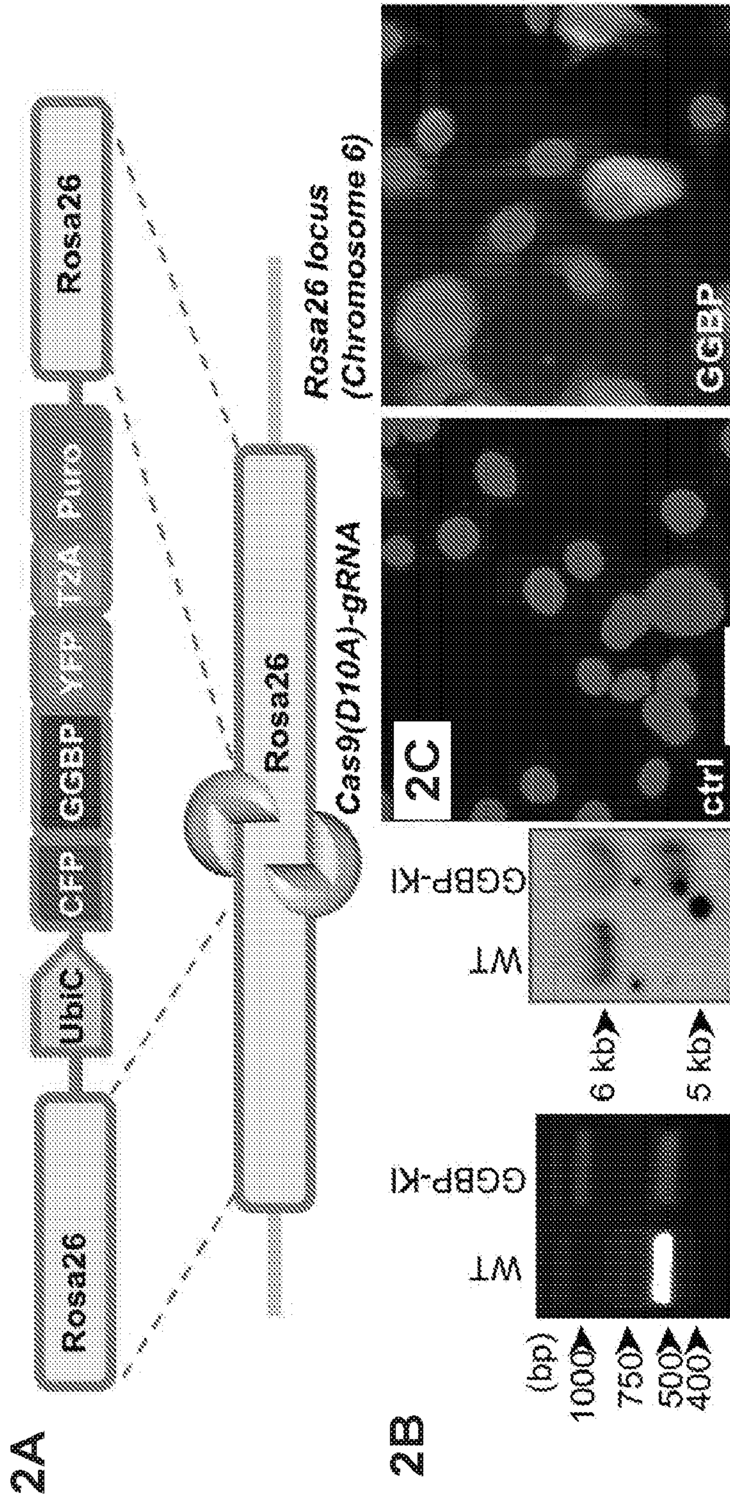
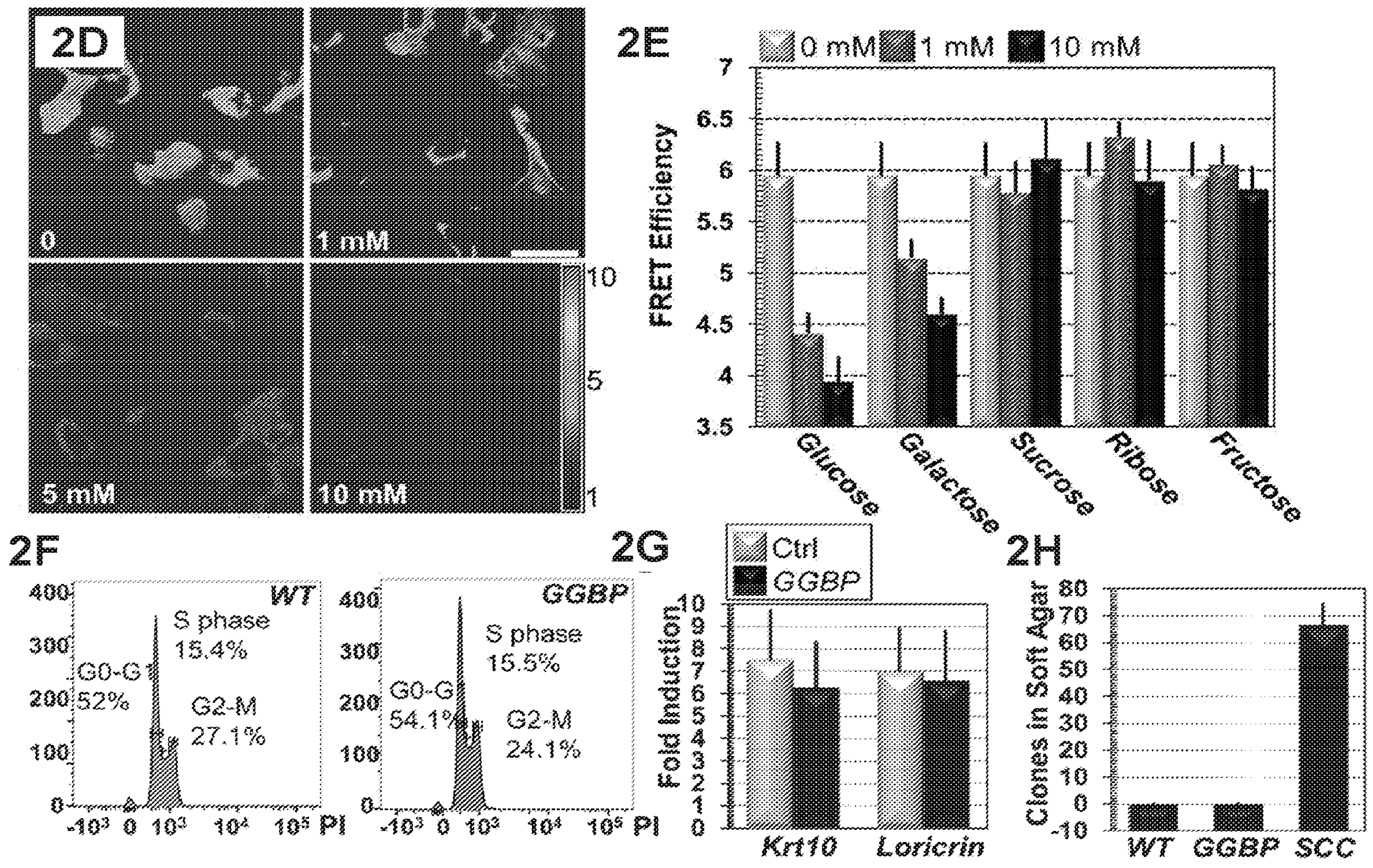


Figure 2



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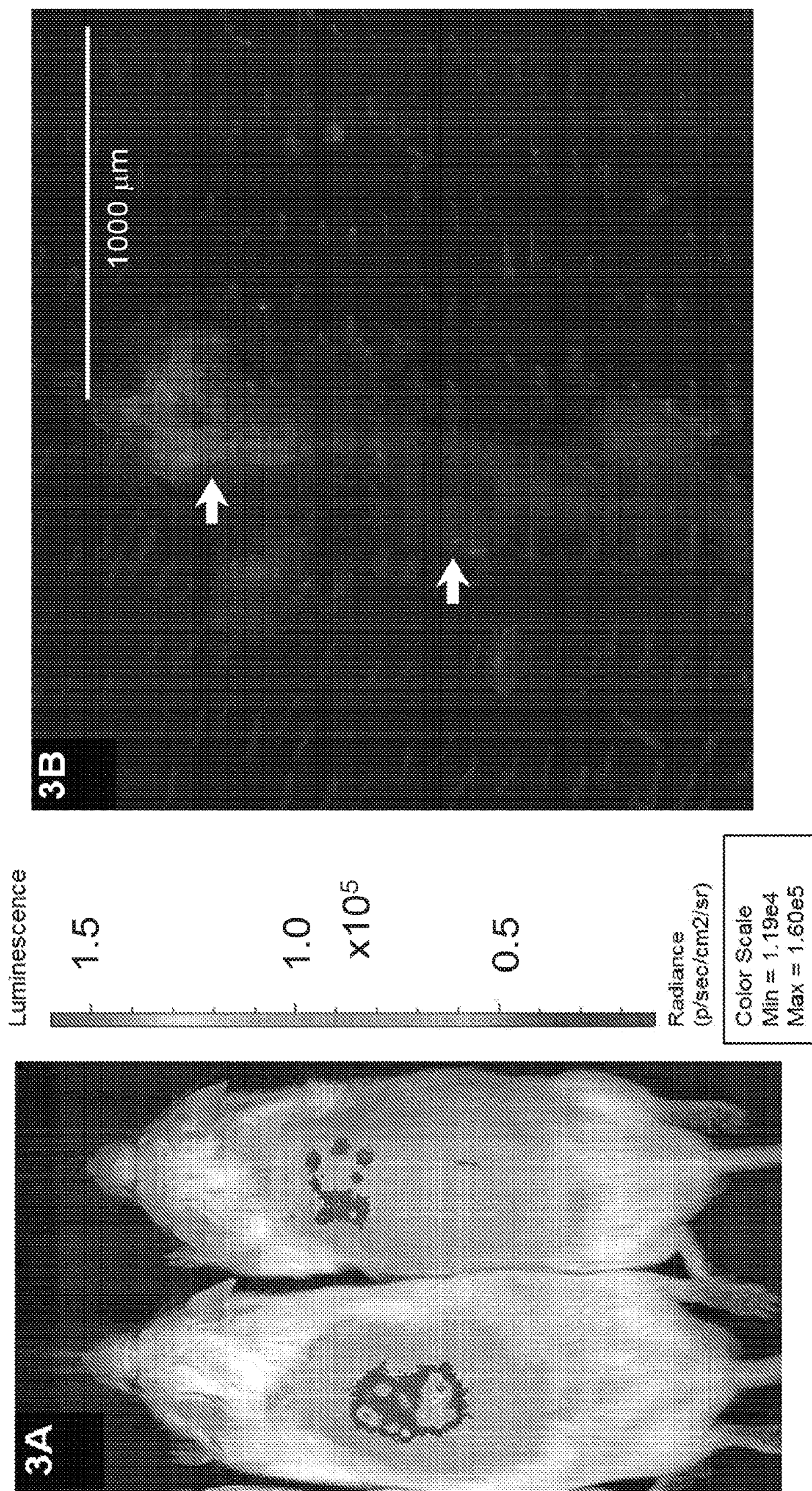


Figure 3

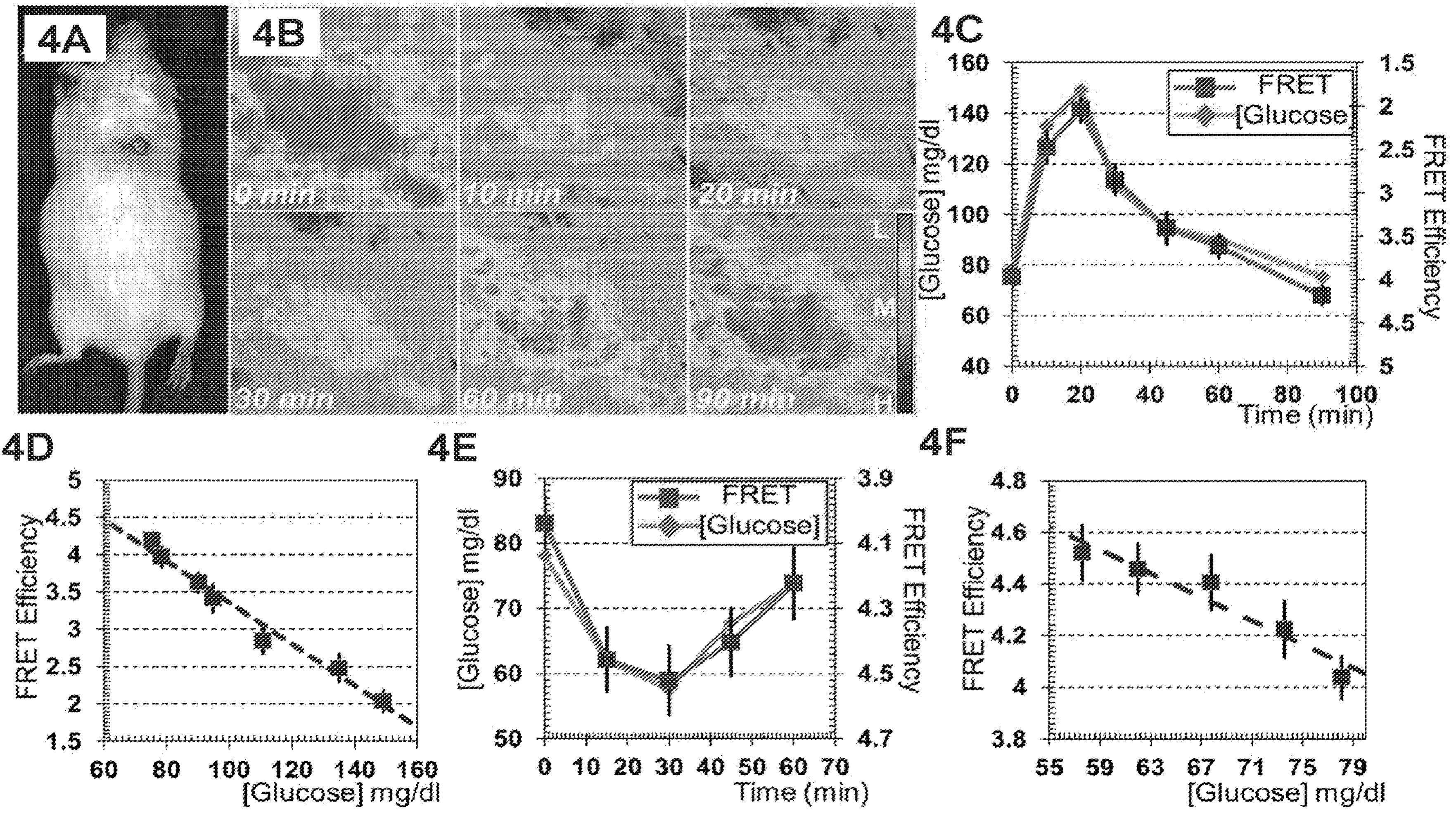
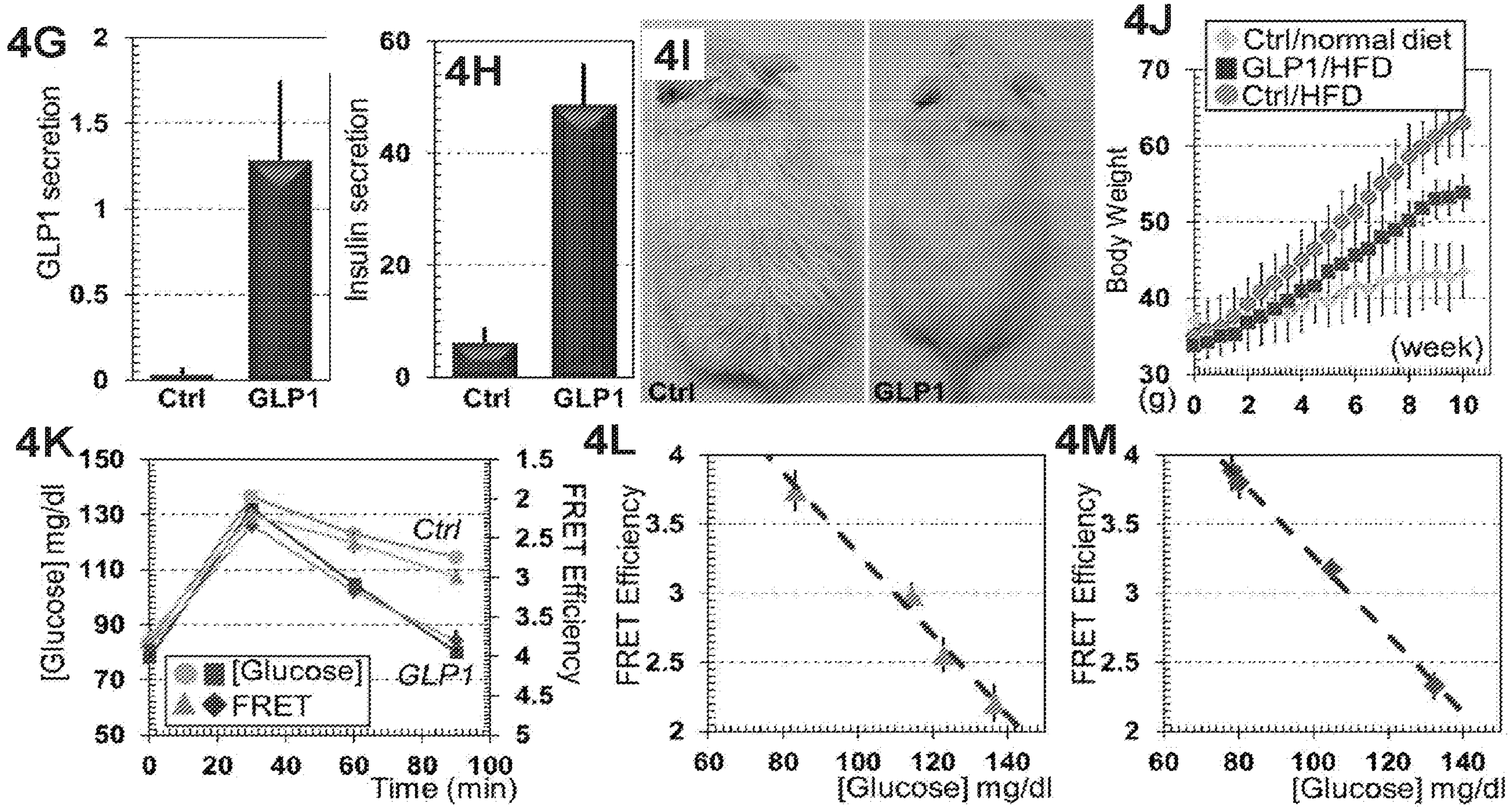


Figure 4



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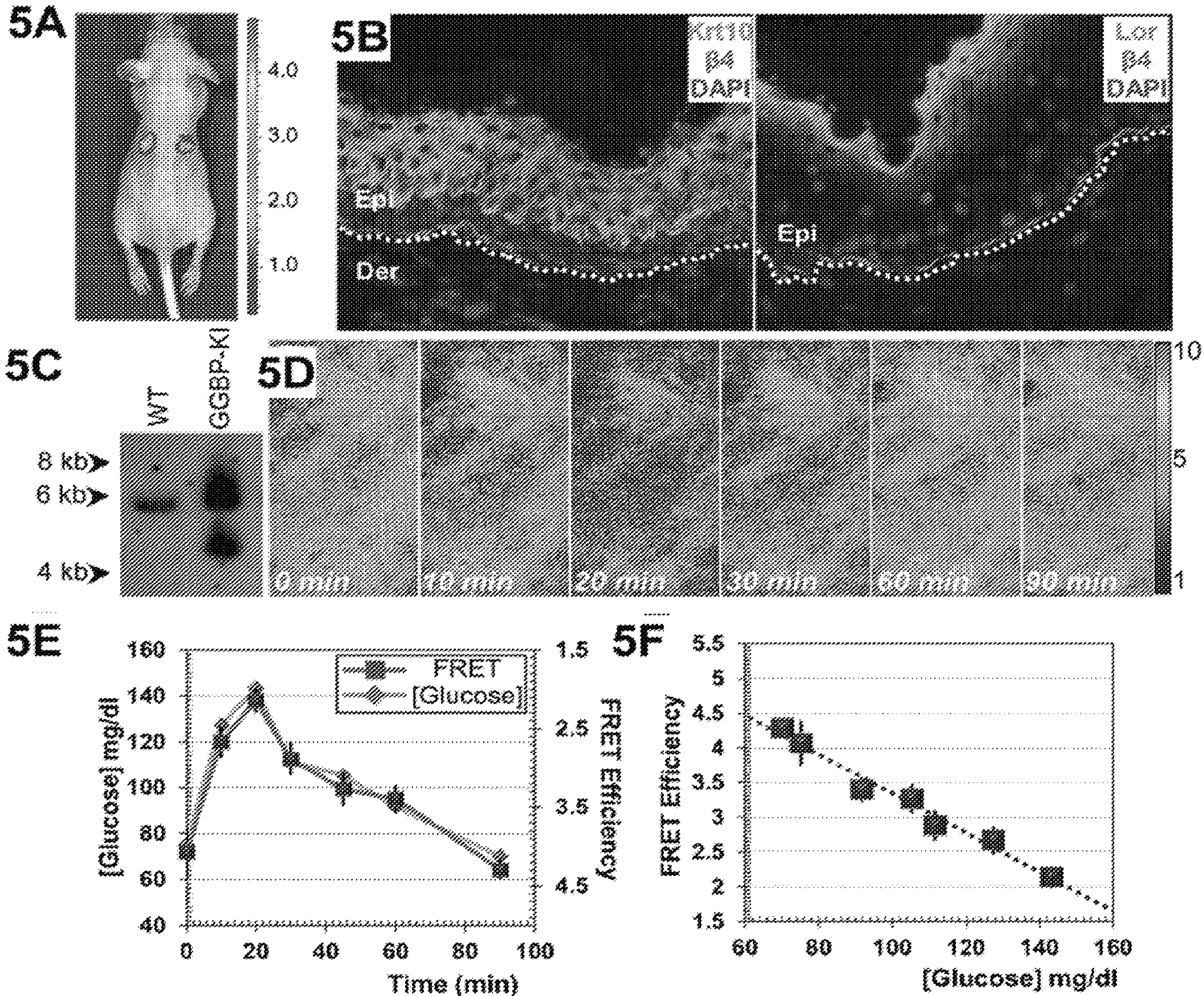

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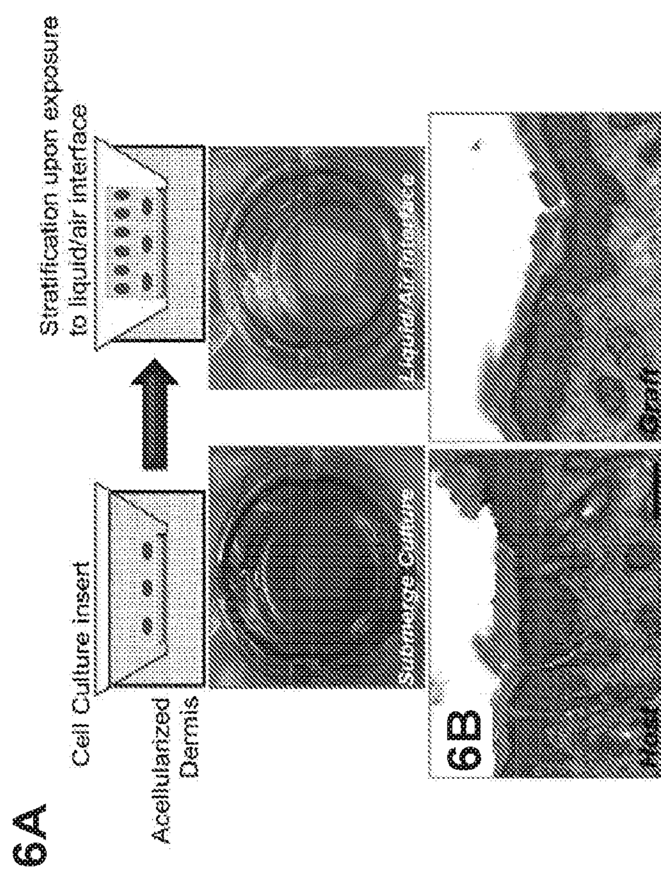

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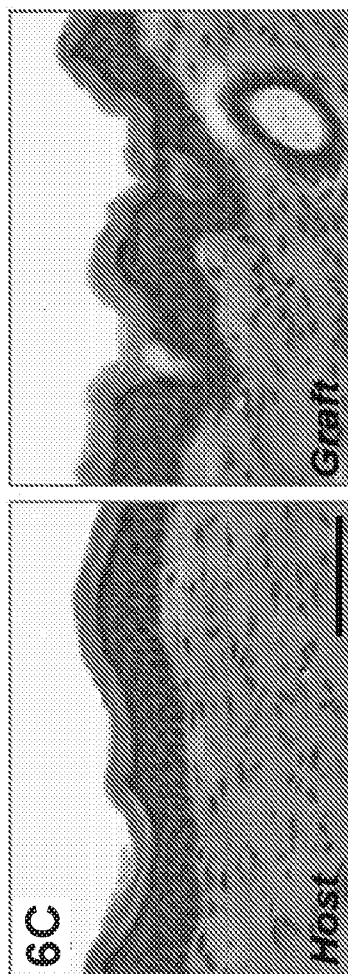

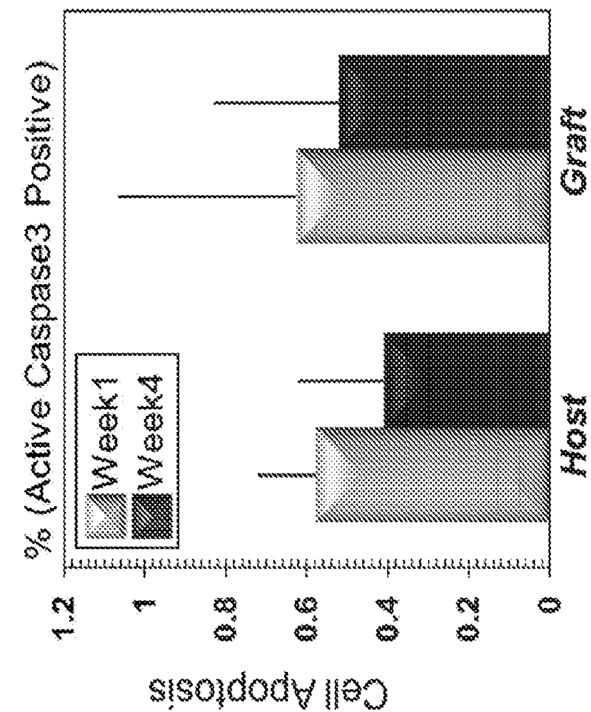

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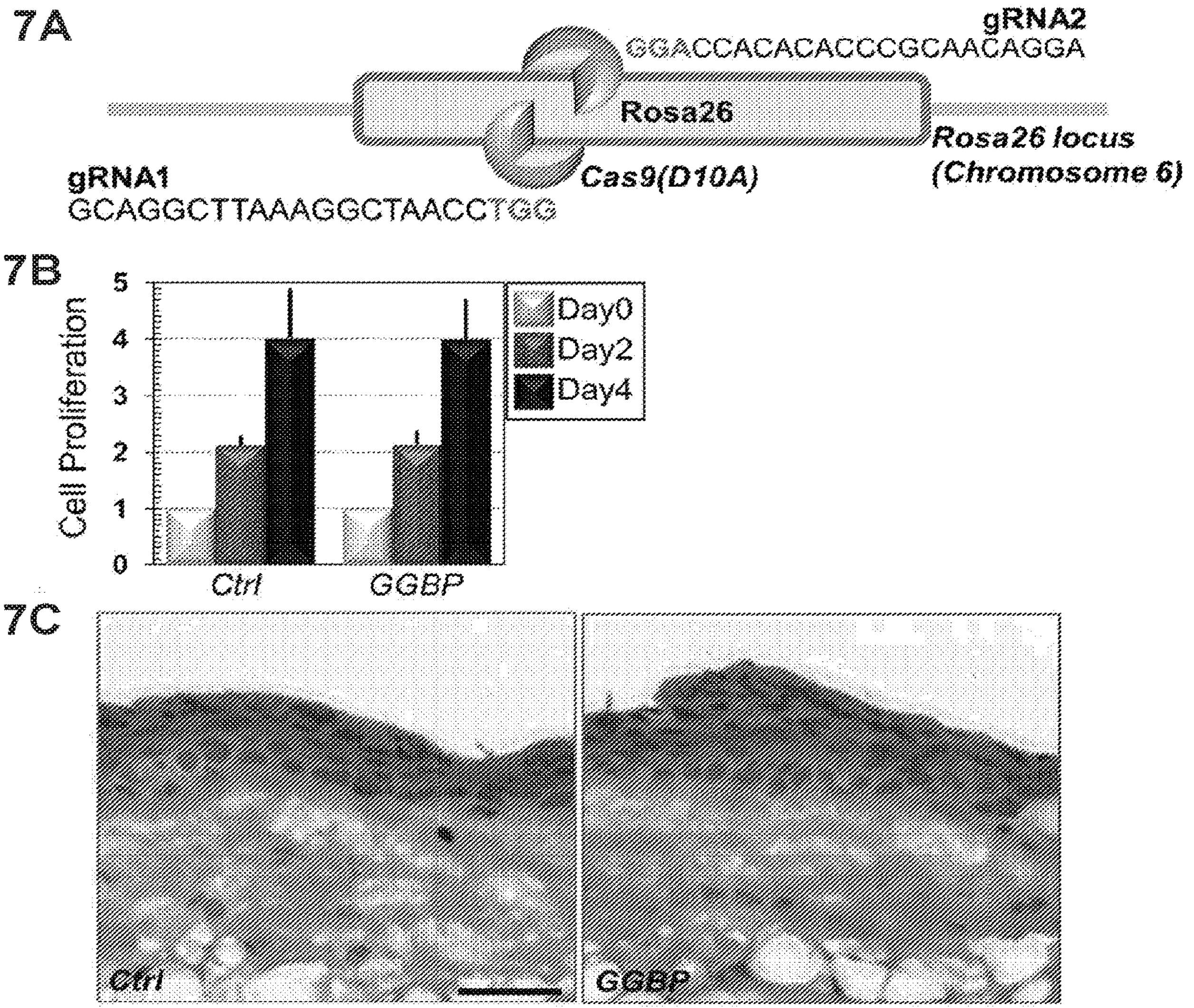

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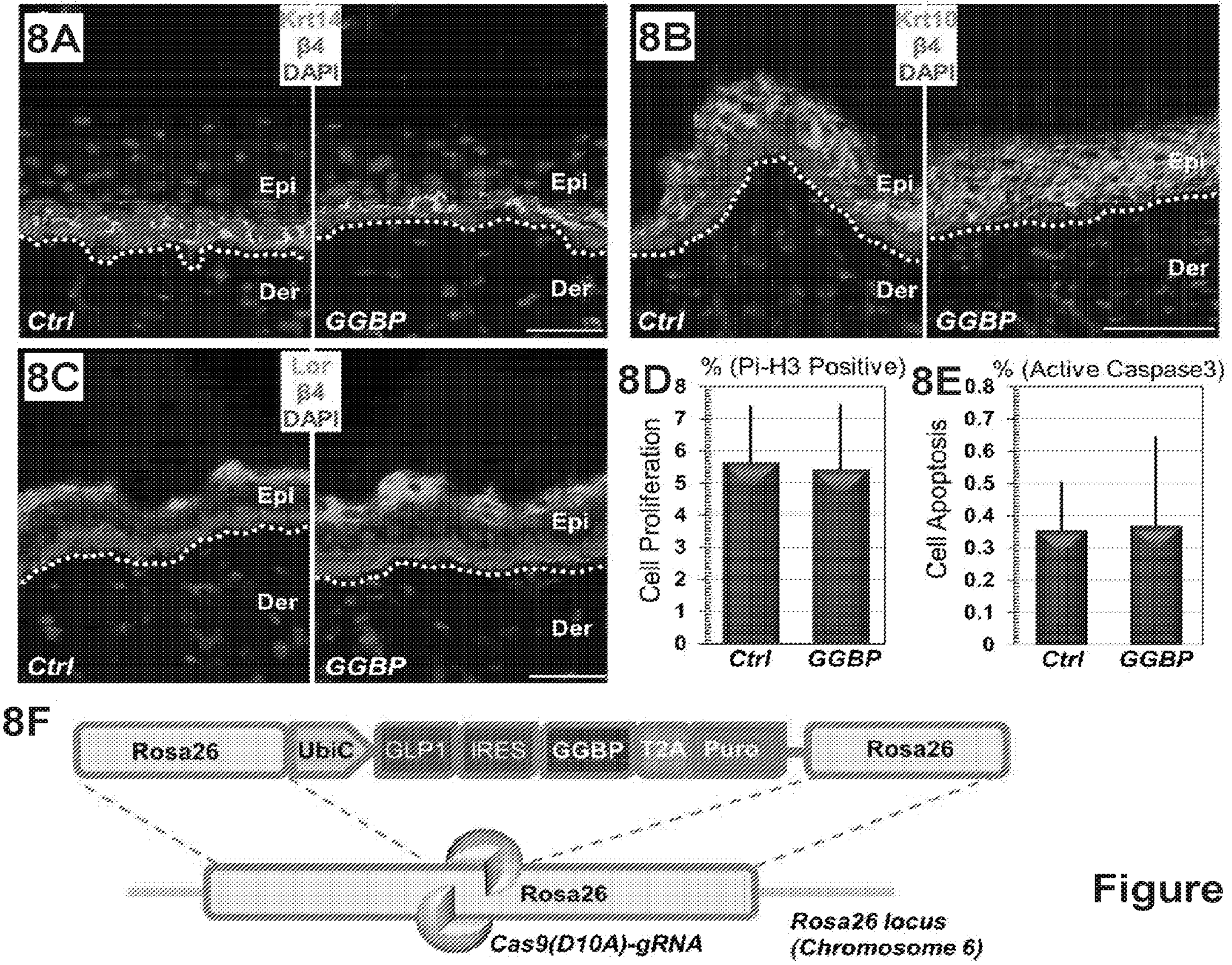

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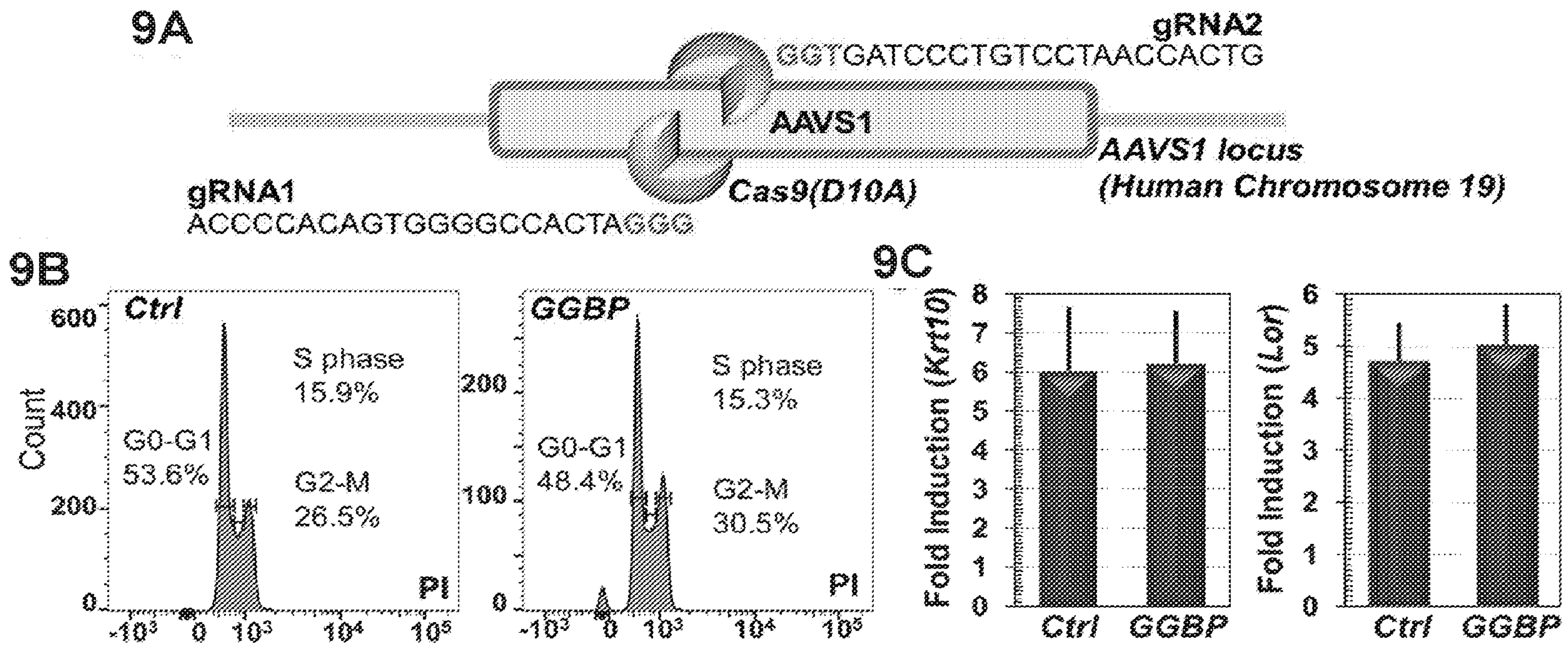

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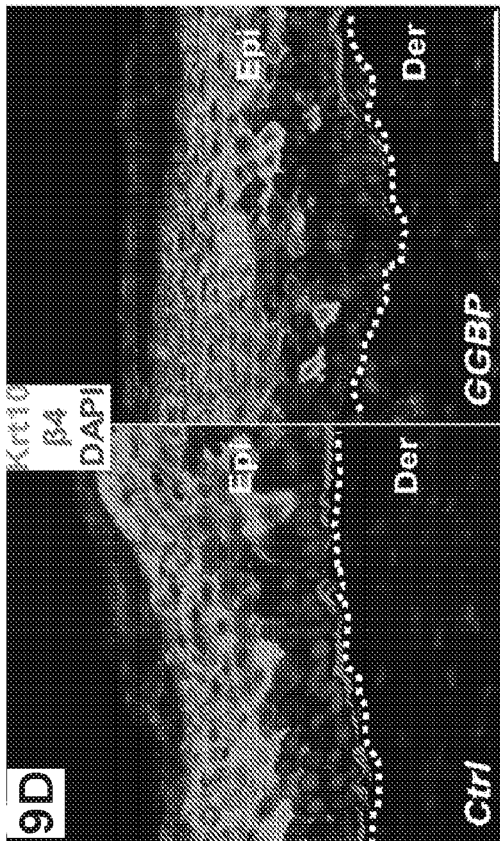

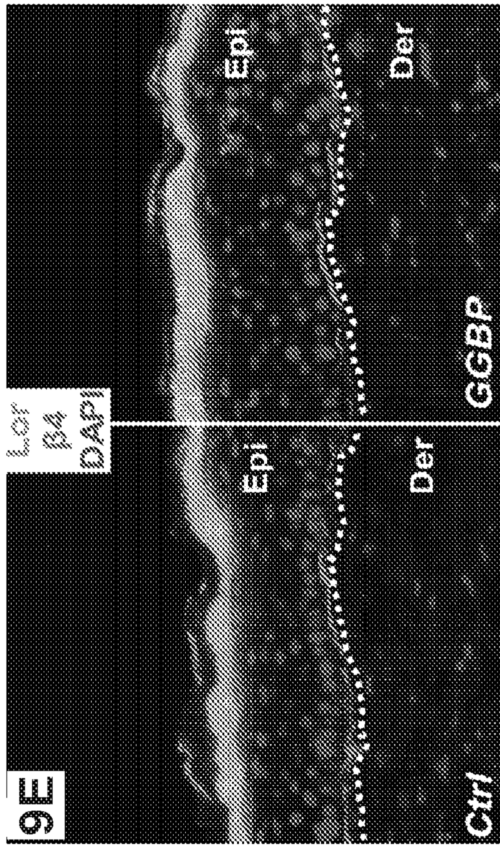

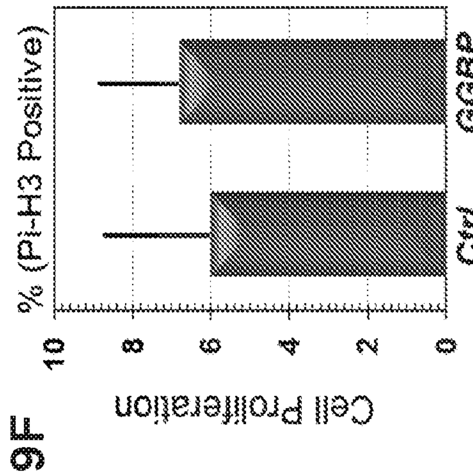

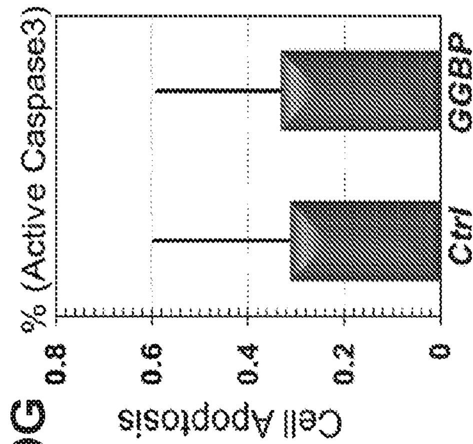

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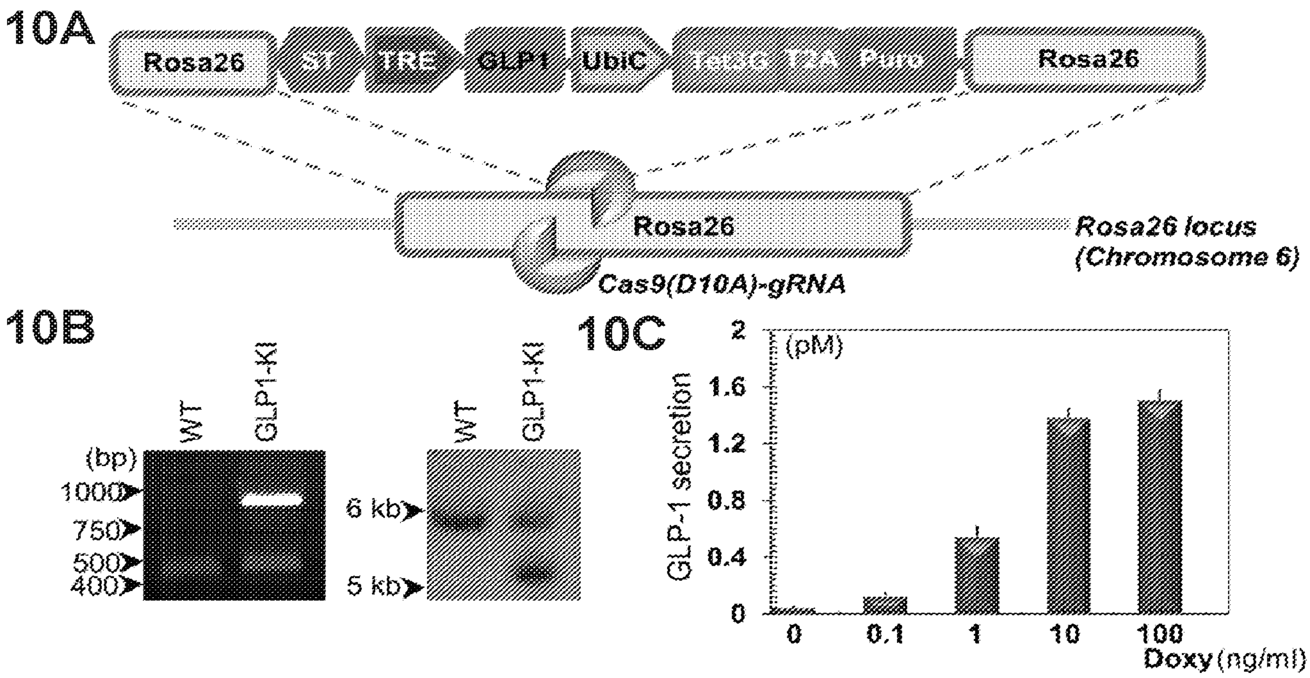

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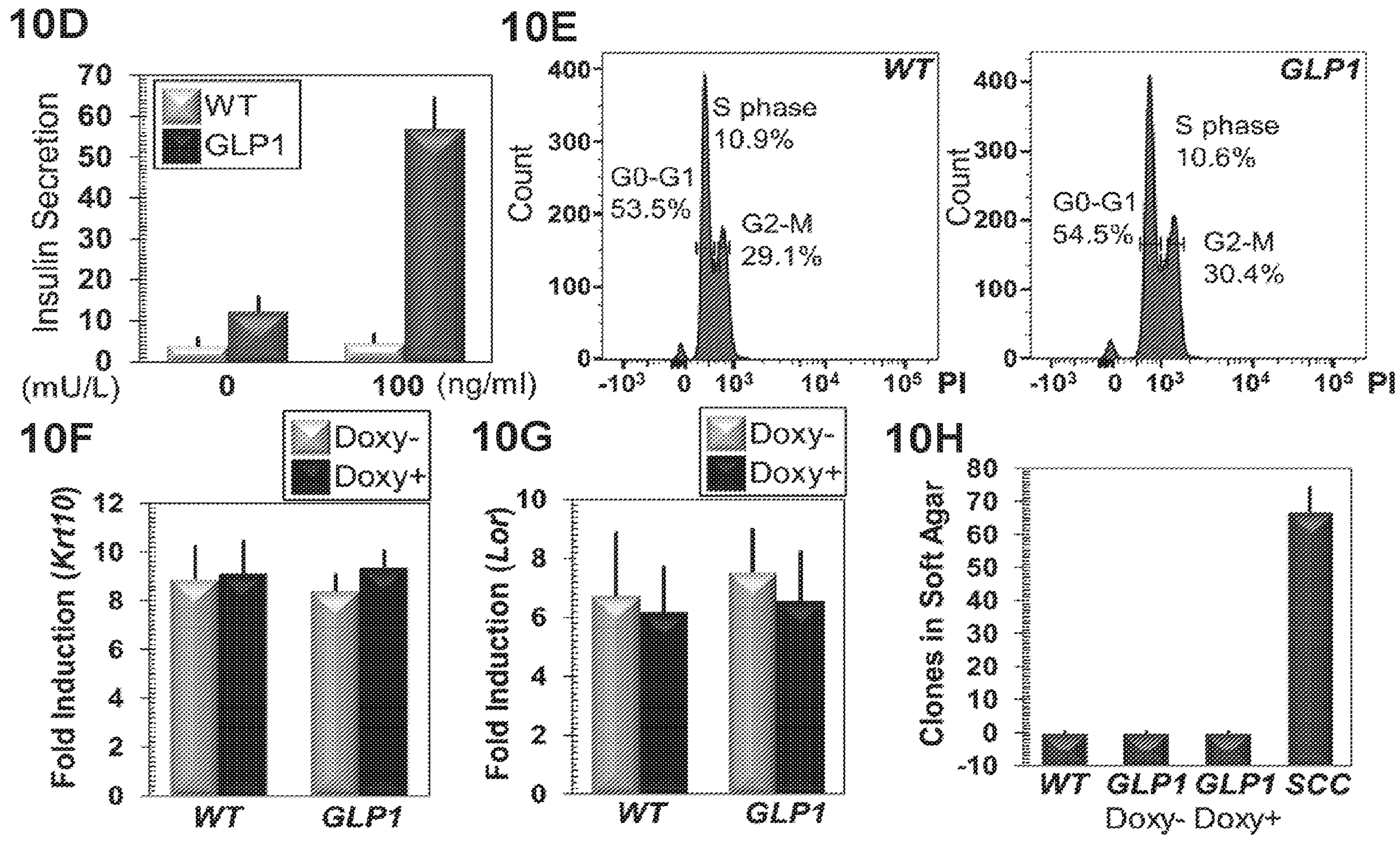

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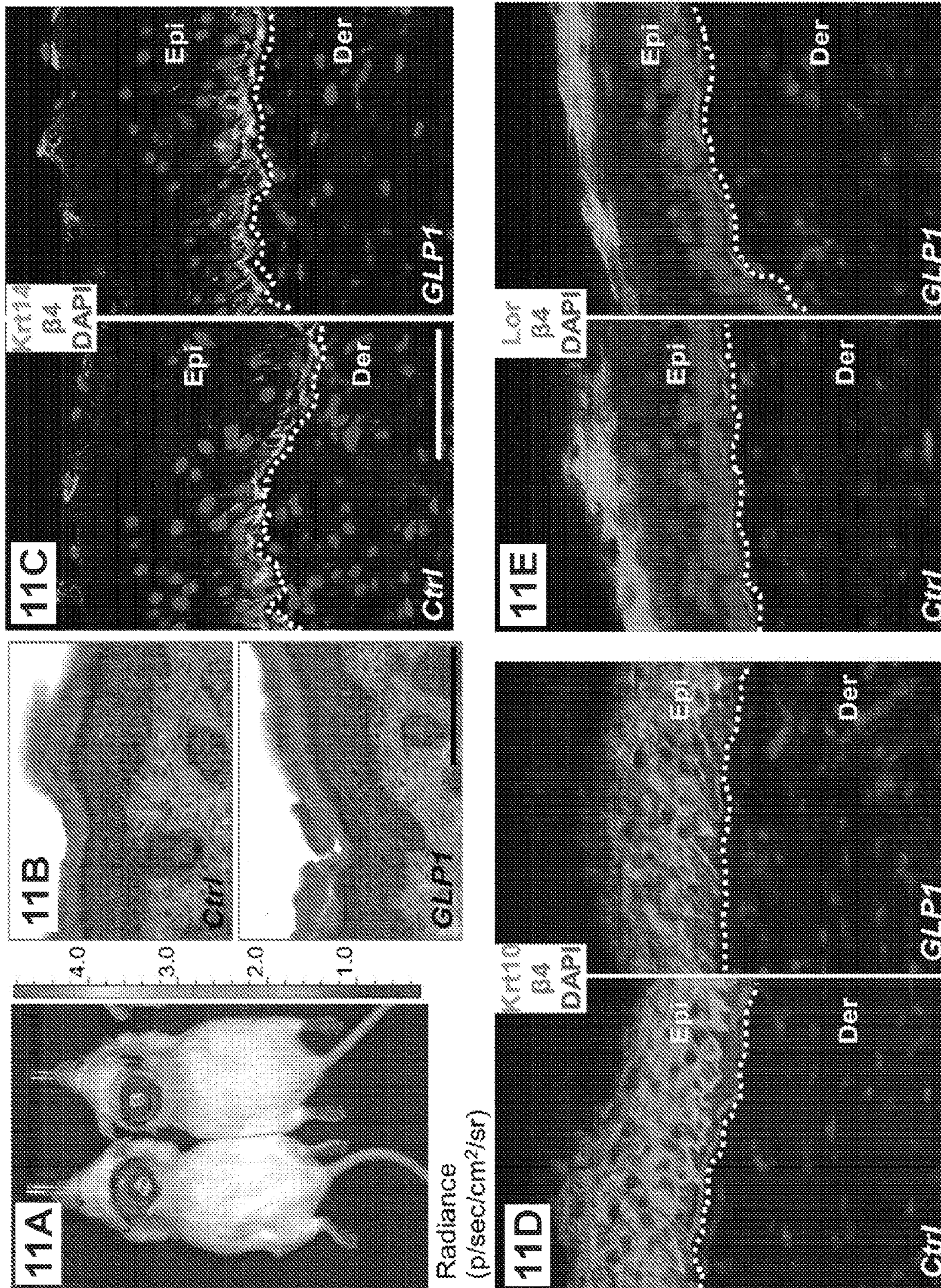

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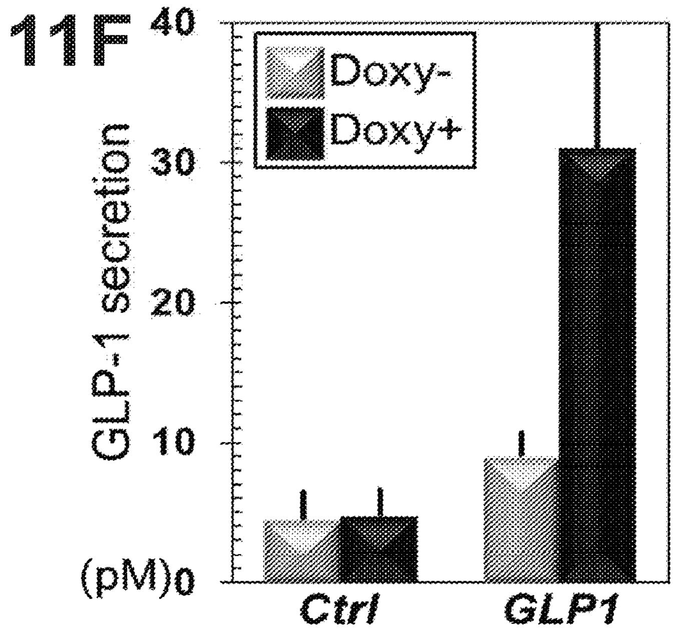

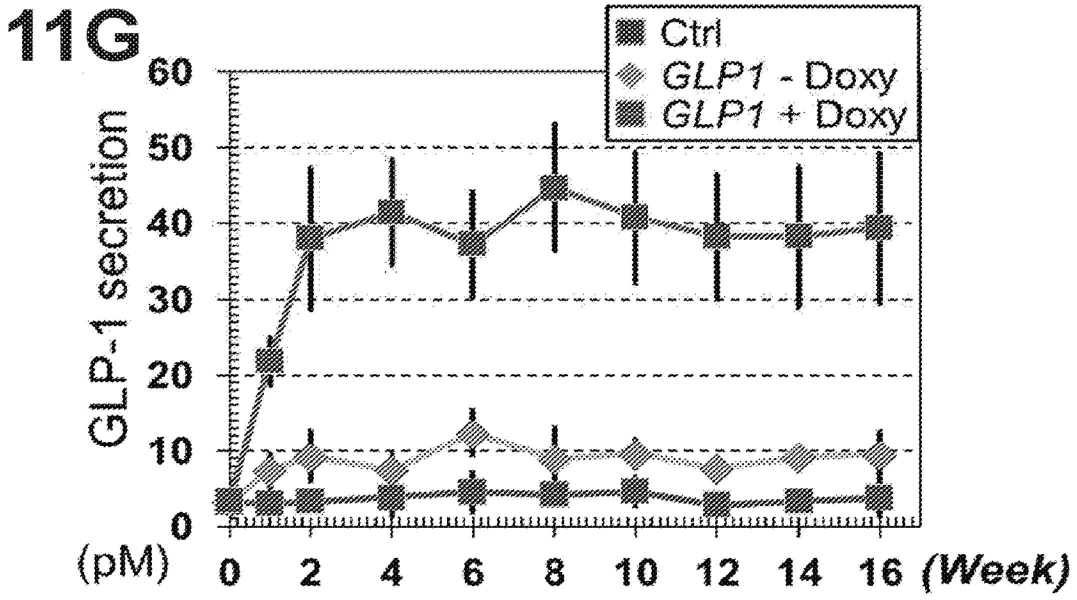

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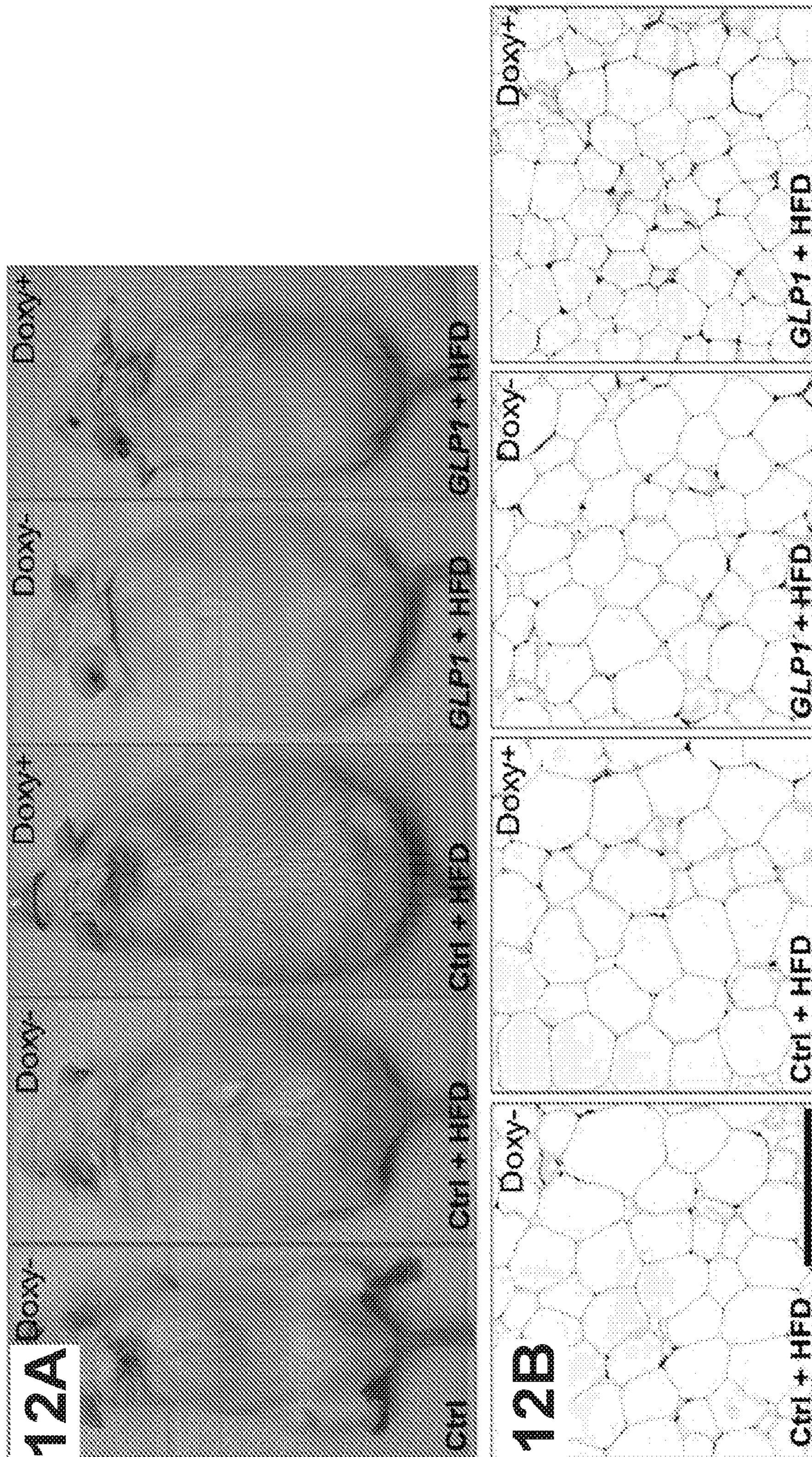

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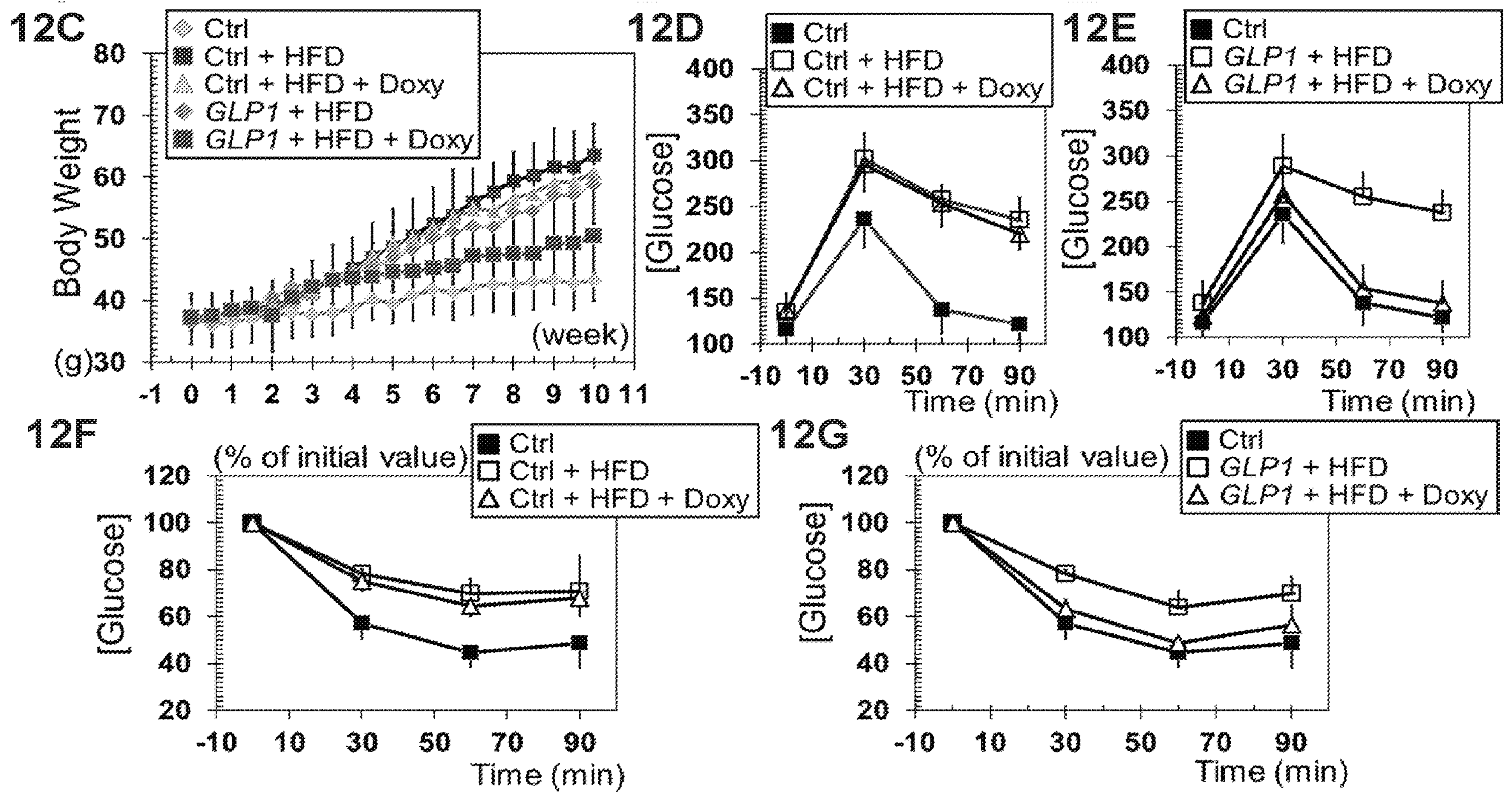

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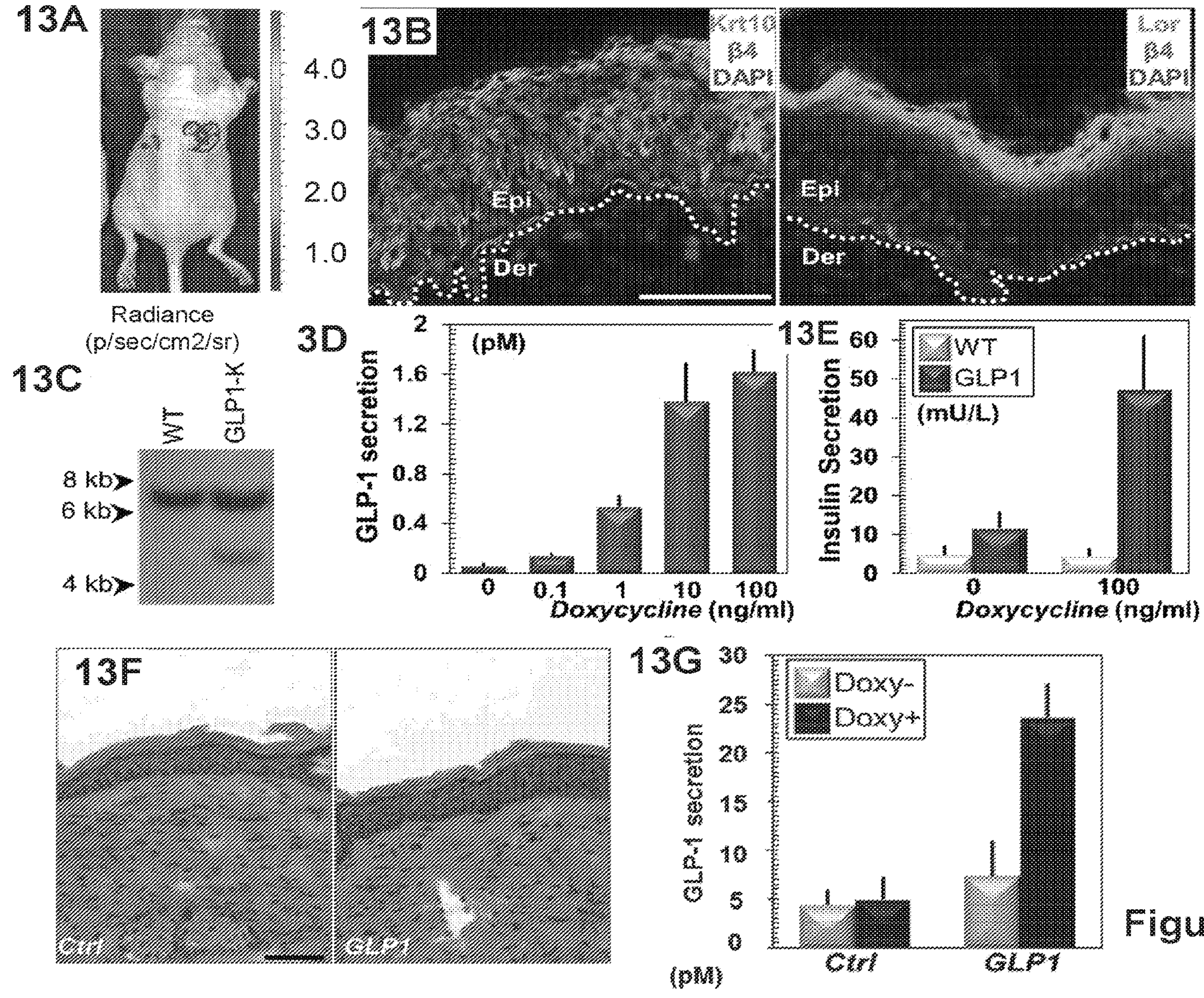
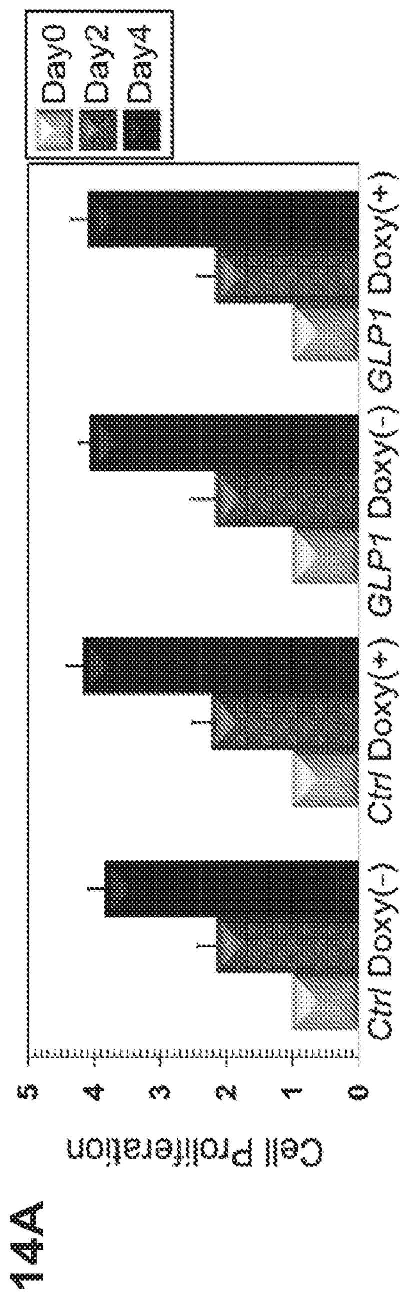


Figure 13

14A



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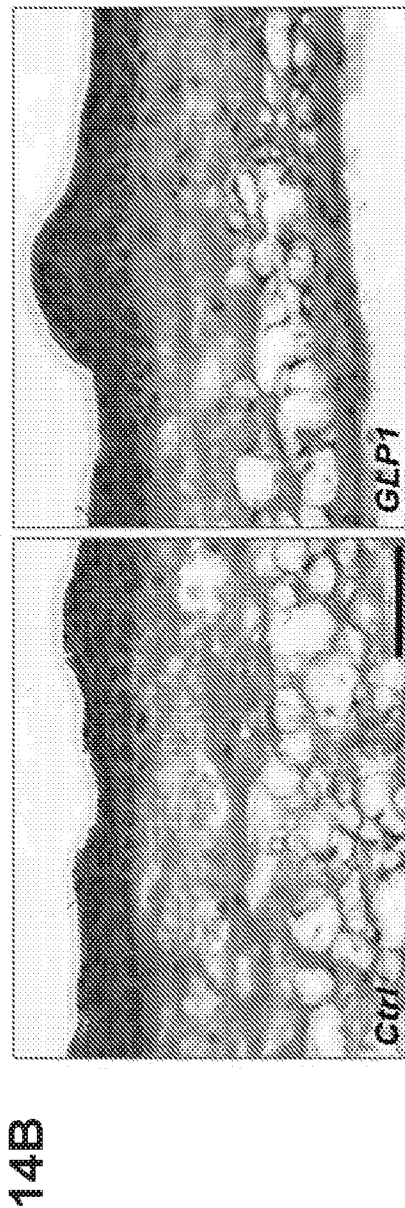

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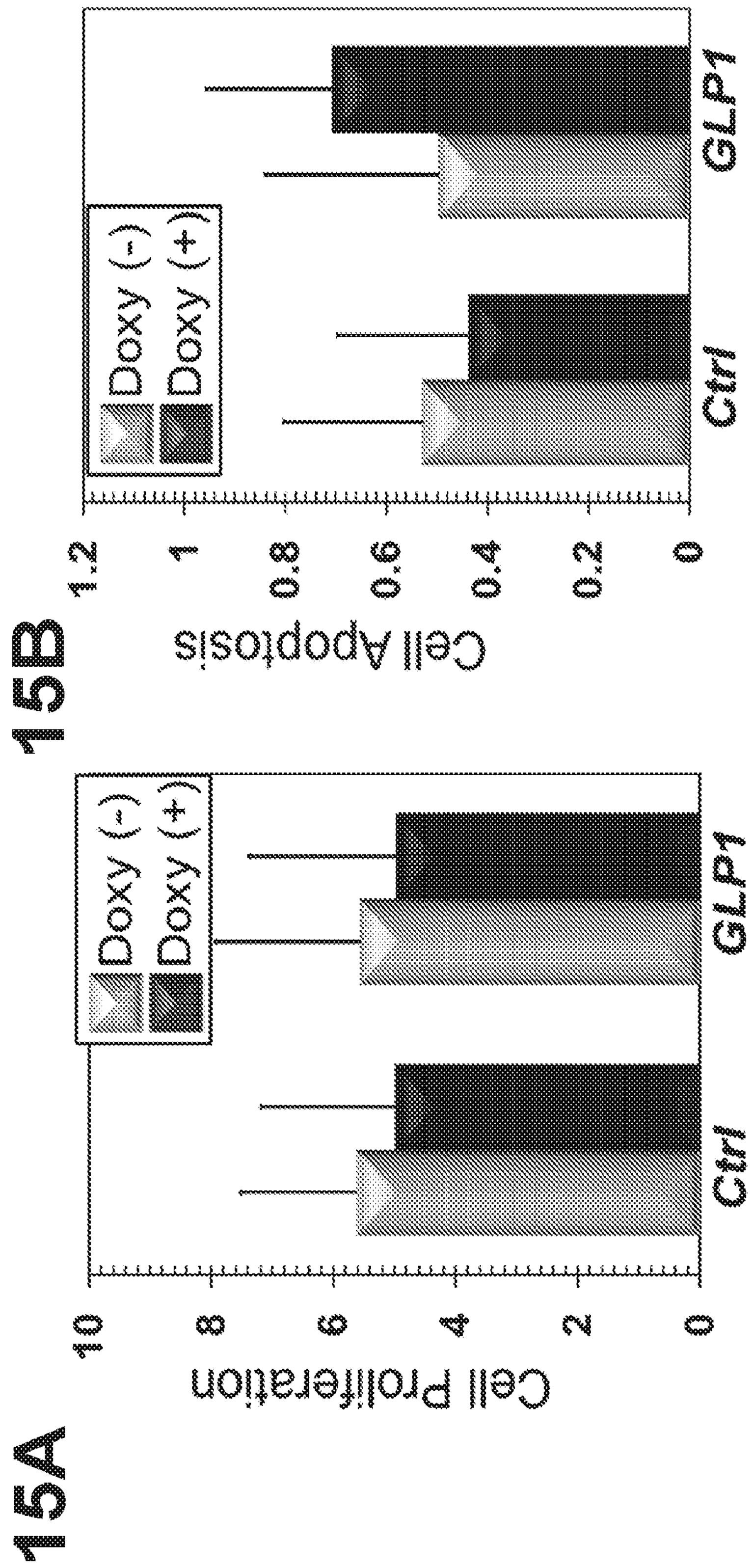

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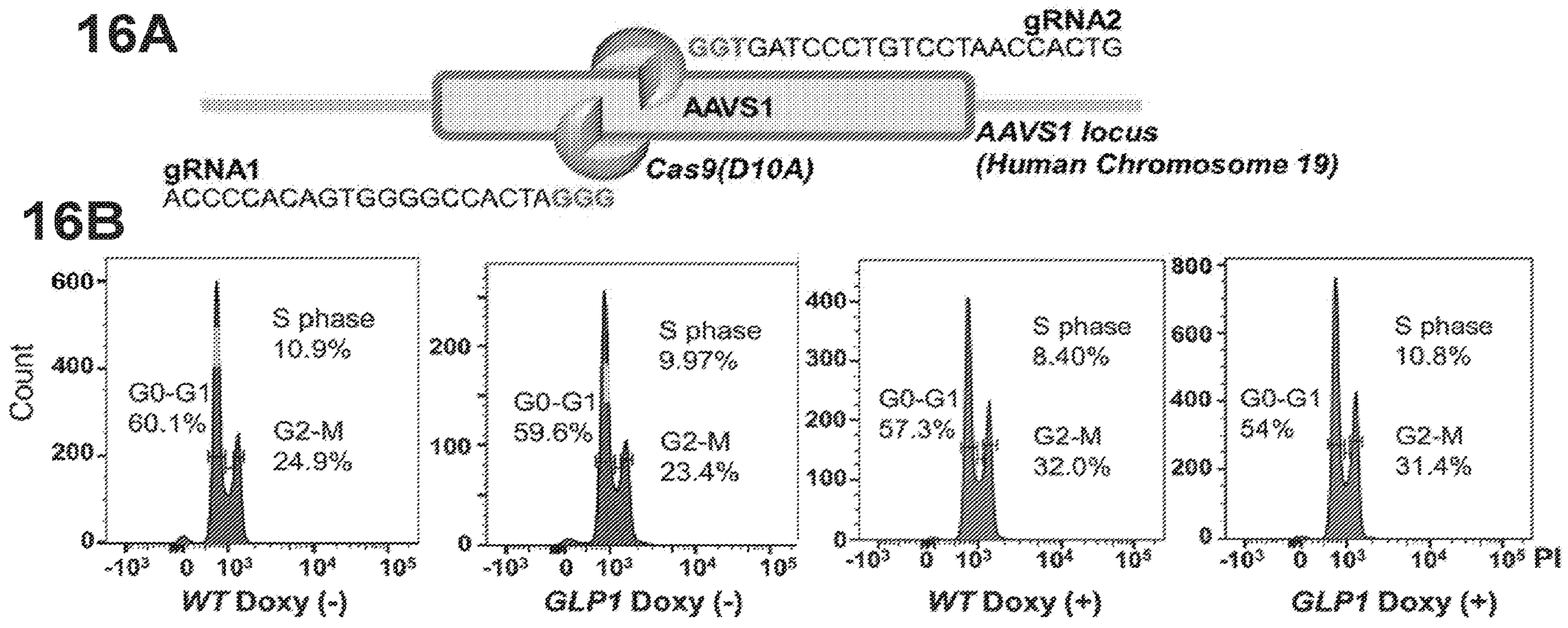

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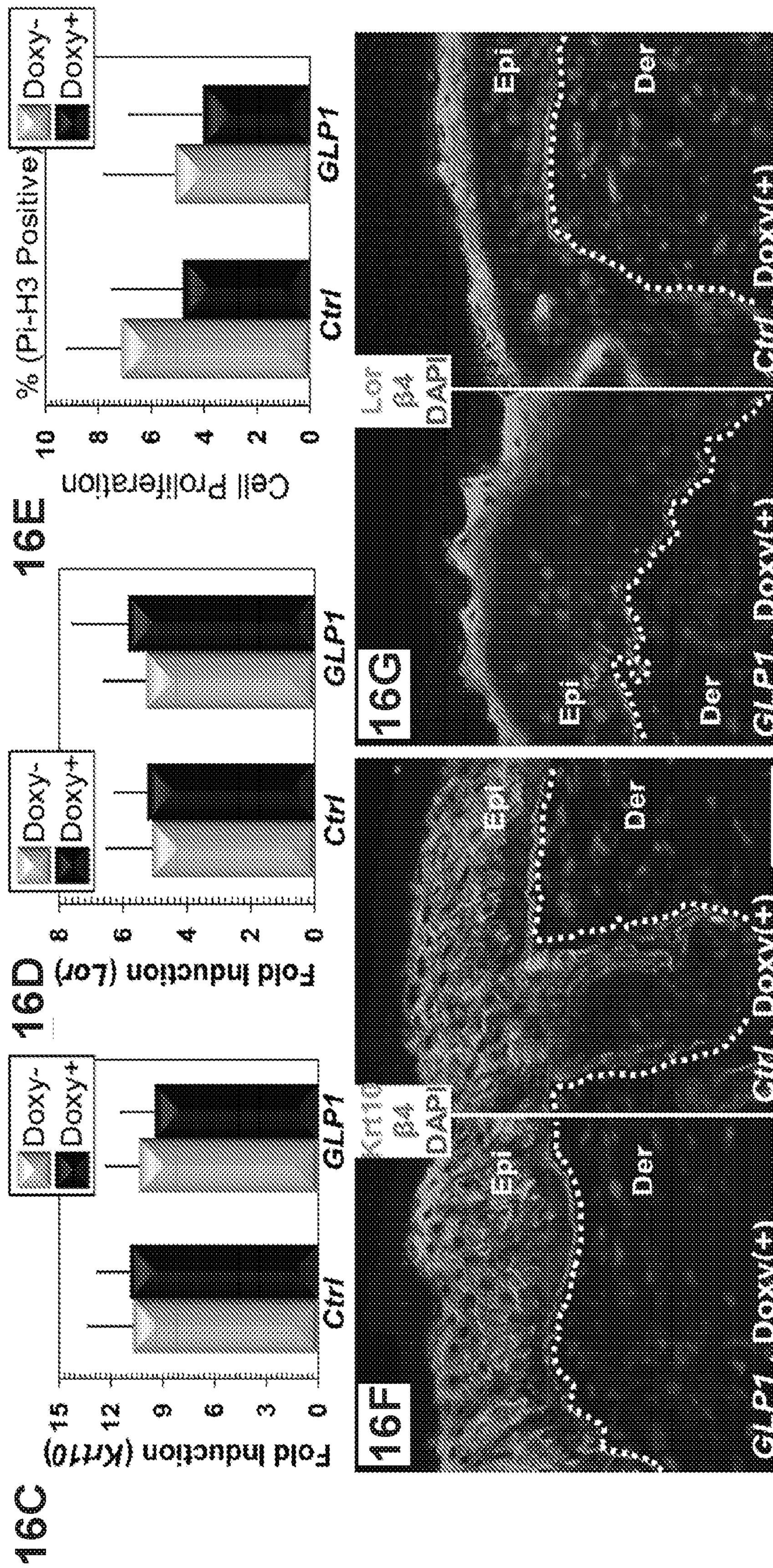

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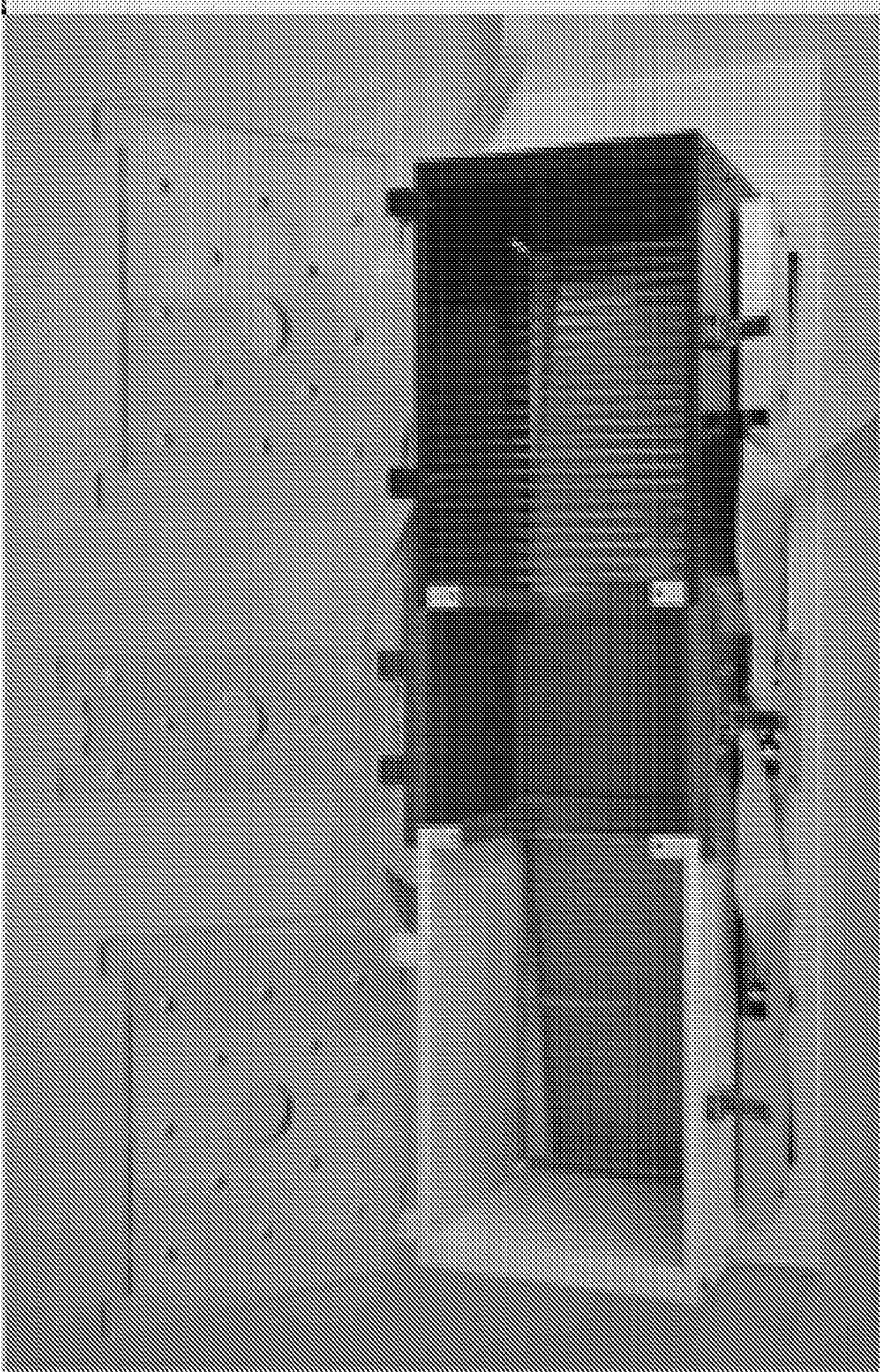

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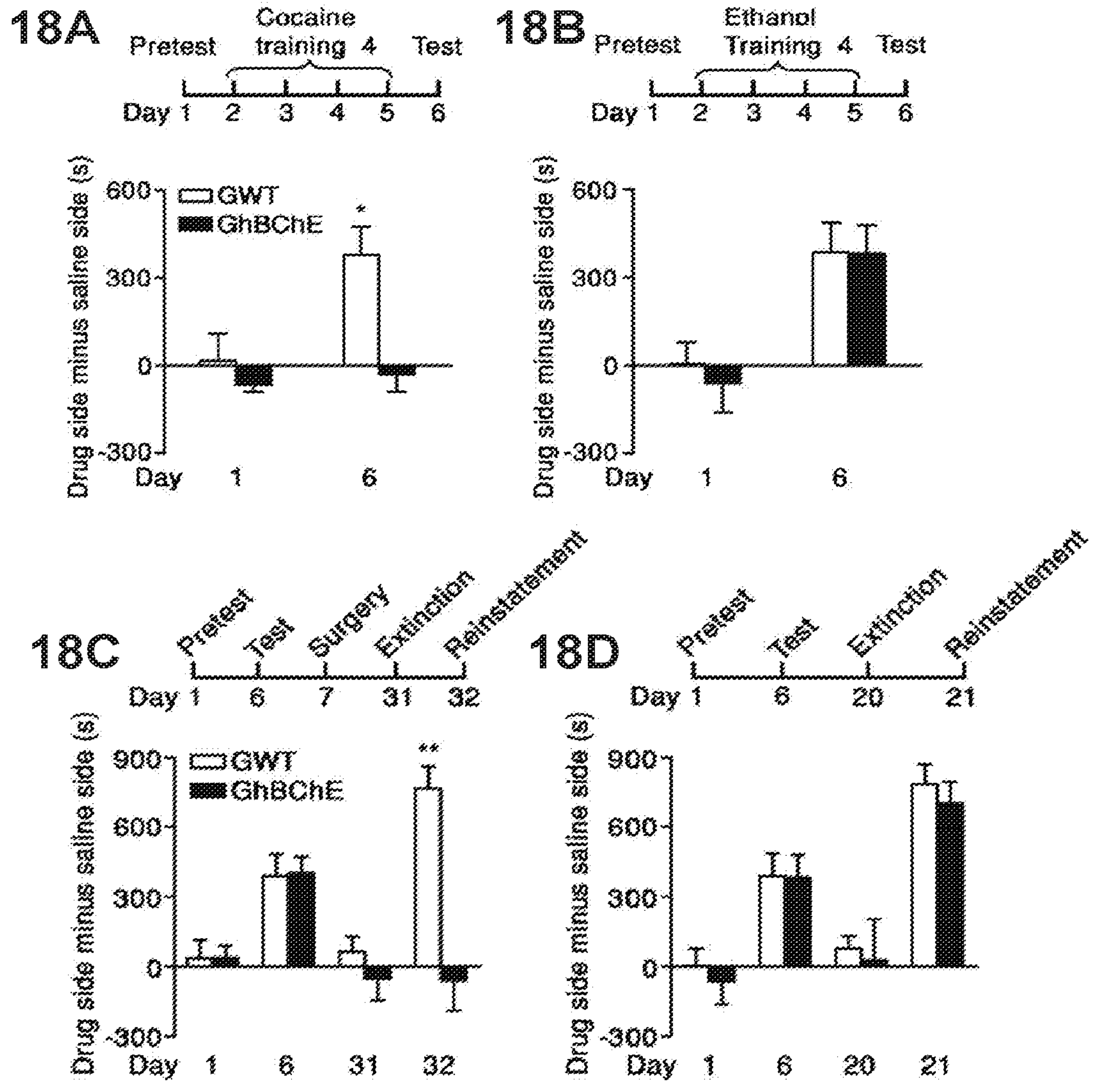

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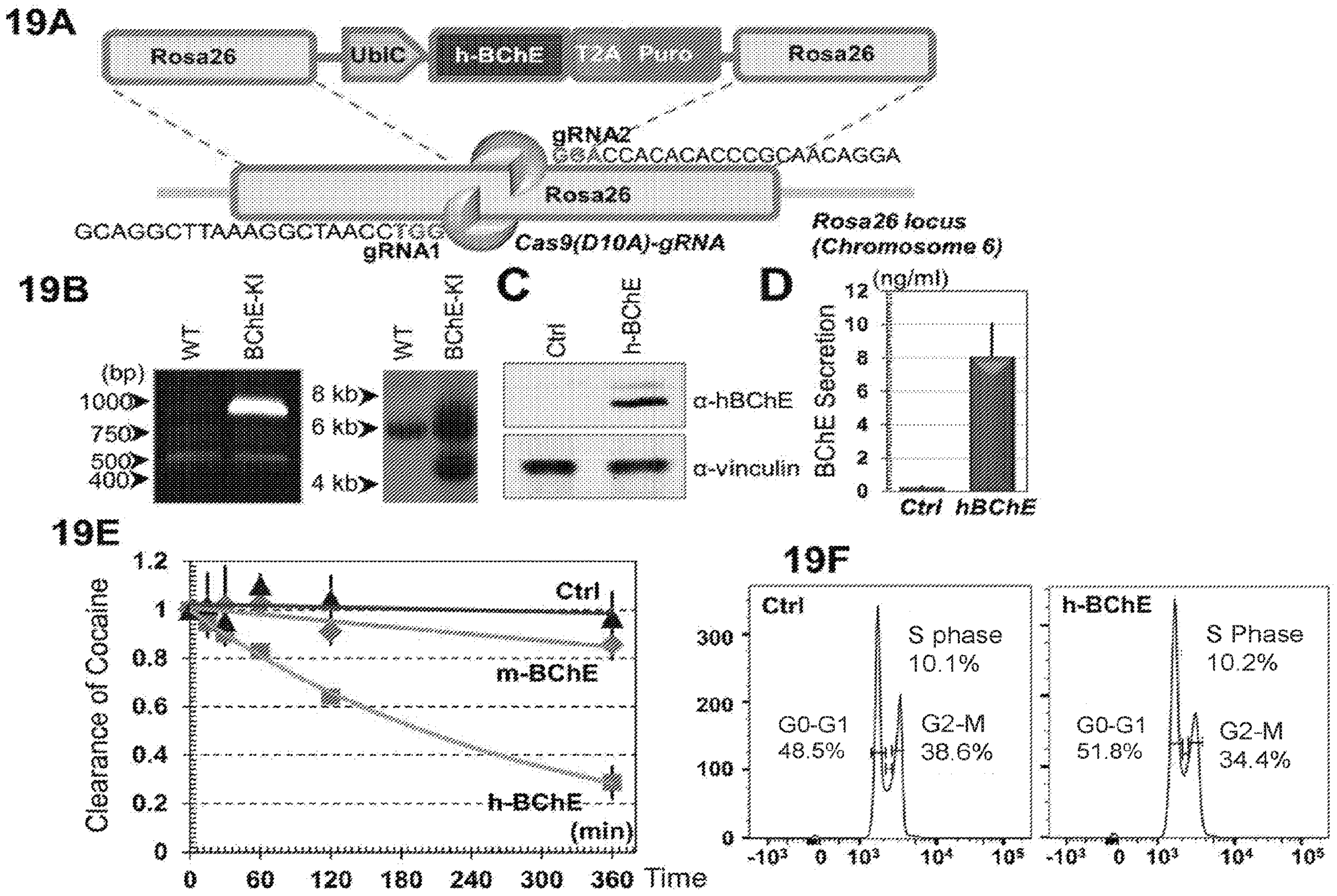

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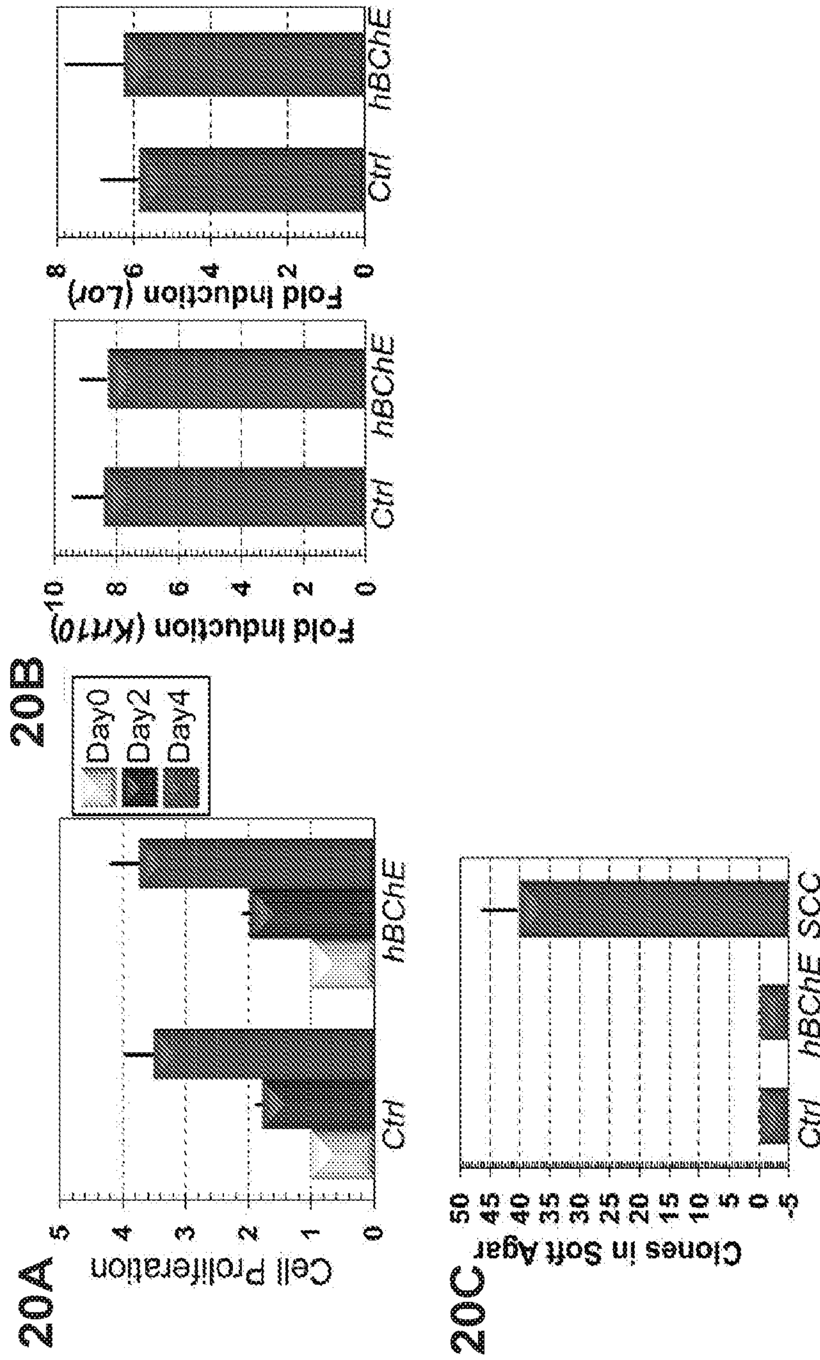

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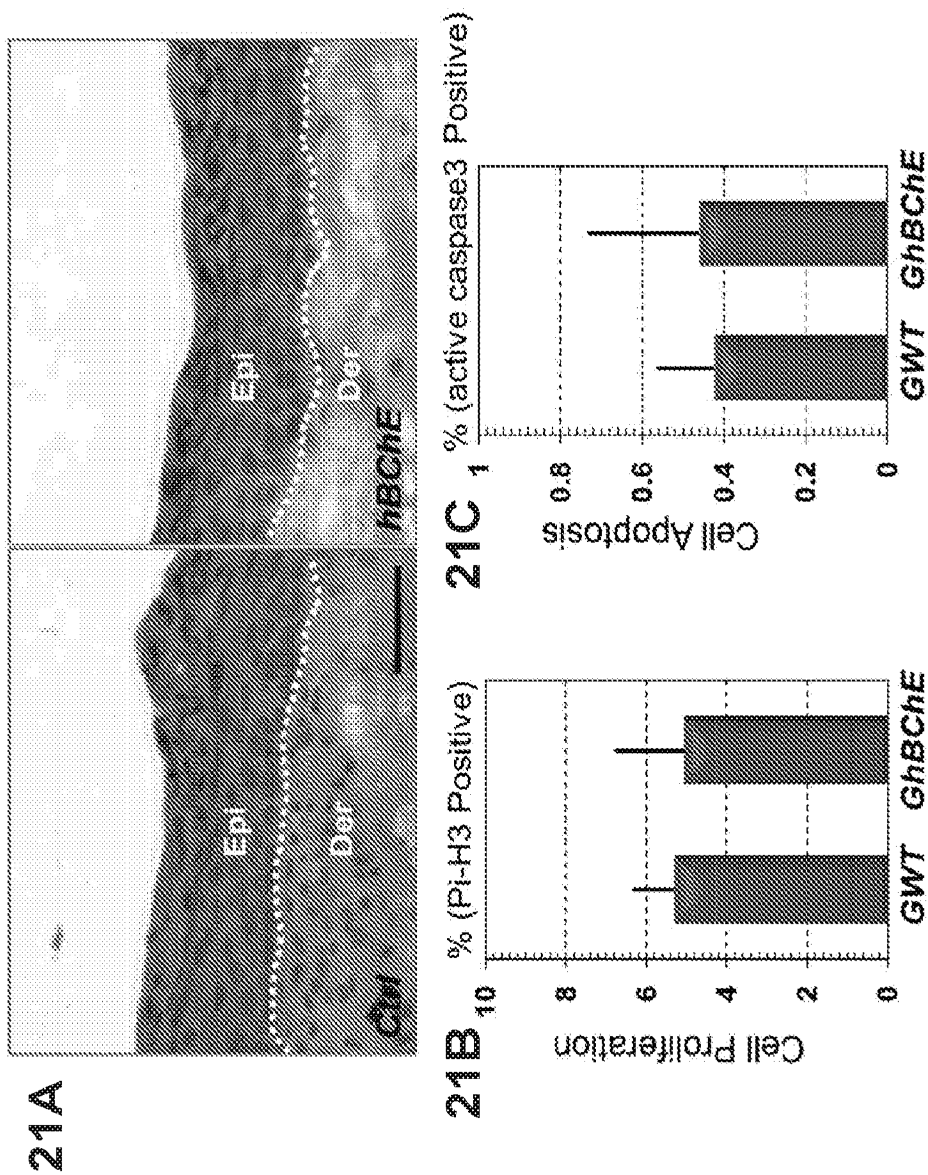

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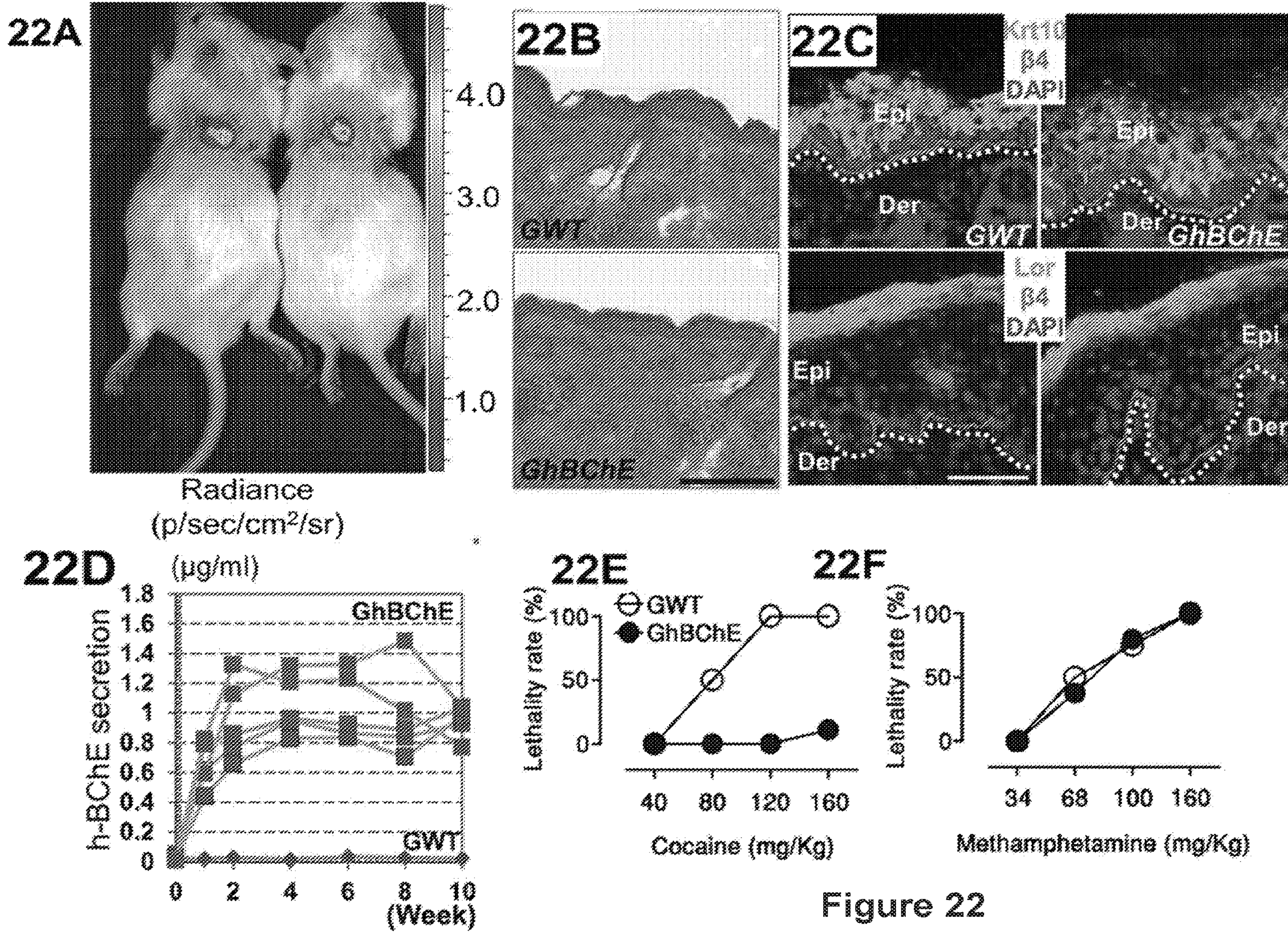

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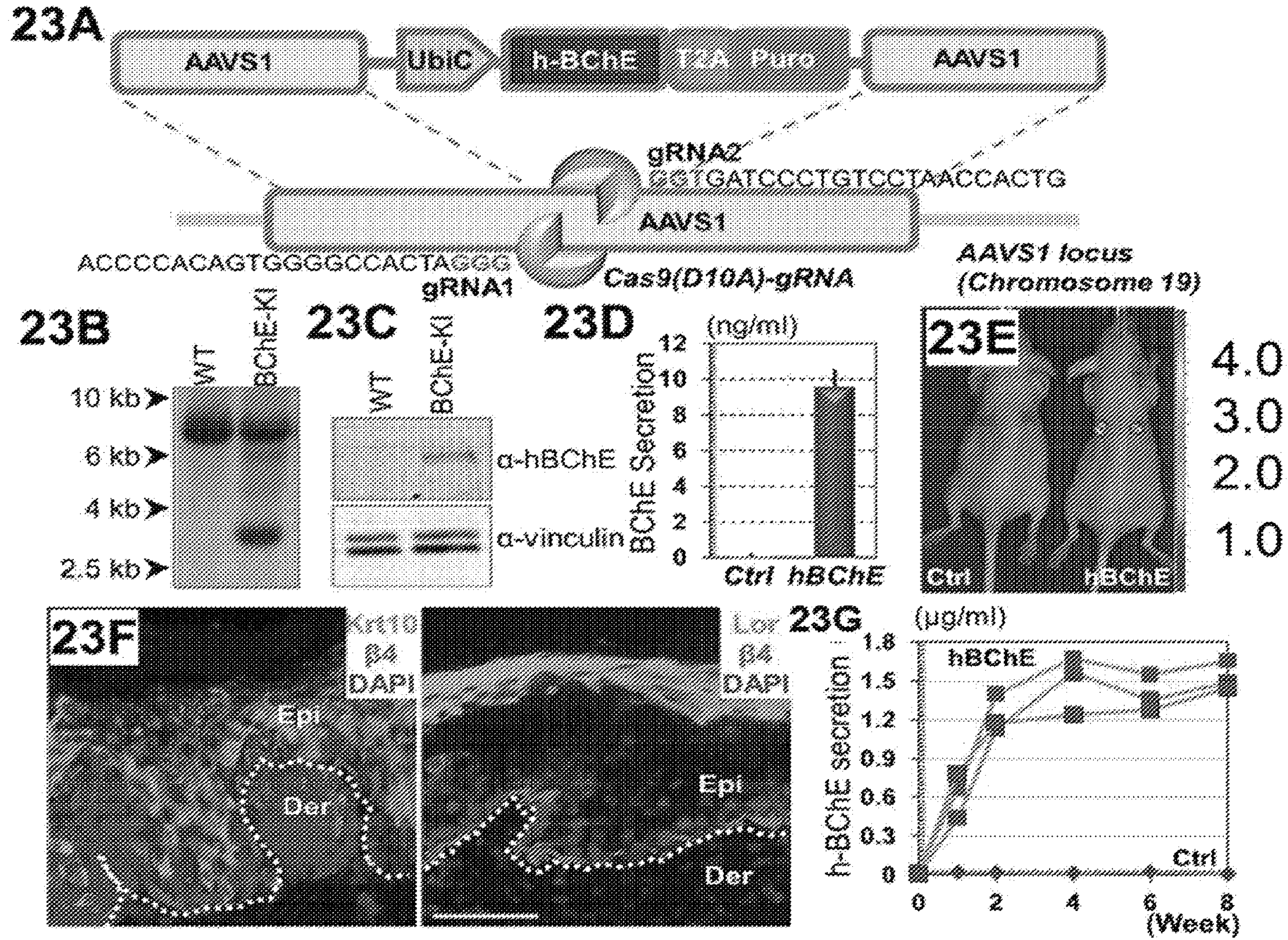

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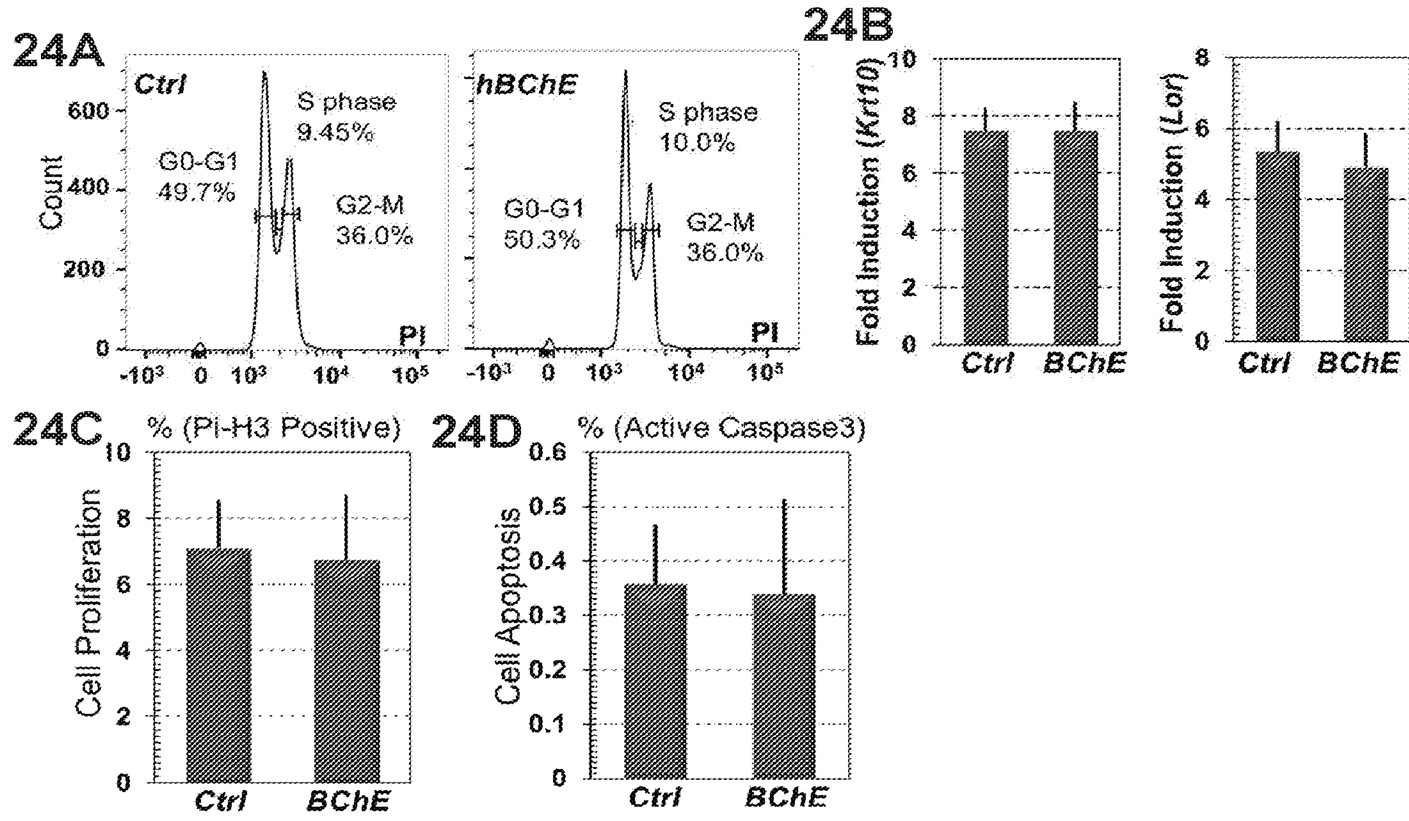
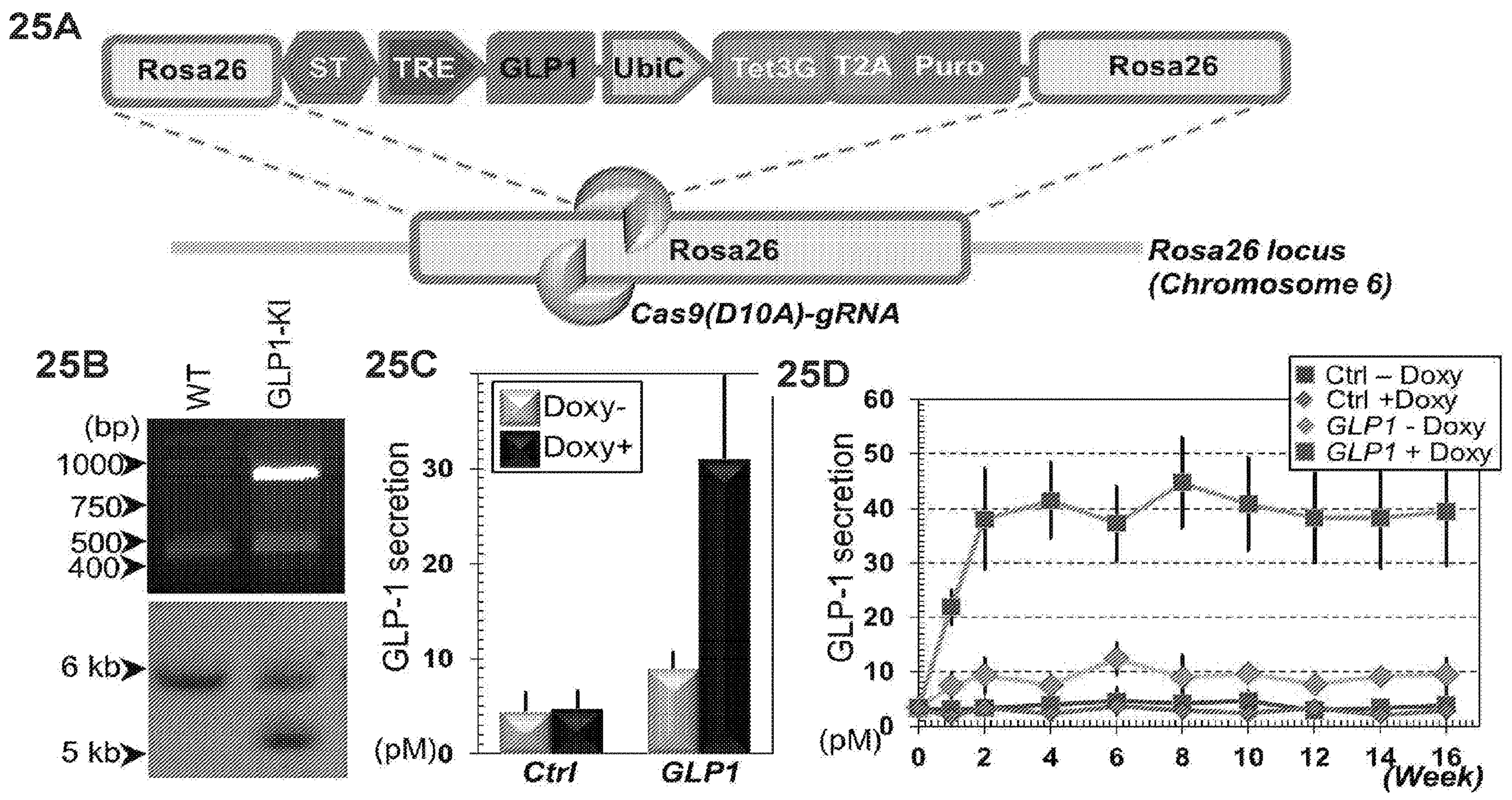


Figure 24



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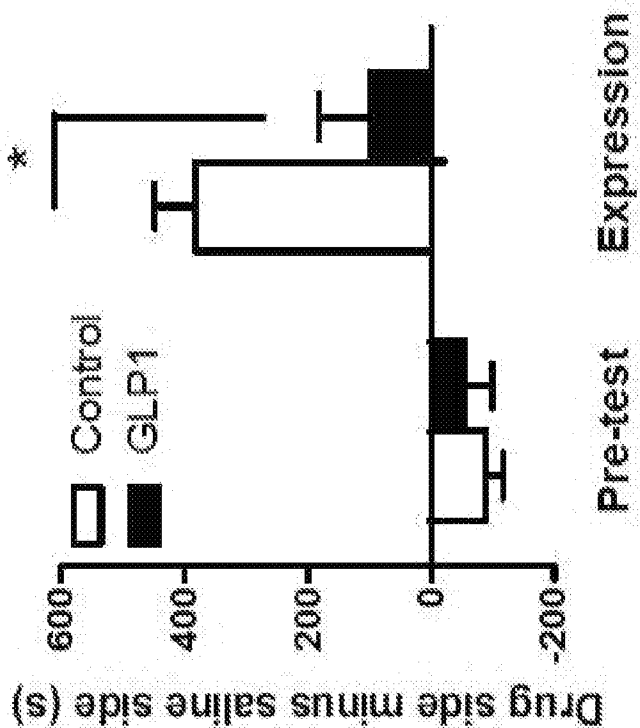
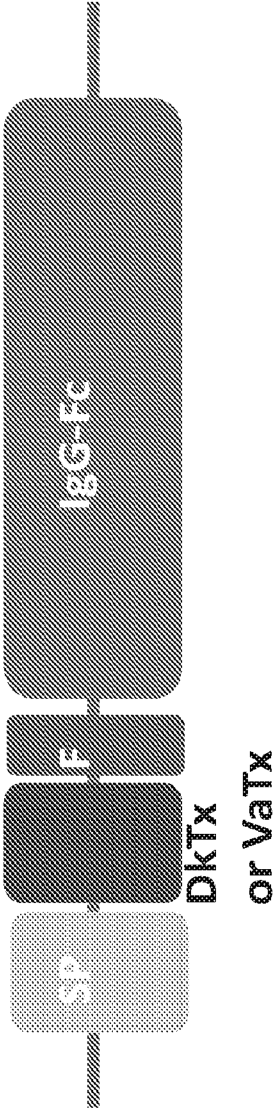


Figure 26



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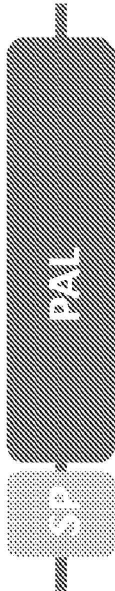

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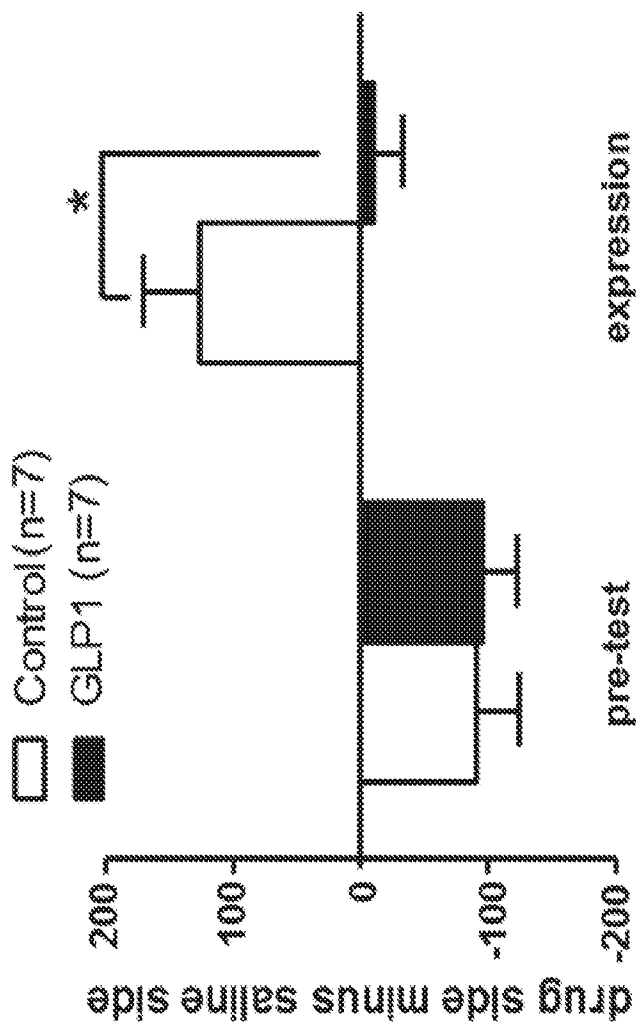

Figure 29

TISSUE ORGANOIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a national stage application under 35 U.S.C. § 371 of International Application No. PCT/US2017/065374, filed Dec. 8, 2017, which claims the benefit of U.S. Provisional Application No. 62/432346, filed Dec. 9, 2016; U.S. Provisional Application No. 62/432350, filed Dec. 9, 2016; and U.S. Provisional Application No. 62/554560, filed Sep. 5, 2017, the disclosures of each of which are explicitly incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant number R01AR063630 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] This disclosure relates to genetically engineered tissue organoids capable of noninvasively reporting sensed biochemical signals in an individual and/or providing a therapeutic agent to the individual to monitor and/or treat a disease or improve an individual's health.

Description of Related Art

[0004] Obesity and diabetes are examples of acute and growing global health concerns that can require careful monitoring and therapeutic intervention (see Ahima, R. S. Digging deeper into obesity. *J Clin Invest* 121, 2076-2079, 2011). Diabetes, in particular, often requires daily monitoring of blood glucose levels. However, such constant blood monitoring can be inconvenient, time consuming, and painful.

[0005] In addition, there are numerous other diseases such as phenylketonuria, substance addiction, and heart disease, to name a few, that exhibit elevated blood concentrations of specific disease-associated indicators or blood factors (e.g., amino acids, illicit chemicals, proteins, lipids, enzymes, metabolites, etc.). However, while certain diseases like diabetes can require constant indicator monitoring (i.e., blood glucose levels), others may only require periodic monitoring such as once a week or once a month or may even only require sporadic, on-demand monitoring.

[0006] Recent advances in blood factor monitoring have provided improved options for individuals, such as diabetics needing to monitor blood glucose levels. For example, implantable glucose monitors enable diabetics to continuously monitor blood glucose levels remotely using compatible smart devices. Indeed, biointegrated sensors can address various monitoring challenges in medicine by transmitting a wide variety of biological signals, including electrophysiological, physiological, and biochemical information continuously (see Kim et al. Flexible and stretchable electronics for biointegrated devices. *Annual review of biomedical engineering* 14, 113-128, 2012).

[0007] Concomitant with the requirements of blood monitoring for some individuals is the need to receive therapeutic agents to counter the underlying disease causing the elevated concentrations of disease-associated indicators. For example, diabetic individuals often require insulin supplementation. Insulin supplementation is typically self-administered by injection, which similar to blood monitoring, can be inconvenient, difficult to remember, time consuming, and painful. Fortunately, as with advances in blood monitoring technology, new biointegrated devices are becoming available that provide greater convenience to individuals requiring periodic, self-administered monitoring and administration of therapeutic agents. For example, devices combining glucose monitors and insulin pumps are available that constantly monitor glucose levels and periodically deliver rapid- or short-acting insulin through a catheter, as required. Further, "smart insulin patches" are being developed that integrate thin polymeric platforms covered with needles with live beta cells.

[0008] There are still other diseases where proteins or metabolites are either non-functional or expressed at insufficient levels but do not increase concentrations of a particular, telltale, disease-associated indicator. For example, hemophilia A and hemophilia B are genetic diseases where individuals express insufficient amounts of clotting factors VIII and IX, respectively. Yet, hemophiliacs can be treated by replacing these missing clotting factors which can be harvested from human blood or made recombinantly.

[0009] While advancements in biointegrated device design and fabrication using novel nano-materials has greatly accelerated development for applications in vivo, including brain, heart, and skin (see Kim et al. and Choi et al. Recent Advances in Flexible and Stretchable Bio-Electronic Devices Integrated with Nanomaterials. *Advanced materials* 28, 4203-4218, 2016), the requisite biomechanical interfaces for these devices still remain problematic. For example, stability, biocompatibility, and potential infection from the implanted devices remain technically challenging to the field. Moreover, current biointegrated devices either require replacement of batteries and/or therapeutic agent reservoirs and/or must be replaced themselves once their useful lifespan has been reached.

[0010] In light of the foregoing, there is a need for improved, stable, and permanent means for constantly monitoring a disease state and/or administering therapeutic agents and that can be physiologically tailored for a specific individual.

SUMMARY OF THE INVENTION

[0011] Provided herein are physiologically-tailored tissue organoids and methods of their use for monitoring and treating diseases.

[0012] In a first aspect, the invention provides a physiologically-tailored tissue organoid that includes a plurality of genetically engineered cells including at least one recombinant gene encoding a reporter molecule. The reporter molecule produces a detectable signal when associated with and/or contacting a predetermined blood factor. In one embodiment, tissue organoid includes a stratified skin graft grown from cells taken from an individual. In another embodiment, the tissue organoid includes a cultured skin graft grown from embryonic stem cells, human induced pluripotent stem cells, epidermal stem cells, or keratinocytes. In one embodiment, when the tissue organoid is biontegrated into an individual, the tissue organoid expresses the reporter molecule in proportion to a concentration of a blood factor in the individual's blood. In a further

embodiment, the blood factor includes a cell, an enzyme, a protein, a polypeptide, an amino acid, a polynucleotide, a nucleic acid, a sugar, a lipid, a metabolite, a synthetic chemical compound, a naturally occurring chemical compound, a mineral, a metal, a bacterium, a virus, a prion, a disease indicator, or combinations thereof. In one embodiment, the physiologically-tailored tissue organoid is biointegrated into an individual. The individual can be an animal.

[0013] In a second aspect, the invention provides a physiologically-tailored tissue organoid includes a plurality of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent. When the therapeutic agent is administered to an individual in need thereof, the therapeutic agent improves the individual's health. In one embodiment, the therapeutic agent comprises an enzyme, a protein, a clotting factor, a vitamin, a peptide, a lipid, a toxin, or a combination thereof. In another embodiment, expression of the therapeutic agent is inducible by an inducer. The inducer can be one or more of heat, cold, light, a protein, a hormone, a lipid, a chemical, a metabolic change, an electric potential or field, and combinations thereof. In one embodiment, when expression of the therapeutic agent is induced, the therapeutic agent is released into the individual's circulation. In another embodiment, the physiologically-tailored tissue organoid can biointegrated into an individual. The individual can be an animal. The predetermined blood factor can be glucose.

[0014] In a third aspect, the invention provides a physiologically-tailored tissue organoid including a plurality of genetically engineered cells comprising at least one recombinant gene encoding a reporter molecule and at least one recombinant gene encoding a therapeutic agent. The therapeutic agent is expressed as a function of expression of the reporter molecule.

[0015] In a fourth aspect, the invention provides a method of monitoring a blood factor including biointegrating a tissue organoid according to any of the preceding aspects or embodiments into an individual and detecting a detectable signal produced by a reporter molecule expressed by the tissue organoid in response to a blood factor. The detectable signal is proportional to a concentration of the blood factor in the individual. In one embodiment of the method, the tissue organoids are biointegrated into an individual by grafting or surgical implantation. In another embodiment of the method, the detectable signal is detected by one or more of a fluorometer, a colorimeter, a bioluminescence monitoring system, and an electrical system with proper electrodes.

[0016] In a fifth aspect, the invention provides a method of treating a disease in an individual in need thereof. The method includes biointegrating a tissue organoid according to any of the preceding aspects or embodiments into an individual and administering a therapeutic agent to the individual. In one embodiment, the individual is a human.

[0017] In a sixth aspect, the invention provides a physiologically-tailored tissue organoid includes a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent. The therapeutic agent comprises at least one of mGLP1 and hBChE, and when the therapeutic agent is administered to an individual in need thereof, the therapeutic agent improves the individual's health. In one embodiment, the therapeutic agent improves the individual's health by reducing the individual's seeking and/or consumption of at least one of nicotine, alcohol, cocaine, and amphetamine.

[0018] In a seventh aspect, the invention provides a method of treating cocaine addiction in an individual in need thereof, the method including contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein the therapeutic agent comprises hBChE. In one embodiment, the tissue organoid is transplanted into the individual. In one embodiment, hBChE is expressed constitutively or by induction with an inducer. In one embodiment, the expression of hBChE hydrolyzes cocaine in the blood of the individual. In one embodiment, the expression of hBChE causes decreased cocaine seeking and/or consumption by the individual.

[0019] In an eighth aspect, the invention provides a method of treating AUD in an individual in need thereof, the method including contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein the therapeutic agent comprises mGLP1 or an analog thereof. In one embodiment, the tissue organoid is transplanted into the individual. In one embodiment, the mGLP1 or the analog thereof is expressed constitutively or by induction with an inducer. In one embodiment, the expression of mGLP1 or the analog thereof causes decreased alcohol seeking and/or consumption by the individual.

[0020] In a ninth aspect, the invention provides a method of treating nicotine addiction in an individual in need thereof, the method including contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein the therapeutic agent comprises mGLP1 or an analog thereof. In one embodiment, the tissue organoid is transplanted into the individual. In one embodiment, mGLP1 or the analog thereof is expressed constitutively or by induction with an inducer. In one embodiment, the expression of mGLP1 or the analog thereof causes decreased nicotine seeking and consumption by the individual.

[0021] In a tenth aspect, the invention provides a method of treating amphetamine addiction in an individual in need thereof, the method including contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein the therapeutic agent comprises mGLP1 or an analog thereof. In one embodiment, the tissue organoid is transplanted into the individual. In one embodiment, mGLP1 or the analog thereof is expressed constitutively or by induction with an inducer. In one embodiment, the expression of mGLP1 causes decreased amphetamine seeking and consumption by the individual.

[0022] In an eleventh aspect, the invention provides a method of treating obesity in an individual in need thereof, the method including contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein the therapeutic agent comprises peptide tyrosine tyrosine (PYY) or an analog thereof. In one embodiment, the tissue organoid is transplanted into the individual. In one embodiment, PYY or the analog thereof is expressed constitutively or by induction

with an inducer. In one embodiment, the expression of PYY causes decreased food seeking and consumption by the individual.

[0023] In a twelfth aspect, the invention provides a method of treating the effects of aging in an individual in need thereof, the method including contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein the therapeutic agent comprises tissue inhibitor of metalloproteinases 2 (TIMP2) or an analog thereof. In one embodiment, the tissue organoid is transplanted into the individual. In one embodiment, TIMP2 or the analog thereof is expressed constitutively or by induction with an inducer. In one embodiment, the expression of TIMP2 causes a decrease in the negative effects of aging in an individual. In one embodiment, the negative effects of aging include memory loss, reduced vascular response, and/or reduced immune response.

[0024] These and other features and advantages of the present invention will be more fully understood from the following detailed description taken together with the accompanying claims. It is noted that the scope of the claims is defined by the recitations therein and not by the specific discussion of features and advantages set forth in the present description.

DESCRIPTION OF DRAWINGS

[0025] FIG. **1**. Development of a mouse-to-mouse cutaneous gene transfer model with immunocompetent hosts. FIG. **1**A: Diagram of the cutaneous gene transfer strategy. Primary epidermal stem cells are isolated and cultured from patients' skin biopsy. The cells are genetically modified with genome editing technology, and the resultant cells are used to generate skin organoids and transplant to the same patient for clinical applications. FIG. **1**B: Images of immunocompetent mice (CD1 strain or C57BL/6J strain) grafted with isogenic skin organoids with (left two panels) or without (right panel, direct grafting) the assistance of skin dome chamber for transplantation. Intravital imaging shows efficient incorporation of grafted cells expressing luciferase (Luc) upon engraftment. Ctrl: control. FIG. **1**C: Sections of CD1 skin with grafted H2B-RFP-expressing keratinocytes were stained with DAPI. Dotted lines denote dermal—epidermal boundaries. Arrow heads denote the boundary between grafted skin and host skin. Scale bar=50 μm. Epi: epidermis, Der: dermis, HF: hair follicle. FIGS. D-F: Sections of grafted skin and adjacent host skin were immunostained with different antibodies as indicated (Krt14: keratin 14, Krt10: keratin 10, Lor: Loricrin, β4: β4-integrin, CD104). Scale bar=50 μm. FIG. **1**G: Proliferation of epidermal cells in host or grafted skin one or four weeks post skin transplantation was determined and quantified by immunohistological staining with antibody against phosphor-histone H3. A significant decrease in cell proliferation was observed over time after skin grafting, most likely due to skin wound healing. There is no significant change in cell proliferation between the grafted skin and adjacent host skin ($P>0.05$). Error bar represents s.d. (standard deviation) unless otherwise indicated. The sample size (n)=7 (7 representative sections obtained from 3 different animals for each group).

[0026] FIG. **2**. Engineering skin epidermal stem cells with CRISPR. FIG. **2**A: Diagram showing the Rosa26 targeting strategy for expression of glucose sensor GGBP. The targeting vector contains two Rosa26 homology arms, flanking the expression cassette for GGBP and a selection marker (puromycin resistant gene, Puro) by a constitutive promoter UbiC (Ubiquitin C promoter). GGBP and Puro are separated by a self-cleavable peptide T2A. FIG. **2**B: Integration of the targeting vector into Rosa26 locus was verified by PCR (left panel) and southern blotting (right panel). Positive clones displayed an additional band of the expected size. FIG. **2**C: Expression of GGBP sensor was confirmed in targeted cells by fluorescence imaging. Scale bar=20 μm. FIG. **2**D: FRET ratio images were pseudocolored to demonstrate glucose-dependent ratio changes in engineered cells. Red indicates high (H) FRET efficiency, and blue represents low (L) efficiency. M: medium FRET efficiency. Integration of the ratio over the entire cells was used to quantify the FRET ratio changes. FIG. **2**E: The FRET ratio change of GGBP reporter was determined in the presence of various monosaccharides or oligosaccharides at different concentration. Note: only glucose and galactose led to significant FRET ratio changes ($P<0.01$). n>6 (individual cells). FIG. **2**F: FACS (fluorescence activated cell sorting) demonstrated similar cell cycle profiles for WT (wild type) and GGBP-expressing epidermal stem cells. PI: propidium iodine. FIG. **2**G: Western blotting analysis of early (Krt10) and late (loricrin) differentiation marker expression in WT and GGBP-expressing cells upon calcium shift. Band intensity was determined by densitometry and fold of induction was quantified. n=4 (4 independent tests). $P>0.05$. FIG. **2**H: WT cells or GGBP cells were tested for anchorage independent growth in soft agar. Note: no growth for WT or GGBP cells, but tumor initiating cells isolated from skin SCC (squamous cell carcinoma) can readily produce colonies in soft agar plate. n=3. $P<0.01$ (between WT and SCC or GGBP and SCC).

[0027] FIG. **3**. Transfecting skin cells in vivo with electroporation. FIG. **3**A: CD1 mice were electroporated intradermally with plasmid DNA encoding luciferase. Expression of luciferase was determined by bioluminescence imaging two days after treatment. FIG. **3**B: CD1 mice were electroporated intradermally with plasmid DNA encoding tdTomato. Expression of red fluorescence protein was determined by intravital imaging with two-photon microscope. Arrows denote tdTomato-expressing cells in skin.

[0028] FIG. **4**. Monitoring changes of blood glucose level with GGBP reporter in vivo. FIG. **4**A: Skin organoids were developed from control or GGBP-producing cells, and transplanted to CD1 mice. FIG. **4**B: Glucose fluctuation was induced in grafted animal with IPGTT (intraperitoneal glucose tolerance test). FRET ratio images were pseudocolored to demonstrate glucose-dependent ratio changes in grafted skin. Red indicates high FRET efficiency, and blue represents low efficiency. FIG. **4**C and **4**D: Correlation of FRET ratio with blood glucose concentration upon IPGTT (intraperitoneal glucose tolerance test). n=9 (integrated FRET value from 9 different fields at each time point). FIG. **4**E and **4**F: Insulin injection was used to induce hypoglycemia in the grafted animals. Intravital imaging with the grafted skin demonstrated the correlation between FRET ratios of GGBP reporter with blood glucose concentration. n=9 (integrated FRET value from 9 different fields at each time point). FIG. **4**G: Secretion of GLP1 in cell culture medium was determined by ELISA (enzyme-linked immunosorbent assay). n=3 (3 independent tests). $P<0.01$. FIG. **4**H: Conditioned

medium was collected from different cell cultures and used to treat starved insulinoma cells. Secretion of insulin in vitro was determined by ELISA. n=4 (4 independent tests). P<0.01. FIG. **4**I: Images of control and GLP1 animals fed with HFD (high fat diet). FIG. **4**J: Body weight change of different cohorts of mice measured from ~10 weeks of age. Note that the HFD induced significant obesity in control mice (P<0.01, between control mice with normal diet or HFD for week 8-10) but that expression of GLP1 inhibited weight gain (P<0.05, between GLP1/HFD and control/HFD groups for week 8-10). n=5 (animals). FIG. **4**K: Correlation of FRET ratio with blood glucose concentration over time upon IPGTT in control and GLP1 mice. n=9 (integrated FRET value from 9 different fields at each time point). FIG. **4**L and **4**M: Correlation of blood glucose concentration with GGBP FRET changes in control (L) and GLP1 (M) mice. n=9 (integrated FRET value from 9 different fields at each time point).

[0029] FIG. **5**. Expression of GGBP reporter in human epidermal stem cells with CRISPR. FIG. **5**A: Image of nude mouse grafted with organotypic human skin culture. Intravital imaging shows efficient incorporation of grafted cells expressing luciferase. FIG. **5**B: Sections of grafted skin and adjacent host skin were immunostained with different antibodies as indicated. Scale bar=50 μm. FIG. **5**C: Integration of the targeting vector into AAVS1 locus was verified by southern blotting. Positive clones display an additional band of the expected size. FIG. **5**D: Glucose fluctuation was induced in grafted animal with an IPGTT (intraperitoneal glucose tolerance test). FRET ratio images were pseudocolored to demonstrate glucose-dependent ratio changes in grafted skin. Red indicates high FRET efficiency, and blue represents low efficiency. FIG. **5**E and **5**F: Correlation of FRET ratio with blood glucose concentration upon IPGTT. n=9 (integrated FRET value from 9 different fields at each time point).

[0030] FIG. **6**. Development of a mouse-to-mouse cutaneous gene transfer model with immunocompetent hosts. FIG. **6**A: Diagram demonstrating the procedure for skin organotypic culture in vitro. Epidermal progenitor cells were plated on top of acellularized dermis, and then exposed to air/liquid interphase to induce differentiation and stratification as skin epidermis in vivo. FIGS. **6**B and **6**C: H/E (haematoxylin and eosin) staining of grafted skin and adjacent host skin one (B) or four (C) weeks after skin transplantation. Scale bar=50 μm. FIG. **6**D: Apoptosis of epidermal cells in host or grafted skin one or four weeks post skin transplantation was determined and quantified by immunohistological staining with antibody against active caspase 3. Error bar represents s.d. (standard deviation) unless otherwise indicated. n=4 (4 independent tests). P>0.05.

[0031] FIG. **7**. Engineering GGBP-producing skin epidermal progenitor cells with CRISPR. FIG. **7**A: Diagram showing Rosa26 targeting strategy for expression of GGBP. Expression vector encoding D10A mutant of cas9 and two gRNAs targeting Rosa26 locus is used to create the cleavage in the chromosomal DNA and enhance integration of GGBP targeting vector. FIG. **7**B: Cell proliferation of control (Ctrl) and GGBP-expressing cells. Fold increase of cell numbers is quantified for all cell types. n=3 (4 independent tests). P>0.05 for each time point between ctrl and GGBP groups. FIG. **7**C: Control or GGBP targeted epidermal progenitor cells can produce similar skin organoids in vitro. Scale bar=50 μm.

[0032] FIG. **8**. Engraftment of GGBP-expressing cells in vivo. FIGS. **8**A-**8**C: Sections of grafted skin were immunostained with different antibodies as indicated (Krt14: keratin 14, Krt10: keratin 10, Lor: Loricrin, β4: β4-integrin, CD104). Dotted lines denote dermal—epidermal boundaries. Epi: epidermis; Der: dermis. Scale bar=50 μm. FIG. **8**D: Proliferation of epidermal cells in control or GGBP skin grafts was determined and quantified by immunohistological staining with antibody against phosphor-histone H3. n=7 (7 sections obtained from 2 animals for each group). P>0.05. FIG. **8**E: Apoptosis of epidermal cells in host or grafted skin one or four weeks post skin transplantation was determined and quantified by immunohistological staining with antibody against active caspase 3. n=6 (6 sections obtained from 2 animals for each group). P>0.05. FIG. **8**F: Diagram showing Rosa26 targeting strategy for expression of GGBP and GLP1 simultaneously. Coding sequences of GGBP and GLP1 is separated by IRES (internal ribosome entry site).

[0033] FIG. **9**. Expression of GGBP in human epidermal progenitor cells with CRISPR. FIG. **9**A: Diagram showing AAVS1 targeting strategy for expression of GGBP. Expression vector encoding D10A mutant of cas9 and two gRNAs targeting AAVS1 locus is used to create the cleavage in the chromosomal DNA and enhance integration of GGBP targeting vector. FIG. **9**B: FACS (fluorescence activated cell sorting) demonstrates similar cell cycle profiles of WT (wild type) and GGBP-expressing epidermal progenitor cells before and after doxycycline treatment. PI: propidium iodine. FIG. **9**C: Western blotting analysis of early (C) and late (D) differentiation marker expression in VVT and GGBP-expressing cells upon calcium shift. Band intensity was determined by densitometry and fold of induction is quantified. Krt10: keratin 10; Lor: loricrin. n=4 (4 independent tests). P>0.05. FIGS. **9**D and **9**E: Sections of grafted skin were immunostained with different antibodies as indicated. Scale bar=50 μm. FIGS. **9**F and **9**G: Proliferation and apoptosis of epidermal cells in control or GGBP skin grafts was determined and quantified by immunohistological staining with antibody against phosphor-histone H3 and active caspase 3 respectively. For proliferation, n=7 (7 sections obtained from 2 animals for each group). P>0.05. For apoptosis, n=6 (6 sections obtained from 2 animals for each group). P>0.05.

[0034] FIG. **10**. Engineering GLP1-producing skin epidermal stem cells with CRISPR. FIG. **10**A: Diagram showing the Rosa26 targeting strategy for expression of GLP1. The targeting vector contains two Rosa26 homology arms, flanking the expression cassette for GLP1. The tetracycline-inducible expression cassette drives expression of Tet3G (tetracycline transactivator) protein and a selection marker (puromycin resistant gene, Puro) by a constitutive promoter UbiC (Ubiquitin C promoter). Tet3G and Puro are separated by a self-cleavable peptide T2A. Expression of the GLP1 fusion protein is controlled by TRE (tet-on) promoter. A transcriptional stop signal (ST) is included in front of the TRE promoter to eliminate the leakage expression of GLP1. FIG. **10**B: Integration of the targeting vector into Rosa26 locus is verified by PCR (left panel) and southern blotting (right panel). Positive clones display an additional band of the expected size. FIG. **10**C: Secretion of GLP1 in cell culture medium is determined by ELISA (enzyme-linked immunosorbent assay) upon stimulation with different amount of doxycycline (Doxy). FIG. **10**D: Conditioned medium is collected from different cell cultures and used to

treat starved insulinoma cells. Secretion of insulin in vitro is determined by ELISA. FIG. **10**E: FACS (fluorescence activated cell sorting) demonstrates similar cell cycle profiles for VVT (wild type) and GLP1-expressing epidermal stem cells after doxycycline treatment. PI: propidium iodine. FIGS. **10**F and **10**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. FIG. **10**H: WT cells or GLP1 cells with or without doxycycline treatment are tested for anchorage independent growth in soft agar. Note no growth for WT or GLP1 cells, but tumor initiating cells isolated from skin SCC (squamous cell carcinoma) can readily produce colonies in soft agar plate.

[0035] FIG. **11**. Stable delivery of GLP1 in vivo through mouse-to-mouse skin transplantation. FIG. **11**A: Images of immunocompetent mice (CD1 strain) grafted with isogenic skin organoids generated from GLP1-expressing cells. Cells are infected with lentivirus encoding Luciferase before grafting, and intravital imaging shows efficient incorporation of grafted cells. FIG. **11**B: Histological examination of grafted GLP1 skin and adjacent host skin as control (Ctrl). Scale bar=50 μm. Epi: epidermis, Der: dermis, HF: hair follicle. FIGS. **11**C-**11**E: Sections of grafted skin and adjacent host skin (ctrl) were immunostained with different antibodies as indicated (Krt14: keratin 14, Krt10: keratin 10, Lor: Loricrin, β4: β4-integrin, CD104). Scale bar=50 μm. FIG. **11**F: Skin organoids are developed from control or GLP1-producing cells, and transplanted to CD1 mice. The level of GLP1 in blood is determined by ELISA. Doxycycline-containing food can activate GLP1 secretion in vivo. FIG. **11**G: CD1 mice are grafted with control or GLP1 skin organoids, and treated with or without doxycycline. Presence of GLP1 in blood is determined by ELISA for 16 weeks after engraftment.

[0036] FIG. **12**. Expression of GLP1 in epidermal stem cells improves body weight and glucose homeostasis in vivo. FIG. **12**A: Images of control and grafted animals fed a regular diet or a HFD (high fat diet). FIG. **12**B: Representative images of white fat tissue histological examinations. Scale bar=100 μm. FIG. **12**C: Body weight change of different cohorts of mice measured from ~10 weeks of age. Note that the HFD induced significant obesity in control mice but that expression of GLP1 by doxycycline stimulation inhibited weight gain. FIGS. **120**-**12**E: IPGTT (intraperitoneal glucose tolerance test) for control (D) and GLP1 grafted (E) animals. Blood glucose concentration as a function of time following intraperitoneal injection of glucose showed improved glucose tolerance in GLP1-expressing mice fed a HFD. FIGS. **12**F and **12**G: ITT (insulin tolerance test). Profile of glucose concentration (percentage of initial value) as a function of time following intraperitoneal injection of insulin shows reduced insulin resistance in GLP1-expressing mice.

[0037] FIG. **13**. Expression of GLP1 in human epidermal stem cells with CRISPR. FIG. **13**A: Image of nude mouse grafted with organotypic human skin culture. Intravital imaging shows efficient incorporation of grafted cells expressing luciferase upon engraftment. FIG. **13**B: Sections of grafted skin and adjacent host skin were immunostained with different antibodies as indicated. Scale bar=50 μm. FIG. **13**C: Integration of the targeting vector into AAVS1 locus is verified by southern blotting. Positive clones display an additional band of the expected size. FIG. **13**D: Secretion of GLP1 into the culture medium was determined by the ELISA upon stimulation with different concentrations of doxycycline (Doxy). FIG. **13**E: Conditioned medium was collected from control and GLP1-expressing cells, cultured in the presence and absence of Doxy, and used to treat starved insulinoma cells. Secretion of insulin in vitro was determined by ELISA. FIG. **13**F: H&E staining of skin organoids developed from control or GLP1-producing human cells, and transplanted to nude mice. FIG. **13**G: Level of GLP1 was determine by ELISA in blood from control and grafted nude mice fed either a standard or Doxycycline-containing food to activate GLP-1 secretion in vivo.

[0038] FIG. **14**. Engineering GLP1-producing skin epidermal progenitor cells with CRISPR. FIG. **14**A: Cell proliferation of control (Ctrl) and GLP1-expressing cells. Fold increase of cell numbers is quantified for all cell types. FIG. **14**B: Control or GLP1 targeted epidermal progenitor cells can produce similar skin organoids in vitro. Scale bar=50 μm.

[0039] FIG. **15**. Stable delivery of GLP1 in vivo through mouse-to-mouse skin transplantation. FIG. **15**A: Proliferation of epidermal cells in control or GLP1 skin grafts was determined and quantified by immunohistological staining with antibody against phosphor-histone H3. FIG. **15**B: Apoptosis of epidermal cells in host or grafted skin one or four weeks post skin transplantation was determined and quantified by immunohistological staining with antibody against active caspase 3.

[0040] FIG. **16**. Expression of GLP1 in human epidermal progenitor cells with CRISPR. FIG. **16**A: Diagram showing AAVS1 targeting strategy for expression of GLP1. Expression vector encoding D10A mutant of cas9 and two gRNAs targeting AAVS1 locus is used to create the cleavage in the chromosomal DNA and enhance integration of GLP1 targeting vector. FIG. **16**B: FACS (fluorescence activated cell sorting) demonstrates similar cell cycle profiles of VVT (wild type) and GLP1-expressing epidermal progenitor cells before and after doxycycline treatment. PI: propidium iodine. FIGS. **16**C and **16**D: Western blotting analysis of early (C) and late (D) differentiation marker expression in VVT and GLP1-expressing cells upon calcium shift. Band intensity was determined by densitometry and fold of induction is quantified. Krt10: keratin 10; Lor: loricrin. FIG. **16**E: Proliferation of epidermal cells in control or GLP1 skin grafts was determined and quantified by immunohistological staining with antibody against phosphor-histone H3. FIGS. **16**F and **16**G: Sections of grafted skin were immunostained with different antibodies as indicated. Scale bar=50 μm.

[0041] FIG. **17**. CPP apparatus. FIG. **17** shows a three compartment CPP apparatus that includes two large conditioning compartments with different colors and floor textures. The CPP apparatus (Med Associates, E. Fairfield, Vt. USA) consisted of two larger chambers (16.8×12.7×12.7 cm), which were separated by a smaller chamber (7.2×12.7×12.7 cm) as previously described (Yan et al, 2013). Each chamber had a unique combination of visual and tactile properties (one large chamber had black walls and a rod floor, the other larger chamber had white walls with a mesh floor, whereas the middle chamber had gray walls and a solid gray floor). Each compartment had a light embedded in a clear, Plexiglas hinged lid. Time spent in each chamber was measure via photobeam breaks and recorded. CPP was

determined on testing days via time spent in the drug-paired side minus time spent in the saline-paired side.

[0042] FIG. **18**. Engraftment of hBChE-expressing cells can attenuate CPP acquisition and reinstatement induced by cocaine. **18**A: After engraftment, GhBChE (grafted hBChE expressing cells) and GWT (grafted wild type cells) mice underwent pretest (Day 1), cocaine conditioning (Day 2 to Day 5) and CPP expression test (Day 6). Data represent mean±SEM (n=9 in each group, treatment×days interaction: $F_{1,16}$=4.94, PG0.05). **18**B: After engraftment, GhBChE and GWT mice underwent pretest (Day 1), ethanol conditioning (Day 2 to Day 5) and CPP testing (day 6). Data represent mean±SEM (n=8 in each group, treatment×days interaction: $F_{1,17}$=0.07, not significant). **PG0.01 compared to pretest (Fisher's t-test). **18**C: Mice acquired similar levels of cocaine CPP after pretest, cocaine conditioning and test and underwent engrafting surgery on Day 7. Following 10 days of recovery, GhBChE and GWT mice underwent extinction till Day 31. During reinstatement on Day 32, GhBChE and GVVT mice were given a cocaine injection and CPP was measured again. Data show mean±SEM (n=8 in each group, treatment×days interaction: $F_{3, 42}$=12.34, P<0.001). 18D: Mice acquired similar levels of ethanol CPP after pretest, ethanol conditioning and test from Day 1 to Day 6, and underwent extinction till Day 20. During reinstatement on Day 21, were given an ethanol injection and CPP was recorded (mean±SEM) (n=8 in each group, treatment×days interaction: $F_{3, 42}$=0.05, not significant). *PG0.05 compared to last extinction (Fishers t-test).

[0043] FIG. **19**. Expression of engineered hBChE via genome editing in skin epidermal stem cells. FIG. **19**A: Targeting strategy for the expression of engineered hBChE. The targeting vector contains two Rosa26 homology arms, flanking the expression cassette for hBChE and a selection marker (puromycin resistant gene, Puro) by a constitutive promoter UbiC (Ubiquitin C promoter). hBChE and Puro were separated by a self-cleavable peptide T2A. gRNA: guide RNA. FIG. **19**B: Integration of the targeting vector into Rosa26 locus was verified by PCR (left panel) and southern blotting (right panel). Positive clones displayed an additional band of the expected size. FIG. **19**C: Confirmation of hBChE expression in targeted cells by immunoblots with different antibodies. FIG. **19**D: Confirmation of secretion of engineered hBChE in the culture media by ELISA. FIG. **19**E: Cocaine hydrolysis activity in vitro. Cell cultured supernatants were collected from cells targeted by hBChE or mBChE. Cocaine hydrolysis activity was examined by a clearance assay in vitro. FIG. **19**F: Cell cycle profiles. FACS (fluorescence activated cell sorting) of control (Ctrl) and hBChE-expressing epidermal stem cells. PI: propidium iodine.

[0044] FIG. **20**. Engineering hBChE-producing skin epidermal progenitor cells with CRISPR. **20**A: Cell proliferation of control (Ctrl) and hBChE-expressing cells. Fold increase of cell numbers is quantified for all cell types. **20**B: Western blotting analysis of early (Krt10: keratin10) and late (Lor: loricrin) differentiation marker expression in WT and hBChE-expressing cells upon calcium shift. Band intensity was determined by densitometry and fold of induction is quantified. **20**C: WT cells or hBChE cells were tested for anchorage independent growth in soft agar. Note no growth for VVT or hBChE cells, but tumor initiating cells isolated from skin SCC (squamous cell carcinoma) can readily produce colonies in soft agar plate.

[0045] FIG. **21**. Stable delivery of engineered hBChE in vivo through mouse-to-mouse skin transplantation. **21**A: Control or hBChE targeted epidermal progenitor cells can produce similar skin organoids in vitro. Scale bar=50 μm. **21**B: Grafted skins were collected from mice grafted with control (GVVT) or hBChE skin organoids (GhBChE). Cell proliferation was determined and quantified by immunohistological staining with antibody against phospho-histone 3. **21**C: Apoptosis of epidermal cells in control or hBChE skin grafts was determined and quantified by immunohistological staining with antibody against active caspase 3.

[0046] FIG. **22**. Engraftment of hBChE-expressing cells can protect against cocaine overdose. **22**A: Skin organoids are developed from control or hBChE-producing cells, and transplanted to the host mice. Cells were infected with lentivirus encoding firefly luciferase before engraftment to allow intravital imaging of the skin grafts. **22**B: Histological examination of grafted skin collected from mice grafted with control (GVVT) or hBChE skin organoids (GhBChE). Scale bar=50 μm. **22**C: Sections of grafted skin were immunostained with different antibodies as indicated (Krt10: keratin 10, a marker for early epidermal differentiation, Lor: Loricrin, a marker for early epidermal differentiation, β4: β4-integrin, CD104, a marker for skin basement membrane). Dashed lines denote the basement of skin. Epi: epidermis, Der: dermis. Scale bar=50 μm. **22**D: Mice are grafted with control or hBChE skin organoids. Presence of hBChE in blood was determined by ELISA for 10 weeks after engraftment (n=5 mice in each group). **22**E: Lethality rates after injection of 40, 80, 120, 160 mg/kg cocaine in GhBChE and GVVT mice (n=8 in each group). **22**F: Lethality rate after injections of 34, 68, 100, 160 mg/kg METH (methamphetamine) in GhBChE and GWT mice (n=8 in each group).

[0047] FIG. **23**. Expression of hBChE in human epidermal stem cells with CRISPR. **23**A: The AAVS1 targeting strategy for expression of engineered hBChE. The targeting vector contains two AAVS1 homology arms, flanking the expression cassette for hBChE and a selection marker (puromycin resistant gene, Puro) by a constitutive promoter UbiC (Ubiquitin C promoter). hBChE and Puro are separated by a self-cleavable peptide T2A. **23**B Integration of the targeting vector into AAVS1 locus is verified by southern blotting. Positive clones display an additional band of the expected size. **23**C: Expression of hBChE is confirmed in targeted cells by immunoblots with different antibodies as indicated. **23**D: Secretion of engineered hBChE in the culture media is confirmed by ELISA. **23**E: Image of nude mouse grafted with organotypic human skin culture. Intravital imaging shows efficient incorporation of grafted cells expressing luciferase (right side) or control cells (left side) upon engraftment. **23**F: Sections of grafted skin were immunostained with different antibodies as indicated. Dashed lines denote the basement of skin. Scale bar=50 μm. **23**G: Mice are grafted with control or hBChE skin organoids. Presence of hBChE in blood was determined by ELISA for 8 weeks after engraftment (n=3 mice in each group).

[0048] FIG. **24**. Expression of hBChE in human epidermal progenitor cells with CRISPR. **24**A: FACS (fluorescence activated cell sorting) demonstrates similar cell cycle profiles for control (Ctrl) and hBChE-expressing epidermal stem cells. PI: propidium iodine. **24**B: Western blotting analysis of early (Krt10: keratin10) and late (Lor: loricrin) differentiation marker expression in WT and hBChE-expressing human epidermal stem cells upon calcium shift.

Band intensity was determined by densitometry and fold of induction is quantified. **24**C: Grafted skins were collected from mice grafted with control or hBChE skin organoids. Cell proliferation was determined and quantified by immunohistological staining with antibody against phospho-histone 3. **24**D: Apoptosis of epidermal cells in control or hBChE skin grafts was determined and quantified by immunohistological staining with antibody against active caspase 3.

[0049] FIG. **25**. Engineering mGLP1-producing skin epidermal stem cells with CRISPR. CRISPR-mediated knockin of DImGLP1 in mouse epidermal progenitor cells and dox-regulated mGLP1 expression. FIG. **25**A: Targeting vector contains two Rosa26 homology arms flanking a dox-responsive expression cassette encoding mGLP1. Expression of Tet3G (a transactivator) and Puromycin resistance (Puro) connected by a T2A peptide is controlled by an Ubiquitin C (Ubi) promoter. ST is a signal to increase TRE promoter specificity. FIG. **25**B: PCR and southern verification of knockin of DImGLP1. FIG. **25**C: Dox-induced mGLP1 expression in plasma of GLP1 mice. FIG. **25**D: Long-term mGLP1 expression in GLP1 mice.

[0050] FIG. **26**. mGLP1 expression on ethanol-induced CPP. GLP1 mice did not exhibit significant ethanol-induced CPP. Following 2 free explorations (Pre-test) on day 1, separate groups of GLP1 and GVVT mice (n=9 each) received alternative ethanol (2 g/kg) and saline i.p. injections twice daily for the next 4 days, as previously described (Chen et al., Dopamine D1 and D3 receptors are differentially involved in cue-elicited cocaine seeking. *J. Neurochem.* 114, 530-541 (2010); Kong et al., Activation of dopamine D3 receptors inhibits reward-related learning induced by cocaine. *Neurosci.* 176, 152-161 (2011)). CPP expression was tested on day 6. Results represent mean±SEM time spent on the drug-paired side minus the saline-paired side. Repeated-measures ANOVA with test days as the within group factor and status of grafting as the between-subject factor were used (Chen et al., 2010; Kong et al., 2011). F value was calculated and Newman-Keuls post-hoc test was performed (Chen et al., 2010; Kong et al., 2011). GLP1 and WT mice were on dox food for the entire duration.

[0051] FIG. **27**. Expression of Spider-derived pain peptides in human epidermal progenitor cells with CRISPR. Diagram showing a contemplated targeting vector for treatment of alcoholism by cutaneous expression of spider derived pain peptides (toxins), DkTx (SEQ ID NO: 36) or VaTx (SEQ ID NO: 35). SP: signal peptide; F: furin cleavage site; IgG-Fc: mouse (SEQ ID NO: 34) or human IgG-Fc (SEQ ID NO: 39) fragment. Contemplated VatX3 and DkTx target vector cassettes are represented by SEQ ID NOS: 37 and 38, respectively.

[0052] FIG. **28**. Expression of PAL in human epidermal progenitor cells with CRISPR. Diagram showing a contemplated targeting vector for treatment of PKU by cutaneous expression of PAL. SP: signal peptide; PAL: coding sequence for PAL.

[0053] FIG. **29** mGLP1 expression on nicotine-induced CPP. Figure legend: GLP1 mice did not exhibit significant nicotine-induced CPP. Following 2 free explorations (Pre-test) on day 1, separate groups of GLP1 and GWT mice (n=7 each) received alternative nicotine (0.4 mg/kg) and saline i.p. injections twice daily for the next 4 days, as previously described (Chen et al., 2010; Kong et al., 2011). CPP expression was tested on day 6. Results represent mean ±SEM time spent on the drug-paired side minus the saline-paired side. Repeated-measures ANOVA with test days as the within group factor and status of grafting as the between-subject factor were used (Chen et al., 2010; Kong et al., 2011). F value was calculated and Newman-Keuls post-hoc test was performed (Chen et al., 2010; Kong et al., 2011). GLP1 and WT mice were on dox food for the entire duration.

DETAILED DESCRIPTION

[0054] All publications, patents, and patent applications cited herein are hereby expressly incorporated by reference in their entirety for all purposes.

[0055] Before describing the present invention in detail, a number of terms will be defined. As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to "a metabolite" means one or more metabolites.

[0056] It is noted that terms like "preferably," "commonly," and "typically" are not utilized herein to limit the scope of the claimed invention or to imply that certain features are critical, essential, or even important to the structure or function of the claimed invention. Rather, these terms are merely intended to highlight alternative or additional features that can or cannot be utilized in a particular embodiment of the present invention.

[0057] For the purposes of describing and defining the present invention it is noted that the term "substantially" as used herein represents the inherent degree of uncertainty that can be attributed to any quantitative comparison, value, measurement, or other representation. The term "substantially" is also used herein to represent the degree by which a quantitative representation can vary from a stated reference without resulting in a change in the basic function of the subject matter at issue.

[0058] Methods well known to those skilled in the art can be used to construct genetic expression constructs, targeting vectors, and genetically engineered cells according to this invention. These methods include in vitro recombinant DNA techniques, synthetic techniques, in vivo recombination techniques, polymerase chain reaction (PCR) techniques, and others. See, for example, techniques as described in Green & Sambrook, 2012, MOLECULAR CLONING: A LABORATORY MANUAL, Fourth Edition, Cold Spring Harbor Laboratory, New York; Ausubel et al., 1989, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, New York, and PCR Protocols: A Guide to Methods and Applications (Innis et al., 1990, Academic Press, San Diego, Calif.).

[0059] As used herein, the terms "polynucleotide," "nucleotide," "oligonucleotide," and "nucleic acid" can be used interchangeably to refer to nucleic acid comprising DNA, RNA, derivatives thereof, or combinations thereof.

[0060] As used herein, the term "genetically engineered" refers to the genetic manipulation of one or more cells, whereby the genome of the one or more cells has been augmented by at least one DNA sequence. Candidate DNA sequences include but are not limited to genes that are not naturally present, DNA sequences that are not normally transcribed into RNA or translated into a protein ("expressed"), and other genes or DNA sequences which one desires to introduce into the one or more cells. It will be

appreciated that typically the genome of genetically engineered cells described herein is augmented through stable introduction of one or more recombinant genes. Generally, introduced DNA is not originally resident in the genetically engineered cell that is the recipient of the DNA, but it is within the scope of this disclosure to isolate a DNA segment from a given genetically engineered cell, and to subsequently introduce one or more additional copies of that DNA into the same genetically engineered cell, e.g., to enhance production of the product of a gene or alter the expression pattern of a gene. In some instances, the introduced DNA will modify or even replace an endogenous gene or DNA sequence by, e.g., homologous recombination, site-directed mutagenesis, and/or genome editing technology, including CRISPR (clustered regularly-interspaced short palindromic repeats), and/or mammalian transposon technology, such as by using the piggyBac™ transposon. In some instances, the introduced DNA is introduced into the recipient via viral vectors, including vectors derived from retrovirus, lentivirus, and adeno-associated virus. In some instances, the introduced DNA is introduced into the recipient skin directly with electroporation without skin stem cell isolation, culture, CRISPR editing, or grafting.

[0061] As used herein, the term "recombinant gene" refers to a gene or DNA sequence that is introduced into a genetically engineered cell, regardless of whether the same or a similar gene or DNA sequence may already be present in such a host. "Introduced," or "augmented" in this context, is known in the art to mean introduced or augmented by the hand of man. Thus, a recombinant gene can be a DNA sequence from another species, or can be a DNA sequence that originated from or is present in the same species, but has been incorporated into a cell by methods to form a genetically engineered cell. It will be appreciated that a recombinant gene that is introduced into a cell can be identical to a DNA sequence that is normally present in the cell being transformed, and is introduced to provide one or more additional copies of the DNA to thereby permit overexpression or modified expression of the gene product of that DNA. Recombinant genes can also be introduced with different driving promoters or associated sequences that can alter the gene's expression level or pattern. Said recombinant genes are particularly encoded by cDNA. Non-coding sequences, such as short hairpin RNAs, microRNAs, or long non-coding RNAs, may also be included.

[0062] It is further contemplated that recombinant genes can be codon optimized to maximize protein expression in genetically engineered cells by increasing the translation efficiency of a particular gene. Codon optimization can be achieved, for example, by transforming nucleotide sequences of one species into the genetic sequence of a different species. Optimal codons help to achieve faster translation rates and high accuracy. As a result of these factors, translational selection is expected to be stronger in highly expressed genes. However, while optimal codon usage is contemplated herein for expression of disclosed proteins, all possible codons are contemplated for use herein for nucleic acids encoding any disclosed protein.

[0063] As used herein, the term "about" refers to ±10% of any particular value.

[0064] As used herein, the terms "or" and "and/or" are utilized to describe multiple components in combination or exclusive of one another. For example, "x, y, and/or z" can refer to "x" alone, "y" alone, "z" alone, "x, y, and z," "(x and y) or z," "x or (y and z)," or "x or y or z."

[0065] As used herein, the term "blood factor" refers to any specific disease-associated indicator or factor that can circulate in the body, such as in the blood and/or lymphatic system. For example, a blood factor can be, without limitation, a cell, an enzyme, a protein, a polypeptide, an amino acid, a polynucleotide, a nucleic acid, a sugar, a lipid, a metabolite, a synthetic chemical compound, a naturally occurring chemical compound, a mineral, a metal, a bacterium, a virus, a prion, a disease indicator, and combinations or variations thereof.

[0066] As used herein, the term "reporter molecule" refers to any compound that can be produced biosynthetically by a genetically engineered host cell. For example, a reporter molecule can be an enzyme, a protein, a polypeptide, an amino acid, a polynucleotide, a nucleic acid, a sugar, a lipid, a metabolite, a synthetic chemical compound, a naturally occurring chemical compound, and combinations thereof. In one particular example, a reporter molecule can be a fluorescent protein, a fret-based biosensor, and/or a bioluminescent protein.

[0067] In one embodiment, the reporter molecule can be inducibly expressed, such as when a blood factor is perceived by the tissue organoid. Induction of expression can also be caused by administration of an inducer, as described herein elsewhere. When expressed by induction, the increased concentration of the reporter molecule functions to "report" the presence of the blood factor in question by producing a detectable signal. "Reporting" can be by any detectable means, such as, for example, fluorescing, producing a FRET signal, producing an electrical signal, and/or undergoing a conformational change.

[0068] Alternatively, a reporter molecule can be constitutively expressed but only "reports" when a signal produced by the reporter molecule is changed as a function of the perception of a blood factor by the reporter molecule. In this context, a change (increase or decrease) in signal can be proportionally associated with an increase in concentration of the blood factor.

[0069] In one embodiment, an external measurement device targeted at a tissue organoid expressing a reporter molecule can be used to noninvasively measure the relative amount of a blood factor in the patient. In one embodiment, it is contemplated that the reporter molecule can directly or indirectly associate with or contact a blood factor to produce a detectable signal that is proportional to the concentration of the blood factor in individual.

[0070] As used herein, the term "therapeutic agent" refers to a substance that when administered to an individual in need thereof, improves the individual's health. For example, a therapeutic agent can be, without limitation, an enzyme, a protein, a clotting factor, a vitamin, a peptide, a lipid, a toxin, a hormone, a polysaccharide, and combinations thereof. In one particular example, a therapeutic agent can be insulin or an analogue thereof that when administered to an individual in need thereof improves the individual's health by regulating the individual's blood sugar levels. In another particular example, a therapeutic agent can be a hormone, such as GLP1, that when administered to an individual in need thereof improves an individual's health by inducing the individual's satiety response, for example, to help regulate food intake. In a further particular example, a therapeutic agent can be an enzyme, such as phenylalanine hydroxylase

(PAH), that when administered to an individual in need thereof improves an individual's health by reducing the concentration of phenylalanine in the patient's blood. Moreover, a therapeutic agent can be any compound that can be produced biosynthetically by a genetically engineered host cell, such as an epidermal cell. Therapeutic agents can include proteins and other substances derived from one species and administered to another species in native and/or modified forms.

[0071] As used herein, the term "individual" refers to any animal. Examples of individuals include humans, domesticated animals, household pets, and other animals without limitation. Further examples of individuals include animals having a disease.

[0072] As used herein, the term "physiologically tailored" refers to a state in which a tissue organoid has been created to be physiologically and/or immunologically compatible with an individual. For example, a physiologically tailored tissue organoid can be a tissue organoid grown from an individual's own cells or from cells that are physiologically compatible and that do not trigger an immune response from the individual when the individual's immune system is exposed to the tissue organoid, such as when the tissue organoid is surgically grafted into or onto the individual or otherwise biointegrated.

[0073] As used herein, the term "tissue organoid" refers to a collection of cells forming a tissue that has been genetically modified and that can be biointegrated in vivo via surgical transplantation or grafting (for example, on an individual's skin). For example, a tissue organoid can be a cultured, stratified skin graft grown from genetically engineered stem cells or keratinocytes taken from an individual. In addition to in vitro construction, it is envisioned that tissue organoids could be constructed in situ on an individual.

[0074] The cultured skin graft can be engineered to express one or more proteins or other molecules of interest under predetermined conditions, such as in response to the presence, absence, or change in levels of one or more blood factors. The protein or other molecule of interest can be a reporter molecule, a therapeutic agent, an inducer, and/or any other molecule or compound that can be produced biosynthetically by a genetically engineered host cell.

[0075] As used herein, the term "inducer" refers to a physical stimulus and/or chemical stimulant that induces expression of one or more genes within a tissue organoid and/or activation and/or release of a reporter molecule or therapeutic agent from a tissue organoid. Non-exclusive examples of inducers can include heat, cold, light, a protein, a peptide, a hormone, a lipid, a chemical, a metabolic change, a metabolite, an electric potential or field, and combinations thereof. Specific examples of inducers include doxycycline, a reporter molecule, and ethanol. It is further contemplated that inducers can induce expression and/or release of a reporter molecule or therapeutic agent in a dose-dependent manner.

[0076] In one embodiment, the present disclosure is directed to a physiologically tailored, biointegratable tissue organoid that can monitor levels of one or more blood factors in an individual. The tissue organoid can be transplanted or grafted onto the individual to provide a permanent or temporary (e.g., for one, two, three, six, twelve, or sixty months or longer) continuous monitor of one or more blood factors and/or source of therapeutic agent(s). For example, the tissue organoid can be a fully stratified skin graft cultured from the individual's own epidermal stem cells that is surgically grafted onto the individual's skin. Once biointegrated into the patient's skin, the tissue organoid forms a part of the patient's skin thereby preventing potential infections by eliminating the need for piercing the skin to monitor blood factor concentration. Further, because the tissue organoid is derived from the patient's own cells, the risk of host rejection of the tissue organoid is reduced.

[0077] Tissue organoids when biointegrated are nourished like any other tissue of the individual and concomitantly are exposed to circulating blood factors. In this context, tissue organoids can be genetically engineered to carry one or more stable genetic modifications (e.g., genome-integrated modifications) such as one or more genes encoding a reporter molecule that are expressed when a blood factor is perceived by or comes in contact with the tissue organoid.

[0078] In another embodiment, a reporter molecule can be constitutively expressed within the tissue organoid but only "reports" by producing a detectable signal (e.g., fluoresces, produces a FRET signal, produces an electrical signal, bioluminesces, produces a colorimetric change, and/or undergoes a conformational change) when associated with and/or contacting a predetermined blood factor. It is contemplated that the reporter molecule may directly or indirectly associate with or contact a blood factor to produce a detectable signal. External measurement devices targeted at the tissue organoid can be used to noninvasively measure the relative amount of perceived blood factor in the patient. In another embodiment, it is contemplated that reporter molecules produced by the tissue organoid are released systemically and can therefore be detected in another part of the body.

[0079] In one specific embodiment, a contemplated tissue organoid is a blood glucose monitor that expresses a reporter molecule in proportion to the relative blood glucose concentration of an individual. For example, the tissue organoid can express a fluorescent or bioluminescent reporter protein in relative proportion to glucose blood concentrations. The relative amount of the reporter protein can externally be measured, such as by a fluorometer or colorimeter and the concentration of the blood factor can therefore be determined. It is further contemplated that an inverse relationship between a reporter molecule and a particular blood factor is possible such that the relative amount of reporter molecule decreases in response to an increase in the blood factor concentration.

[0080] It is further contemplated that a tissue organoid could produce a reporter molecule that can be detected by measuring the reporter molecule in sweat, tears, mucus, plasma, urine, feces, or combinations thereof.

[0081] In another embodiment, the present disclosure is directed to a physiologically tailored, biointegratable tissue organoid that can express a therapeutic agent that passes the epidermal/dermal barrier to reach the circulation and have a therapeutic effect.

[0082] In a specific embodiment, the tissue organoid can be induced to express a therapeutic agent constitutively or by administration of an inducer to the individual. For example, a tissue organoid can express GLP1 upon stimulation with an inducer, such as doxycycline in a dose-dependent manner. In this way, the amount of therapeutic agent expressed by a tissue organoid can be tailored to an individual's specific need at a particular time.

[0083] In one embodiment, a tissue organoid expresses both a reporter molecule and a therapeutic agent.

[0084] Genetic Constructs

[0085] Tissue organoids can include genetically engineered cells capable of expressing reporter molecules and/or therapeutic agents. Genes encoding reporter molecules and/or therapeutic agents can be stably introduced into the genomes of cells using any technology that permits genome editing, such as CRISPR. However, other approaches are contemplated herein. When using CRISPR for genetically engineering cells, any integration locus suitable for genome editing can be used. Examples of integration loci include AAVSI (adeno-associated virus integration site 1), HPRT1 (hypoxanthine phosphoribosyltransferase-1), and/or human Rosa26 locus. Use of the HPRT1 locus offers the advantage that correctly integrated cells can be selected based on their resistance to 6-TG (2-amino-6-captopurine). Further, CRISPR targeting vectors can incorporate a drug resistance gene (puromycin; "puro") for cell selection, which may elicit an immune reaction in vivo. If this occurs, the targeting vector could be modified so that a puro coding sequence would be flanked with two LoxP sites. The puro sequence can be removed in vitro by transient expression of Cre recombinase after selection of targeted clones.

[0086] Cell Selection and Tissue Growth

[0087] Suitable cells that can be used for tissue organoid construction include epidermal stem cells, such as those isolated from human skin. Other sources for epidermal stem cells include induced human pluripotent stem cells. Further examples of suitable cells include embryonic stem cells and human induced pluripotent stem cells.

[0088] Once isolated, the stem cells can be transfected with a targeting vector carrying one or more reporter molecule coding genes and/or one or more therapeutic agent coding genes and selected for correct integration of the targeting vector by Southern blot and other available methods. Correctly integrated genetically engineered epidermal stem cells can then be induced to differentiate to form stratified skin tissue when seeded on decellularized dermis and exposed to an air/liquid interface within a cell culture insert. Once grafts are ready, they can be transplanted to donor patients with well-established protocols.

[0089] Transplantation

[0090] Tissue organoids can be implanted into skin of individuals via known surgical procedures, such as skin grafting. Other suitable procedures include direct application of engineered skin stem cells to patient skin. Once implanted, the tissue organoids can be allowed to heal and fully biointegrate into the individual's skin.

[0091] Reporter Molecule Detection

[0092] Detection of reporter molecules of biointegrated tissue organoids can be performed by any means known in the art suitable for detecting the reporter molecule to be measured. For example, fluorometers and/or colorimeters can be used to measure changes in fluorescence, color, or luminescence associated with reporter molecule expression. Further, intravital bioluminescence imaging can be performed using a bioluminescence monitoring system, such as Xenogen. Additional examples of measurement devices that can be used to measure reporter molecules include electrical systems with proper electrodes.

[0093] Tissue Organoid Standardization

[0094] Once a tissue organoid is biointegrated into an individual, for example, a blood glucose monitoring tissue organoid, the response of the organoid to blood glucose concentrations can be standardized such that a given reporter response is indicative of a specific blood glucose concentration. This can be accomplished by measuring different blood glucose concentrations using a standard blood glucose meter and associating the specific concentrations measured with the relative reporter molecule signals at each concentration. One example of such as standardization test is the intraperitoneal glucose tolerance test. Similar clinical standardization techniques can be performed for tissue organoids that are engineered to detect other specific blood factors. It is envisioned that once the tissue organoid "read-out" is correlated to blood factor concentration, no further direct blood testing will be necessary.

[0095] Therapeutic Platform Development

[0096] The present disclosure is also directed to tissue organoids for treating multiple medical issues simultaneously or as needed. For example, a physiologically tailored, biointegratable tissue organoid is envisioned that can express one or more therapeutic agents designed to address an individual's specific medical needs and thereby form a biointegratable therapeutic platform. For example, therapeutic platforms can be designed to express multiple therapeutic agents such as 2, 3, 4, 5, 6, 7, 8, 9, 10, or more therapeutic agents at one time or at different times depending upon an individual's needs. For example, each therapeutic agent to be expressed by the therapeutic platform can be individually and separately induced by an inducer such that for each therapeutic agent to be expressed, a separate inducer must be introduced to cause expression of the therapeutic agent. Alternatively, therapeutic agents that could advantageously be expressed at the same time, such as 2 or more therapeutic agents, can each be inducible by the same inducer.

[0097] Further, while some embodiments described herein are directed to expression of therapeutic agents for treatment of an existing illness, disease, and/or deficiency or absence of a required physiological substance (e.g., an enzyme, a protein, a clotting factor, a vitamin, a peptide, a lipid, etc.), it is further envisioned that a therapeutic agent can be expressed in anticipation of a physiological insult or stress. For example, tissue organoids made to express a therapeutic agent to counter the effects of a harmful chemical or substance could be induced to express the therapeutic agent in anticipation of exposure to the harmful chemical. In this way, the therapeutic agent has been expressed in the individual before the harmful chemical or substance is encountered by the individual. In another example, tissue organoids can be made to express a therapeutic agent in anticipation of blood loss, a low oxygen environment, and/or other physiological insult.

[0098] In another embodiment, the present disclosure is directed to physiologically tailored, biointegratable tissue organoids that can express therapeutic agents with multiple therapeutic effects. For example, a tissue organoid can be designed to express a single therapeutic agent, such as GLP1 that can be used to combat both alcohol abuse and nicotine abuse.

[0099] In one particular embodiment, it is envisioned that a tissue organoid designed to express GLP-1 (an anti-alcohol and anti-nicotine therapeutic agent) and BChE (an anti-cocaine therapeutic agent) can be biointegrated into an individual suffering from substance abuse or that is at risk for a relapse to eliminate or minimize the addictive effects of alcohol, nicotine, and cocaine at the same time.

[0100] In a further embodiment, GLP-1 analogs are contemplated for use herein. For example, contemplated analogs for use herein include Exendin-4 and Exendin-based therapies (e.g., Exenatide and Exenatide LAR), DPP-IV-resistant GLP-1 analogs (e.g., albiglutide), semaglutide (NN9535), liraglutide, taspoglutide, dulaglutide (GLP-1Fc, Trulicity®) (LY2189265), and derivatives thereof (see Gupta, V. Glucagon-like peptide-1 analogues: An overview, *Indian J Endocrinol Metab.* 17(3): 413-421 (2013)).

[0101] Relatedly, it is envisioned that pharmaceutical compositions including one or more inducers can be administered to an individual to cause expression of one or more therapeutic agents that are inducible by the administered inducers. For example, a formulation in a pharmaceutically-acceptable form, such as an oral, parenteral, inhalable, and/or topical medication, can contain 2 or more inducers each specific for a separate therapeutic agent to be expressed by a tissue organoid. In this way, tissue organoids can be designed to express multiple therapeutic agents and tailored therapeutic agent expression can be obtained.

[0102] In one particular example, a tissue organoid can be biointegrated into an individual where the tissue organoid is designed to express 5 different therapeutic agents, TA1, TA2, TA3, TA4, and TA5, each upon induction by a separate inducer, I1, I2, I3, I4, and I5, respectively. When needed, the individual can be administered a pharmaceutical composition including inducers I2, I3, and I5, for example, which causes expression of therapeutic agents TA2, TA3, and TA5. Alternatively, the individual can be administered a pharmaceutical composition including inducers I1, I2, and I4, for example, which causes expression of therapeutic agents TA1, TA2, and TA4. Multiple variations of therapeutic agent induction are envisioned without limitation. Moreover, multiple variations of pharmaceutical dosage forms are contemplated such as immediate release, delayed release, and/or extended release forms. In this way, a particular pharmaceutical composition can include, for example, inducers I1, I2, I3, I4, and I5, where inducers I1 and I5 are formulated for immediate release, inducers I2 and I3 are formulated for delayed release, and inducer II4 is formulated for extended release. Additional dosage forms such as implantable depots are contemplated. Combinations of any inducers in any dosage form or formulation are contemplated herein without limitation.

[0103] It is further envisioned that a tissue organoid can be designed to express multiple therapeutic agents where expression of one or more of the therapeutic agents is inducible and expression of one or more of the therapeutic agents is constitutive.

[0104] It is further envisioned that a tissue organoid can be cultured that include multiple populations of transformed cells where each population is designed to express a different therapeutic agent than the other populations within the tissue organoid. Any number of separate populations of cells is envisioned.

[0105] The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

[0106] The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only and are not taken as limiting the invention.

Example No. 1: Development of an Intrinsic Skin Sensor for Blood Glucose Level with CRISPR-Mediated Genome Editing in Epidermal Stem Cells

Introduction

[0107] Current integrated biosensors exhibit limitations in stability, biocompatibility, and increased risk of infection. One possibility to overcome these limitations is to transform a small portion of endogenous tissue into a biointegrated, long-lasting sensor for physiological and biochemical signals via genome editing technology (see Hsu et al. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157, 1262-1278 (2014); Maeder et Genome-editing Technologies for Gene and Cell Therapy. *Mol Ther* 24, 430-446 (2016); and Wright et al. Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering. *Cell* 164, 29-44 (2016)). In this regard, the human skin and skin epidermal stem cells (see Blanpain et al. Epidermal stem cells of the skin. *Annu Rev Cell Dev Biol* 22, 339-373 (2006) and Watt, F. M. Mammalian skin cell biology: at the interface between laboratory and clinic. *Science* 346, 937-940 (2014)) have several unique advantages, making them particularly suited for genetic engineering and applications in vivo (FIG. **1**A).

[0108] The procedure to isolate and culture primary epidermal stem cells is well established. Cultured epidermal stem cells can be induced to stratify and differentiate in vitro, and transplantation of epidermal autografts is minimally invasive, safe, and stable in vivo. Autologous skin grafts have been clinically used for treatment of burn wounds for decades (see Carsin, H. et al. Cultured epithelial autografts in extensive burn coverage of severely traumatized patients: a five year single-center experience with 30 patients. *Burns: journal of the International Society for Burn Injuries* 26, 379-387 (2000) and Coleman et al. Cultured epidermal autografts: a life-saving and skin-saving technique in children. *Journal of pediatric surgery* 27, 1029-1032 (1992)). In this example, tissue organoids derived from genome-edited epidermal stem cells are shown to be useful for continuous monitoring of blood glucose level in vivo.

Experimental Procedures

[0109] Reagents and Plasmid DNA Constructions

[0110] Guinea pig anti K5, rabbit anti K14, rabbit anti K10 and Loricrin antibodies were generous gifts from Dr. Elaine Fuchs at the Rockefeller University. Rat monoclonal β4-integrin (CD104, BD 553745) was obtained from BD Pharmingen® (Franklin lakes, N.J.). Ser10 pho-histone antibody was obtained from EMD Millipore® (06-570, Billerica, Mass.). Cleaved caspase-3 antibody was obtained from Cell Signaling Technology® (#9661, Danvers, MA). Insulin ELISA kit was obtained from EMD Millipore® Corp. (EZRMI-13K, Billerica, MA). GLP-1 ELISA kit was obtained from Sigma® (RAB0201-1kt, St. Louis, Mo.). Other chemicals or reagents were obtained from Sigma®, unless otherwise indicated.

[0111] Lentiviral vector encoding Luciferase (SEQ ID NO: 1) and H2B-RFP (SEQ ID NO: 2) has been described before (Liu et al., 2015; Yue, 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 (SEQ ID NO: 3); and gGA ATT CTA ATG CCA ACT TTG TAC (SEQ ID NO: 4), using gBlock as a template. Rosa26—targeting gRNA is constructed with primers: ACA CCG GCA GGC TTA AAG GCT AAC CG (SEQ ID NO: 5), AAA ACG GTT AGC CTT TAA GCC TGC CG (SEQ ID NO: 6), ACA CCG AGG ACA ACG CCC ACA CAC Cg (SEQ ID NO: 7), AAA ACG GTG TGT GGG CGT TGT CCT CG (SEQ ID NO: 8). AAVS1—targeting gRNA is constructed with primers: ACA CCG TCA CCA ATC CTG TCC CTA GG (SEQ ID NO: 9), AAA ACC TAG GGA CAG GAT TGG TGA CG (SEQ ID NO: 10), ACA CCG CCC CAC AGT GGG GCC ACT AG (SEQ ID NO: 11), AAA ACT AGT GGC CCC ACT GTG GGG CG (SEQ ID NO: 12). 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 (SEQ ID NO: 13), GAC TAG TCC CGG GGG ATC CAC CGG TCA GGA ACA GGT GGT GGC GGC CC (SEQ ID NO: 14), CGG GAT CCA CCG GTG AGG GCA GAG GAA GCC TTC TAA C (SEQ ID NO: 15), TCC CCC GGG TAC AAA ATC AGA AGG ACA GGG AAG (SEQ ID NO: 16), GGA ATT CAA TAA AAT ATC TTT ATT TTC ATT ACA TC (SEQ ID NO: 17), CCT TAA TTA AGG ATC CAC GCG TGT TTA AAC ACC GGT TTT ACG AGG GTA GGA AGT GGT AC (SEQ ID NO: 18). AAVS1 targeting vector (SEQ ID NO: 40) 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 (SEQ ID NO: 19), CCC AAG CTT CCA TAG AGC CCA CCG CAT CCC C (SEQ ID NO: 20), CAG GGT CTA GAC GCC GGA TCC GGT ACC CTG TGC CTT CTA GTT GC (SEQ ID NO: 21), GGA TCC GGC GTC TAG ACC CTG GGG AGA GAG GTC GGT G (SEQ ID NO: 22), CCG CTC GAG AAT AAA ATA TCT TTA TTT TCA TTA CAT C (SEQ ID NO: 23), GCT CTA GAC CAA GTG ACG ATC ACA GCG ATC (SEQ ID NO: 24). Genotyping primers for CRISPR mediated knockin: GAG CTG GGA CCA CCT TAT ATT C (SEQ ID NO: 25), GGT GCA TGA CCC GCA AG (SEQ ID NO: 26), GAG AGA TGG CTC CAG GAA ATG (SEQ ID NO: 27).

[0112] Culture of Mouse and Human Primary Keratinocytes

[0113] Primary mouse keratinocytes were isolated from the epidermis of newborn 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 3. Cells were cultured in E-media supplemented with 15% serum and a final concentration of 0.05 mM Ca^{2+}.

[0114] Primary human neonatal epidermal keratinocytes were obtained from Thermo Fischer® (C0015C), and cultured with Epilife® medium (Thermo Fischer, M-EPICF-500) with manufacturer's recommended procedures. Calcium shift was performed to induce differentiation of primary keratinocytes by increasing the calcium concentration in culture media to 1.5 mM.

[0115] Cells are routinely screened for the presence of mycoplasma using the ATCC Universal Mycoplasma Detection Kit (Catalogue # 30-1012K). Cells are screened every 6 months and any mycoplasma contamination will result in the cells being discarded and replaced with previous, mycoplasma-free passages.

[0116] Cell Cycle Analysis:

[0117] Propidium iodide (PI) staining followed by flow cytometry were used to determine the effect of cell cycle profiles. Mouse and human epidermal cells were cultured in two 6 cm cell culture dish for 24 hours, 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 hour. Then, the fixed cells were centrifuged at 500 g at 4° C. for 10 minutes, followed by PBS wash for two times. The cells were then treated with 75 μg RNAse A in 100 μL PBS and incubated at 37° C. for 1 hour. After incubation, the cells were collected by centrifuging at 500 g at 4° C. for 10 minutes, followed by another PBS wash. The cell pellet was re-suspended in 200 μL PBS, in addition of PI solution at a final concentration of 25 ng/μL. After staining, the cells were analyzed immediately using flow cytometer BD FACSCanto™ II (BD Biosciences, San Jose, Calif.) 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).

[0118] Protein Biochemical Analysis

[0119] Western blotting was performed as described previously (Blanpain et al.). Briefly, equal amounts of the cell lysates were separated on a SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a nitrocellulose membrane. The immunoblot was incubated with Odyssey® blocking buffer (Li-Cor) at room temperature for 1 h, 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 h 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).

[0120] Skin Organoid Culture and Transplantation

[0121] Decellularized dermis (circular shape with 1cm diameter) was prepared by EDTA treatment of newborn mouse skin (Maeder et al.). An aliquot containing 1.5×10^6 cultured keratinocytes was seeded onto the dermis in cell culture insert. After overnight attachment, the skin culture was exposed to air/liquid interface.

[0122] For grafting with skin organoids, CD1 (isogenic mouse keratinocyte transplantation) males or Nude (human keratinocyte transplantation) females with the ages of 6-8 weeks were anesthetized. A silicone chamber bottom with the interior diameter of 0.8 cm and the exterior diameter of 1.5 cm 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 0.2 mg α-CD4 (GK1.5) and 0.2 mg α-CD8 (2.43.1) antibodies was administered intraperitoneally for skin grafting.

[0123] Obesity Induced by High Fat Diet and Glucose Tolerance Test

[0124] Male CD-1 mice with skin transplants 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-Sery (Frenchtown, N.J.). Body weight and food intake were measure biweekly.

[0125] For glucose tolerance testing, an intraperitoneal glucose tolerance test (IPGTT) was performed on mice fed an HFD for 10 weeks. Mice were fasted for 6 h 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, 10, 20, 30, 60 and 90 minutes using glucose test strips and glucose meters.

[0126] To induce hypoglycemia, CD1 mice with skin grafts were fasted for 4 h and injected (2 U/kg, i.p.) with insulin purchased from Sigma (St. Louis, Mo.). Blood glucose levels were determined thereafter at 0, 15, 30, 45, and 60 minutes.

[0127] Intravital Imaging of Mice

[0128] Optical imaging was performed in the integrated small animal imaging research resource (iSAIRR) at the University of Chicago. Bioluminescence images were acquired on an IVIS Spectrum (Caliper Life Sciences®, Alameda, Calif.) after animal was injected with luciferin (100 mg/kg). Acquisition and image analysis were performed with Living Image 4.3.1 software.

[0129] Wound healing in grafted skin were imaged by multiphoton microscope in the light microscopy center at the University of Chicago. Images were analyzed with Image J software.

[0130] Histology and Immunofluorescence

[0131] 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 in Wright et al. Antibodies were diluted according to manufacturer's instruction, unless otherwise indicated.

[0132] Statistical Analysis

[0133] Statistical analysis was performed using Excel or OriginLab software. Box plots are used to describe the entire population without assumptions on the statistical distribution. A student t test was used to assess the statistical significance (P value) of differences between two experimental conditions (2 tailed distribution unless specified). All experiments were repeated at least three times, unless otherwise specified. For all figures, statistical tests are justified and meet the assumption of the tests. The variance between different test groups that are being statistically compared is similar.

[0134] For all the experiments, the sample size was chosen based upon our preliminary test and previous research. There is no sample exclusion for all the in vitro analysis. For in vivo experiments, animals that died before the end of the experiment were excluded. The exclusion criteria is pre-established. No randomization or blinding was used in this study.

Results and Discussion

[0135] Despite the potential clinical applications, research in skin epidermal stem cells has been greatly hampered by the lack of an appropriate model. Although it has been shown that mouse skin or human skin can be transplanted onto immunodeficient mice, the lack of an intact immune system in this model forecloses prediction of potential outcomes of this procedure in vivo. Immune clearance of engineered cells has been a major complication for somatic gene therapy (see Collins, M. & Thrasher, A. Gene therapy: progress and predictions. *Proceedings. Biological sciences/ The Royal Society* 282 (2015)). Additionally, it remains technically challenging to perform skin organoid culture with mouse epidermal stem cells and generate mouse skin substitute for transplantation. To resolve these issues, a new organotypic culture model with mouse epidermal stem cells in vitro was developed by culturing the cells on top of acellularized mouse dermis (FIG. **6**A) (see Liu, H. et al. Regulation of Focal Adhesion Dynamics and Cell Motility by the EB2 and Hax1 Protein Complex. J Biol Chem 290, 30771-30782 (2015) and Yue, J. et al. In vivo epidermal migration requires focal adhesion targeting of ACF7. Nat Commun 7, 11692 (2016).

[0136] Tissue Organoids

[0137] Exposure to the air/liquid interface can induce stratification of cultured epidermal cells to generate a skin-like organoid in vitro. Transplantation of such cultured skin organoids to nude hosts leads to efficient skin engraftments (see Liu, H et al. 2015 and Yue, J et al. 2016). Using a modified surgical procedure and skin graft maintenance protocol, engrafting the isogenic mouse skin substitute onto an immunocompetent host (CD1 and C57BL/6J strains) with or without the silicone dome chamber for skin transplantation (FIG. **1**B and FIGS. **6**B and **6**C) was achieved.

[0138] Grafted cells in immunocompetent hosts readily expressed exogenous genes, such as Luciferase and Histone H2B-RFP (FIG. **1**B-C), which were transduced to the cells with lentivirus. The grafted tissue exhibited normal skin stratification (FIG. **1**D-F) when stained for basal epidermal stem cells (Keratin 14) or early (Keratin 10) and later (Loricrin) skin differentiation markers. In addition, skin grafts displayed similar cell proliferation and cell death when compared with adjacent host skin (FIG. **1**G and FIG. **6**D). Importantly, the tissue organoid with expression of exogenous gene, such as Luciferase and H2B-RFP, was stable in vivo for more than 5 months in immunocompetent hosts, as determined by intravital bioluminescence imaging and tissue histology. Together, these results demonstrate that tissue organoids are a new model for epidermal stem cell engineering and transplantation, and strongly suggest that genetically modified skin epidermal cells are not immunogenic and well tolerated in vivo.

[0139] Engineered Blood Glucose Monitors

[0140] A biointegrated sensor for noninvasive monitoring of blood glucose level in vivo could remove the need for diabetic patients to draw blood multiple times a day. Additionally, continuous monitoring of glucose allows patients to better maintain blood glucose levels by altering insulin dosage or diet according to the prevailing glucose values. Currently, most continuous glucose monitoring sensors are enzyme electrodes or microdialysis probes implanted under skin. These sensors usually require oxygen for activity, and are insufficiently stable in vivo and poorly accurate under low glucose condition. Presence of interfering electroactive substances in tissues can also cause impaired responses and signal drift in vivo, which necessitate frequent calibrations of current sensors. A fluorescence-based glucose sensor in skin would likely be more stable, have improved sensitivity,

and resolve the issue of electrochemical interference from the tissue (see Pickup et al. Fluorescence-based glucose sensors. *Biosensors & bioelectronics* 20, 2555-2565 (2005)).

[0141] To engineer epidermal stem cells for glucose sensing, intracellular expression of a sugar binding protein, glucose/galactose-binding protein (GGBP) (Jeffery, C. J. Engineering periplasmic ligand binding proteins as glucose nanosensors. *Nano reviews* 2, 2011) was examined. GGBP transports glucose within the periplasm of *E. coli,* and binding with glucose can lead to a large conformational change in the protein (Jeffery, 2011). This property of GGBP has been exploited to develop protein sensors for glucose based on FRET (fluorescence resonance energy transfer) or bioluminescence imaging (see Fehr et al. In vivo imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J Biol Chem* 278, 19127-19133, (2003); Saxl et al. A fluorescence lifetime-based fibre-optic glucose sensor using glucose/galactose-binding protein. The Analyst 136, 968-972 (2011); Teasley Hamorsky et al. A bioluminescent molecular switch for glucose. Angewandte Chemie 47, 3718-3721 (2008); Tian et al. Structure-based design of robust glucose biosensors using a Thermotoga maritima periplasmic glucose-binding protein. Protein science: a publication of the Protein Society 16, 2240-225 (2007) and Veetil et al. A glucose sensor protein for continuous glucose monitoring. Biosensors & bioelectronics 26, 1650-1655 (2010). WT (wild type) GGBP has very high glucose binding affinity (K_d=0.2 μm). To generate a probe corresponding to physiologically relevant range of glucose, a CFP/YFP FRET sensor with A213R/L238S double mutant of GGBP (SEQ ID NO: 28) (K_d =10 mM) (Amiss, T. J., Sherman, D. B., Nycz, C. M., Andaluz, S. A. & Pitner, J. B. Engineering and rapid selection of a low-affinity glucose/galactose-binding protein for a glucose biosensor. Protein science: *a publication of the Protein Society* 16, 2350-2359) was engineered via CRISPR-mediated genome editing in mouse epidermal stem cells. DNA vectors encoding the D10A mutant of Cas9 (CRISPR associated protein 9) (Ran, F. A. et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154, 1380-1389 (2013) (SEQ ID NO: 29), two gRNAs (guide RNA) (SEQ ID NO: 30), (SEQ ID NO: 31) targeting the mouse Rosa26 locus, and a Rosa26-targeting vector (SEQ ID NO: 32) were developed. The targeting vector contained two homology arms for the Rosa26 locus, flanking an expression cassette that encoded a GGBP fusion protein (FIG. **2**A and FIG. **7**A) (SEQ ID NO: 33).

[0142] 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 (FIG. **2**B). Engineered epidermal cells exhibited robust expression of GGBP fusion proteins in the cytosol (FIG. **2**C).

[0143] To test whether intradermal electroporation would also be an effective means of introducing a reporter, CD1 mice were electroporated intradermally with plasmid DNA encoding luciferase and tdTomato and expression was assayed by bioluminescence imaging (FIG. **3**A) and intravital imaging with a two-photon microscope (FIG. **3**B), respectively. Electroporation resulted in expression of both luciferase and tdTomato.

[0144] To test glucose sensing in vitro, the cells were exposed to medium containing increasing amounts of glucose. Quantification of FRET ratio by microscopic imaging showed an excellent correlation of FRET ratio with extracellular glucose concentration, ranging from 0-10 mM (FIG. **2**D). Further analysis revealed that the GGBP reporter responded to extracellular glucose or galactose, but not to other sugars, such as sucrose, fructose, or ribose (FIG. **2**E). The intracellular GGBP probe responded to the change of glucose concentration rapidly. The FRET ratio changed within 30 seconds after replacement of the medium, and remained stable in the same medium. Together, these results indicate that epidermal stem cells expressing a GGBP reporter can faithfully sense the extracellular glucose concentration.

[0145] Expression of the GGBP fusion protein in epidermal cells did not significantly change cell proliferation (FIG. **2**F and FIG. **7**B) or differentiation (FIG. **2**G) in vitro. To confirm that modified epidermal cells are not tumorigenic, anchorage-independent growth of cells was assayed and confirmed that epidermal stem cells with GGBP targeting could not grow in suspension (FIG. **2**H). As a positive control, cancer initiating cells isolated from mouse SCC (squamous cell carcinoma) exhibited robust colony formation in soft agar medium (FIG. **2**H). Expression of GGBP reporter did not affect the ability of epidermal stem cells to stratify. When subjected to skin organoid culture, the targeted cells readily produced stratified epithelial tissue (FIG. **7**C).

[0146] To investigate the potential applicability in vivo of the engineered blood glucose monitor, a skin organoid culture was prepared with engineered epidermal stem cells, and the organoid was grafted onto CD1 host animals (FIG. **4**A). No significant rejection of the skin grafts was observed after transplantation. The grafted organoids exhibited normal epidermal stratification, proliferation and cell death (FIGS. **8**A-**8**E). To test the glucose sensing capability in vivo, an intraperitoneal glucose tolerance test (IPGTT) was performed in grafted animals. Fasted animals received a bolus of glucose intraperitoneally. Fluorescence (FRET) change in the grafted skin was monitored by intravital imaging (FIG. **4**B), and blood glucose level was measured by a commercial glucose monitoring system (Bayer Contour) with blood samples taken from the snipped tail. FIGS. **4**C and **4**D show the correlation between the measured glucose concentration and the FRET ratio changes over time. The FRET ratio exhibited a nearly linear correlation with the glucose concentration in vivo (FIG. **4**D, R^2=0.977). In contrast, traditional glucose sensors cannot accurately measure low glucose level in vivo.

[0147] To test the skin sensor for lower glucose concentration, we induced hypoglycemia by insulin administration to fasted animals. Intravital imaging of the grafted skin again showed excellent correlation of the FRET ratio changes with glucose level (FIG. **4**E and **4**F). Together, these results illustrate that engineered skin organoid with GGBP reporter can accurately sense the blood glucose level in vivo.

[0148] Engineered Tissue Organoids

[0149] If an engineered blood glucose monitor could be engineered to produce or regulate insulin levels in response to blood glucose levels, it could approximate an "artificial

endocrine pancreas" that would automatically maintain glucose level in patients. Thus, introduction of an expression cassette into epidermal stem cells that encodes both a GGBP reporter and a therapeutic protein could achieve continuous glucose monitoring and diabetes treatment with a single skin transplantation. GLP1 (glucagon-like peptide 1) is released from the gut upon food intake and acts both as a satiety signal to reduce food consumption and as an incretin hormone to stimulate insulin release and inhibit glucagon secretion. Indeed, GLP1 receptor agonists have previously been used to treat type 2 diabetes. Therefore, a new Rosa26 targeting vector containing an expression cassette that encodes a GLP1 and mouse IgG-Fc fragment (for enhanced stability and secretion of GLP1) fusion protein together with the GGBP reporter was developed (FIG. **8**F).

[0150] Engineered epidermal cells exhibited robust GLP1 production and secretion (FIG. **4**G). The secreted GLP1 fusion protein was functional as the conditioned medium significantly induced secretion of insulin when added to cultured insulinoma cells (FIG. **4**H).

[0151] To examine the potential applicability in diet-induced obesity and diabetes, GLP1/GGBP-expressing cells and control cells (GGBP alone) were transplanted into two cohorts of CD1 adult male mice. The mice were fed a high fat diet (HFD) to induce obesity in grafted animals. Compared with animals on a regular chow diet, the HFD greatly accelerated body weight gain in mice grafted with control cells. By contrast, GLP1 expression led to significant inhibition in body weight increase (FIG. **4**I; quantified in FIG. **4**J).

[0152] IPGTT was performed to examine glucose homeostasis. Expression of GLP1 significantly reduced glycemic excursion in vivo as determined by both direct measurement of blood glucose or intravital imaging of the GGBP reporter (FIGS. **4**K-**4**M). Noninvasive monitoring with the GGBP reporter exhibited excellent correlation with conventional glucose measurement in both diabetic animals and GLP1-treated animals (FIGS. **4**L and **4**M).

[0153] Human Tissue Organoids

[0154] To test the feasibility of glucose sensing with human epidermal stem cells, human skin organoids were cultured from primary epidermal keratinocytes isolated from human newborn foreskin. The human epidermal keratinocytes 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 (FIG. **5**A). The grafted tissue shows normal skin stratification when stained for early or late epidermal differentiation markers (FIG. **5**B).

[0155] For CRISPR-mediated genome editing, vectors encoding two gRNAs targeting the human AAVS1 (adeno-associated virus integration site 1) locus and an AAVS/-targeting vector (FIG. **9**A) that encodes the GGBP reporter protein were developed. Human epidermal keratinocytes were electroporated with the targeting vector together with plasmids encoding Cas9 and the gRNAs. Clones were isolated and correct integration was confirmed by southern blotting analysis (FIG. **5**C).

[0156] Expression of the GGBP fusion protein did not significantly change cell proliferation (FIG. **9**B) or differentiation (FIG. **9**C) in vitro. The engineered cells stratified and formed skin organoids in vitro, which were successfully transplanted onto nude hosts. Grafted tissue organoids exhibited normal epidermal stratification and proliferation in vivo (FIGS. **9**D-**9**G). IPGTT and intravital imaging of the GGBP reporter were performed and a similar correlation of FRET ratio changes in the grafted skin with blood glucose level was observed (FIG. **5**D-**5**F). These data indicate that these human tissue organoids could be used for monitoring of blood glucose levels in humans.

[0157] In this study, technical hurdles were overcome to establish a unique mouse-to-mouse skin transplantation model with immunocompetent hosts. The results provide key evidence supporting the feasibility of cutaneous monitoring of blood glucose level in vivo. The same platform can be also exploited for development of other biosensors and ex vivo cutaneous gene therapy for stable delivery of therapeutic proteins in vivo, such as GLP1, providing a promising treatment for many otherwise terminal or severely disabling diseases (see Christensen, R. et al. Skin genetically engineered as a bioreactor or a 'metabolic sink'. *Cells, tissues, organs* 172, 96-104, (2002) and Del Rio, M. et al. Current approaches and perspectives in human keratinocyte-based gene therapies. *Gene therapy* 11 Suppl 1, S57-63, (2004)).

Example No. 2: Treatment of Diabetes and Obesity with CRISPR-Mediated Genome Editing in Epidermal Stem Cells

Introduction

[0158] In this report, by combining CRISPR-mediated genome editing with epidermal stem cell platform, a skin graft with controllable release of GLP1 (glucagon-like peptide-1) was developed and demonstrated therapeutic effect in vivo by reducing glycemic excursions in diet-induced obese and diabetic mice. 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 (see Ross et al. 2010, Sandoval et al. 2015). However, native GLP1 must be delivered through a parenteral route to achieve its effect as 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 in vivo in order to treat diabetes (Prud'homme et al. 2007; Rowzee et al. 2011).

[0159] The recent development of genome editing technology, including CRISPR (clustered regularly-interspaced short palindromic repeats) system, has made it possible to perform precise genetic engineering, providing an ideal tool for somatic gene therapy (Cox et al., 2015; Hotta et al. 2015; 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.

[0160] Skin epidermal stem cells (Blanpain and Fuchs, 2006; Watt, 2014) have several unique advantages, making them particularly suited for somatic gene therapy ex vivo: i) Human skin is the largest and most accessible organ in the body, offering availability for collection of epidermal stem cells with well-established procedures (Rasmussen et al., 2013; Rheinwald and Green, 1975, 1977). 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. Cultured epidermal stem cells can 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). Comparing 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; Coleman and Siwy, 1992). Somatic gene therapy with epidermal stem 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. iv) Epidermal stem 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; Kustikova et al. 2010). v) Epidermal stem 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 stem cells can achieve long term and stable transplantation in human patients without eliciting significant immune reaction (Centanni et al., 2011; Zaulyanov and Kirsner, 2007). vi) 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.

[0161] This example demonstrates that genome-edited epidermal stem cells can be exploited for robust, controllable delivery of GLP1 in vivo and effective treatment of diabetes and obesity in a clinically-relevant setting.

Experimental Procedures

[0162] Reagents and Plasmid DNA Constructions

[0163] Guinea pig anti K5, rabbit anti K14, rabbit anti K10 and Loricrin antibodies were generous gifts from Dr. Elaine Fuchs at the Rockefeller University. Rat monoclonal β4-integrin (CD104, BD 553745) was obtained from BD Pharmingen (Franklin lakes, N.J.). Ser10 pho-histone antibody was obtained from EMD Millipore (Billerica, Mass.). Cleaved caspase-3 antibody was obtained from Cell Signaling Technology (Danvers, Mass.). Insulin ELISA kit was obtained from EMD Millipore Corp (Billerica, Mass.). GLP-1 ELISA kit was obtained from Sigma (St. Louis, Mo.). Other chemicals or reagents were obtained from Sigma, unless otherwise indicated.

[0164] Lentiviral vector encoding Luciferase (SEQ ID NO: 1) and H2B-RFP (SEQ ID NO: 2) has been described before (Liu et al., 2015; Yue, 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 (SEQ ID NO: 3); and gGA ATT CTA ATG CCA ACT TTG TAC (SEQ ID NO: 4), using gBlock as a template. Rosa26—targeting gRNA is constructed with primers: ACA CCG GCA GGC TTA AAG GCT AAC CG (SEQ ID NO: 5), AAA ACG GTT AGC CTT TAA GCC TGC CG (SEQ ID NO: 6), ACA CCG AGG ACA ACG CCC ACA CAC Cg (SEQ ID NO: 7), AAA ACG GTG TGT GGG CGT TGT CCT CG (SEQ ID NO: 8). AAVS1—targeting gRNA is constructed with primers: ACA CCG TCA CCA ATC CTG TCC CTA GG (SEQ ID NO: 9), AAA ACC TAG GGA CAG GAT TGG TGA CG (SEQ ID NO: 10), ACA CCG CCC CAC AGT GGG GCC ACT AG (SEQ ID NO:11), AAA ACT AGT GGC CCC ACT GTG GGG CG (SEQ ID NO: 12). 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 (SEQ ID NO: 13), GAC TAG TCC CGG GGG ATC CAC CGG TCA GGA ACA GGT GGT GGC GGC CC (SEQ ID NO: 14), CGG GAT CCA CCG GTG AGG GCA GAG GAA GCC TTC TAA C (SEQ ID NO: 15), TCC CCC GGG TAC AAA ATC AGA AGG ACA GGG AAG (SEQ ID NO: 16), GGA ATT CAA TAA AAT ATC TTT ATT TTC ATT ACA TC (SEQ ID NO: 17), CCT TAA TTA AGG ATC CAC GCG TGT TTA AAC ACC GGT TTT ACG AGG GTA GGA AGT GGT AC (SEQ ID NO: 18). AAVS1 targeting vector (SEQ ID NO: 40) 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 (SEQ ID NO: 19), CCC AAG CTT CCA TAG AGC CCA CCG CAT CCC C (SEQ ID NO: 20), CAG GGT CTA GAC GCC GGA TCC GGT ACC CTG TGC CTT CTA GTT GC (SEQ ID NO: 21), GGA TCC GGC GTC TAG ACC CTG GGG AGA GAG GTC GGT G (SEQ ID NO: 22), CCG CTC GAG AAT AAA ATA TCT TTA TTT TCA TTA CAT C (SEQ ID NO: 23), GCT CTA GAC CAA GTG ACG ATC ACA GCG ATC (SEQ ID NO: 24). Genotyping primers for CRISPR mediated knockin: GAG CTG GGA CCA CCT TAT ATT C (SEQ ID NO: 25), GGT GCA TGA CCC GCA AG (SEQ ID NO: 26), GAG AGA TGG CTC CAG GAA ATG (SEQ ID NO: 27).

[0165] Skin Organoid Culture and Transplantation

[0166] Decellularized dermis (circular shape with 1 cm diameter) was prepared by EDTA treatment of newborn mouse skin (Prunieras et al., 1983). An aliquot of 1.5×10^6 cultured keratinocytes was seeded onto the dermis in a cell culture insert. After overnight attachment, the skin culture was exposed to air/liquid interface.

[0167] 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 0.8 cm and exterior diameter of 1.5 cm 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 immediately 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 0.2 mg α-CD4 (GK1.5) and 0.2 mg α-CD8 (2.43.1) antibodies was administered intraperitoneally for skin grafting.

[0168] Histology and Immunofluorescence

[0169] Skin or wound samples were embedded in OCT, frozen, sectioned, and fixed in 4% formaldehyde. For par-

affin 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. Antibodies were diluted according to manufacturer instructions, unless otherwise indicated.

[0170] Cell Cycle Analysis:

[0171] Propidium iodide (PI) staining followed by flow cytometry were used to determine the effect of cell cycle profiles. Mouse and human epidermal cells were cultured in two 6 cm cell culture dish for 24 hours, 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 hour. Then, fixed cells were centrifuged at 500 g at 4° C. for 10 minutes, followed by 2× PBS wash. Cells were then treated with 75 μg RNAse A in 100 μl PBS and incubated at 37° C. for 1 hour. After incubation, the cells were collected by centrifuging at 500 g at 4° C. for 10 minutes, followed by another PBS wash. The cell pellet was resuspended in 200 μl PBS with PI solution at a final concentration of 25 ng/μl. After staining, the cells were analyzed immediately using flow cytometer BD FACSCanto™ II (BD Biosciences, San Jose, Calif.) 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).

[0172] Protein Biochemical Analysis

[0173] Western blotting was performed to assess protein biochemistry. Briefly, equal amounts of cell lysates were separated on an 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 h, 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 h at room temperature. Blots were washed three times with TBST again. Visualization and quantification were carried out with the LI-COR Odyssey scanner and software (LI-COR Biosciences).

[0174] Obesity Induced by High Fat Diet and Glucose and Insulin Tolerance Tests

[0175] 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-Sery (Frenchtown, N.J.). Body weight and food intake were measure biweekly.

[0176] For glucose and insulin tolerance tests, an intraperitoneal glucose tolerance test (IPGTT) was performed on mice fed an HFD for 10 weeks. Mice were fasted for 6 h 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 IPGTT. Mice were fasted for 4 h 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.

[0177] Statistical Analysis

[0178] Statistical analysis was performed using Excel or OriginLab software. Box plots were used to describe the entire population without assumptions on the statistical distribution. A student t test was used to assess the statistical significance (P value) of differences between two experimental conditions.

Results

[0179] Ectopic expression of GLP1 in epidermal stem cells via CRISPR-mediated genome editing

[0180] By genetic engineering of skin epidermal stem cells, skin can potentially be transformed into an in vivo reactor that produces GLP1 in a controllable manner (FIG. **1**A). Although skin stem cells are very susceptible for manipulation with viral vectors, viral infection could lead to genotoxicity and may raise significant safety concern for the potential gene therapy (Kotterman et al., 2015; Kustikova et al., 2010). The CRISPR system presents a novel approach to carry out site-specific modification of target genomes non-virally (Cox et al., 2015; Hotta and Yamanaka, 2015; Wright et al., 2016).

[0181] To test CRISPR-mediated genome editing in mouse epidermal stem cells, DNA vectors encoding the D10A mutant of Cas9 (CRISPR associated protein 9) (Ran et al., 2013), two gRNAs (guide RNA) targeting the mouse Rosa26 locus, and a Rosa26-targeting vector were developed. 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 (FIG. **10**A and FIG. **2**A). Fusion with IgG-Fc enhances the stability and secretion of GLP-1 when ectopically expressed in epidermal cells (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 (FIG. **10**A).

[0182] 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 (FIG. **10**B). Engineered epidermal cells exhibited robust GLP1 production upon stimulation with doxycycline in a dose-dependent manner (FIG. **10**C). The secreted GLP1 fusion protein was functional as the conditioned medium can significantly induce secretion of insulin when added to insulinoma cells cultured in vitro (FIG. **10**D). Expression of GLP1 fusion protein in epidermal cells did not significantly change cell proliferation (FIG. **10**E and FIG. **14**A) or differentiation (FIG. **10**F and **10**G) in vitro.

[0183] To confirm that modified epidermal cells were not tumorigenic, the potential anchorage-independent growth of cells was examined, and the results indicated that epidermal stem cells with GLP1 targeting cannot grow in suspension with or without doxycycline stimulation (FIG. **10**H). 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 (FIG. **10**H). Expression of GLP1 did not affect the ability of epidermal stem cells to stratify. When subjected to skin organoid culture, the targeted cells readily produced stratified epithelial tissue (FIG. **14**B).

[0184] Stable Delivery of GLP1 In Vivo Through Skin Transplantation

[0185] To investigate the potential therapeutic effect of GLP1 in vivo, skin organoid cultures with epidermal keratinocytes targeted with a GLP1-expression vector or a control vector, and transplant the organoids to CD1 host animals (FIGS. **11**A and **11**B) were prepared. No significant rejection of the skin grafts has been observed after transplantation, suggesting that the targeted epidermal stem cells were well tolerated immunologically in vivo. Grafted skin exhibited normal epidermal stratification, proliferation and cell death regardless of doxycycline treatment (FIGS. **11**C-**11**E, and FIGS. **15**A and **15**B). When fed with food containing doxycycline, the mice that were grafted with GLP1-expressing cells displayed significantly enhanced level of GLP1 in the blood (FIG. **11**F). Expression of GLP1 in grafted animals was stable for more than 3 months (FIG. **11**G). 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), these results confirm that a skin-derived therapeutic protein can cross the basement membrane barrier and achieve a systematic effect in vivo.

[0186] Cutaneous gene transfer with GLP1 can achieve therapeutic effects in vivo

[0187] To examine the potential effect in diet-induced obesity and diabetes, GLP1-expressing cells and control cells were grafted onto two cohorts of CD1 adult mice, and a high fat diet (HFD) was used to induce obesity in the grafted animals. Doxycycline was applied to half of the animals to induce expression of GLP1. To minimize gender difference, only male animals were used. Compared with animals on regular chow diet, 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 (FIG. **12**A and quantified in FIG. **1**C). Consistently, histological examination of white fat tissue demonstrated that HFD progressively increased the size of adipocytes and induced a significant level of adipocyte hypertrophy at the end of the experiment in the control groups (FIG. **12**B). By contrast, induction of GLP1 expression dramatically suppressed this effect (FIG. **12**B).

[0188] To examine glucose homeostasis, IPGTT (intraperitoneal glucose tolerance test) and ITT (insulin tolerance test) were performed. HFD resulted in decreased glucose tolerance in control mice or GLP1-grafted mice without doxycycline treatment FIGS. **12**D and **12**E). By contrast, expression of GLP1 significantly reduced glycemic excursion in vivo (FIGS. **12**D and **12**E). Consistent with glucose homeostasis analysis, expression of GLP1 in grafted skin significantly reduced insulin resistance compared with control mice or GLP1 mice without doxycycline treatment (FIGS. **12**F and **12**G). Together, these 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.

[0189] Cutaneous Delivery of GLP1 with Human Epidermal Stem Cells

[0190] To test the feasibility of cutaneous gene therapy with human epidermal stem cells, human skin organoids were cultured from primary epidermal keratinocytes isolated from human newborn foreskin. When infected with lentivirus, the grafted human cells exhibited robust expression of the exogenous Luciferase gene (FIG. **13**A). The grafted tissue showed normal skin stratification when stained for early or late epidermal differentiation markers (FIG. **13**B).

[0191] To examine CRISPR-mediated genome editing in human epidermal cells, vectors encoding two gRNAs targeting human AAVS1 (adeno-associated virus integration site 1) locus, and an AAVS1-targeting vector (FIG. **16**A) that harbors a tetracycline-inducible expression cassette encoding the GLP-1 and IgG-Fc fragment fusion protein were used. 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 (FIG. **13**C). Like mouse cells, engineered human epidermal cells exhibited strong GLP1 production upon dose-dependent stimulation with doxycycline (FIG. **13**D). The GLP1 fusion protein secreted from these cells induced insulin secretion in vitro (FIG. **13**E), confirming the release of functional GLP1 from these cells.

[0192] Expression of the GLP1 fusion protein in human cells did not significantly change cell proliferation (FIG. **16**B) or differentiation (FIGS. **16**C and **16**D) in vitro. The engineered cells stratified and formed skin organoids in vitro, which were transplanted to nude hosts (FIG. **13**F). Grafted skin exhibited normal epidermal stratification and proliferation regardless of doxycycline treatment (FIGS. **16**E-**16**G). When fed with food containing doxycycline, significant secretion of GLP1 was detected in the blood from the nude mice that were grafted with GLP1-expressing cells (FIG. **13**G).

Discussion

[0193] Somatic gene therapy provides a promising therapeutic approach for treatment of a variety of otherwise terminal or severely disabling diseases (Collins and Thrasher, 2015). Skin epidermal stem cells represent an ideal platform for ex vivo gene therapy, allowing efficient genetic manipulation with minimal risk of tumorigenesis or other detrimental complications in vivo (Christensen et al., 2002; Del Rio et al., 2004). In addition to expression of therapeutic agent (e.g., hormones and/or protein factors), such as GLP-1, ectopic expression of metabolic enzymes in skin epidermal cells can also transform engineered tissue organoids into a potential "metabolic sink" for correction of various metabolic disorders (Christensen et al., 2002). Thus, the applicability of such a cutaneous gene therapy platform is very broad. Because of the minimally invasive and safe nature of skin transplantation, should an efficient preclinical model for cutaneous gene therapy be established and encouraging results be obtained from animal models, human clinical tests could start relatively easily.

[0194] Diabetes is a major health issue worldwide (Ahima, 2011; Ashcroft and Rorsman, 2012). The peptide hormone GLP1 has the essential requisite properties to maintain homeostatic levels of glucose in order to effectively treat diabetes. Compounds that elongate half-life of endogenous GLP1 or synthetic GLP1 receptor agonists have already been clinically used for adjunctive antidiabetic treatments (Ross and Ekoe, 2010; Sandoval and D'Alessio, Physiology of proglucagon peptides: role of glucagon and GLP-1 in health and disease. *Physio. Rev.* 95, 513-548 (2015)). Somatic gene transfer that can stably deliver GLP1 to the patients has been proposed as a more effective way for

diabetes treatment (Prud'homme et al., 2007; Rowzee et al., 2011). In this regard, skin constitutes a tempting target organ, providing a long-lasting, safe, and affordable way for GLP1 delivery. Here, controllable release of GLP1 was demonstrated in engineered tissue organoids and proven to be therapeutically effective in vivo, thus laying the essential groundwork for developing novel therapeutic approach for combating obesity and diabetes.

[0195] Gene therapy-derived products can be recognized as foreign antigens by the host immune system, which may mount an immune response leading to the clearance of genetically modified cells (Collins and Thrasher, 2015). However, the model developed in this study provides a unique approach. 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 the engineered tissue organoids described here (generated from epidermal stem 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). The present results demonstrate for the first time that grafted skin stem cells expressing therapeutic proteins can be efficiently and stably grafted to host mice with intact immune systems. The present results further demonstrate the potential of cutaneous gene therapy for the treatment of various human diseases in the future.

Example No. 3: Tissue Organoids for Treating Cocaine Addiction

Introduction

[0196] Drug addiction is characterized by the development of compulsive drug-seeking and taking and a high likelihood of relapse when an addicted individual is exposed to drugs or drug-associated cues, even long after abstention (Kalivas et al. Drug addiction as a pathology of staged neuroplasticity. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* 33: 166-180 (2008); Koob et al. Neurocircuitry of addiction. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* 35: 217-238 (2010); O'Brien et al. Conditioning factors in drug abuse: can they explain compulsion? *Journal of psychopharmacology* 12: 15-22 (1998)). Cocaine is a commonly abused drug that causes significant morbidity and mortality. Although a variety of pharmacological targets and behavioral interventions have been explored, there are currently no FDA-approved medications for treating cocaine use or relapse in users, and there are no effective interventions for the acute emergencies that result from cocaine overdose (Heard et al. Mechanisms of acute cocaine toxicity. The open *pharmacology journal* 2: 70-78, (2008); Zimmerman, J. L. Cocaine intoxication. *Critical care clinics* 28: 517-526 (2012)). We recently demonstrated that the epidermal progenitor cells of the skin can be readily genome edited in vitro using CRISPR (clustered regularly interspaced short palindromic repeats) and transplanted back into donor mice (see Liu, H et al., 2015; Yue, J et al., 2016; Yue et al. Engineered epidermal progenitor cells can correct diet-induced obesity and diabetes. *Cell stem cell* (2017)). This unique ex vivo platform can provide a long-lasting, effective, and safe way for somatic gene delivery. Here, we report that this skin stem cell-based platform for long-term delivery of a cocaine hydrolase in vivo can efficiently and specifically protect against cocaine-seeking and acute overdose.

[0197] BChE (butyrylcholinesterase) is a natural enzyme that is present in hepatocytes and plasma and hydrolyzes its normal substrate acetylcholine. BChE can also hydrolyze cocaine at low catalytic efficiency into benzoic acid and ecgonine methylester, which are low in toxicity and rewarding properties, i.e., they are not addictive substances. Recent advances in protein engineering have greatly enhanced catalytic potency and substrate specificity of BChE for cocaine hydrolysis, and for protecting against cocaine-induced behaviors, including acquisition and reinstatement of IVSA (intravenous self-administration). The modified hBChE (E30-6) has more than 4400 times higher catalytic efficiency (k_{cat}/K_M) than wild-type (VVT) hBChE with significantly reduced activity for acetylcholine (see Zheng et al. A highly efficient cocaine-detoxifying enzyme obtained by computational design. *Nat Commun.* 5: 3457 (2014)). However, purified recombinant hBChE has a short half-life in vivo after i.v. injection, making it useful only for acute treatment of cocaine abuse. In recent clinical trials, an hBChE-albumin fusion protein, TV-1380, was ineffective in facilitating cocaine-abstinence in dependent individuals, most likely due to the short half-life of the protein and also the inefficient intramuscular route of injection (Cohen-Barak et al. Safety, pharmacokinetics, and pharmacodynamics of TV-1380, a novel mutated butyrylcholinesterase treatment for cocaine addiction, after single and multiple intramuscular injections in healthy subjects. *Journal of clinical pharmacology* 55: 573-583 (2015); Gilgun-Sherki et al. Placebo-controlled evaluation of a bioengineered, cocaine-metabolizing fusion protein, TV-1380 (AlbuBChE), in the treatment of cocaine dependence. *Drug and alcohol dependence* 166: 13-20 (2016)). Therefore, the ability to stably deliver engineered hBChE in vivo to allow continuous activity could lead to cocaine abstinence in dependent individuals and prevent establishment of cocaine dependence in others.

Experimental Procedures

[0198] Reagents and Plasmid DNA Constructions

[0199] Unless indicated to the contrary, reagents and experimental procedures follow those described in Example No. 2 above. Human BChE quantikine ELISA kit was obtained from R&D systems (Minneapolis, Minn.).

[0200] Human (SEQ ID NO: 41) and mouse BChE (SEQ ID NO: 43) with point mutations {Zheng, 2014 #794} were codon-optimized and synthesized from IDT (Integrated DNA Technology, Coralville, Iowa), and PCR amplified with primers A-D, respectively: GCT CTA GAG CCA CCA TGC AGA CTC AGC ATA CCA AGG (SEQ ID NO: 47), CGG GAT CCA CCG GTT TAG AGA GCT GTA CAA GAT TCT TTC TTG (SEQ ID NO: 48), CCC AAG CTT GCC ACC ATG CAT AGC AAA GTC ACA ATC (SEQ ID NO: 49), ACG CGT CGA CTT AGA GAC CCA CAC AAC TTT CTT TCT TG (SEQ ID NO: 50).

[0201] Skin Organoid Culture and Transplantation

[0202] Skin organoid culture and transplantation was performed following the procedures described in Example No. 2 above.

[0203] Engraftment

[0204] Engraftment followed the procedures described in Example No. 2 above unless otherwise indicated below.

[0205] Cocaine-Induced Behaviors

[0206] For all behavioral experiments except where noted, C57BL/6J mice were used. Roughly equal numbers of adult male and female mice were group-housed until surgery. Mice were maintained under controlled temperature and humidity conditions on a 12 h:12 h light:dark cycle (lights on at 7:00). Water and food were available ad libitum. Mice weighed around 25-30 g at the beginning of the experiments. All procedures followed National Institutes of Health Guide for the Care and Use of Laboratory Animal and were approved by the University of Chicago Institutional Animal Care and Use Committee.

[0207] Drug: Cocaine HCl and methamphetamine HCl (Sigma-Aldrich, Saint Louis, MO) were dissolved in sterile saline and delivered intraperitoneally at appropriates doses in a volume of 10 mL/kg. Ethanol (Sigma-Aldrich, Saint Louis, Mo., 95%, density=0.816) was prepared in 20% (v/v) diluted in sterile saline and delivered intraperitoneally at appropriate doses. Vehicle (sterile saline) was intraperitoneally administered at 10 mL/kg as a control.

[0208] CPP apparatus: The CPP apparatus (FIG. **17**; Med Associates, E. Fairfield, Vt., USA) consisted of two larger chambers (16.8×12.7×12.7 cm), which were separated by a smaller chamber (7.2×12.7×12.7 cm) as previously described (Yan et al., 2013). Each chamber had a unique combination of visual and tactile properties (one large chamber had black walls and a rod floor, the other larger chamber had white walls with a mesh floor, whereas the middle chamber had gray walls and a solid gray floor). Each compartment had a light embedded in a clear, Plexiglas hinged lid. Time spent in each chamber was measure via photobeam breaks and recorded. CPP was determined on testing days via time spent in the drug-paired side minus time spent in the saline-paired side.

[0209] Acquisition of CPP: A biased CPP procedure was used similar to that from a previous study (Yan et al., 2013). Acquisition of CPP consisted of three sequential procedures—pretest, conditioning, and test. After 7-12 days of recovery from engrafting surgeries, mice underwent pretest on Day 1, where mice were allowed to freely explore the entire chamber for 20 min once daily. Mice that spent more than 500 s in the grey compartment or more than 800 s in either of the large compartments were excluded from the study. Following the pretest day, mice underwent conditioning and testing on Days 2-5. Starting on Day 2, mice received an i.p. injection of drug (10 mg/kg cocaine in cocaine CPP or 2 g/kg ethanol in ethanol CPP) and were confined to the white chamber for 30 min. At least 5 hours after in the same day, mice received an i.p. injection of saline and were confined to the black compartment for 30 min. On Day 6 (test day) mice were allowed to explore the entire chambers for 20 min and time spent in each area was recorded.

[0210] Extinction and reinstatement of CPP: Following CPP acquisition, mice underwent extinction, in which the procedure was identical to that in the test day. In each extinction day, mice were allowed to explore the entire chambers for 20 min and time spent in each area was recorded. Extinction was performed until the CPP decreased to a level that was not different from that of the pretest in consecutive two days. On the following day of the last extinction, mice underwent reinstatement procedures, in which mice that were trained for cocaine CPP received an i.p. injection of 15 mg/kg cocaine, and mice that were trained for ethanol CPP received an i.p. injection of 1 g/kg ethanol. Immediate after injection, mice were allowed to explore the entire chambers for 20 min and time spent in each area was recorded.

[0211] Acute drug overdose test: Two weeks after grafting surgery, 4 groups of GhBChE and 4 groups of GWT mice (n=8 each in each group) received i.p. injections of cocaine at 40, 80, 120, and 160 mg/kg. As a control, 4 groups of GhBChE and 4 groups of GWT mice (n=8 each) received i.p. injections of methamphetamine (METH) at 34, 68 (LD50), 100, and 160 mg/kg. Two each of GhBChE and GVVT mice with CD1 mice as hosts were also used to videotape acute cocaine (80 mg/kg) induced behaviors. Mice were monitored for 2 h following injection and percent of cocaine- and METH-induced lethality was calculated.

[0212] Specific methods: One group of GhBChE and one group of GVVT mice (n=9 in each group) were trained for cocaine CPP 7 days after engraftment (FIG. **18**A). Mice underwent pretest on Day 1, four days of cocaine conditioning day 2 to Day 5, and CPP test on Day 6. One group of GhBChE and one group of GWT mice (n=8 in each group) were trained for ethanol CPP 7 days after engraftment (FIG. **18**B). Mice underwent pretest on Day 1, four ethanol conditioning day 2 to Day 5, and CPP test on Day 6. Two groups of drug-naïive wildtype mice (n=8 in each group) were trained for cocaine CPP from day 1 to Day 6 (FIG. **18**C). On the following day, mice underwent engrafting surgery. The behavioral procedure resumed after engraftment surgery from Day 18. Extinction was performed from Day 18 to Day 31. On Day 32, mice underwent reinstatement induced by i.p. injection of cocaine. One group of GhBChE and one group of GVVT mice (n=8 in each group) were trained for ethanol CPP from day 1 to Day 6. Extinction was performed from Day 7 to Day 20 (FIG. **18**D). On Day 21, mice underwent reinstatement procedure induced by i.p. injection of ethanol.

[0213] Statistical Analysis

[0214] Statistical analysis was performed using Excel or OriginLab software. Box plots are used to describe the entire population without assumptions on the statistical distribution. A student t test was used to assess the statistical significance (P value) of differences between two experimental conditions. For cocaine behavioral analysis, CPP results were analyzed using repeated-measures ANOVA with within factor time (testing days) and between factor treatment (engraftment). Significant effects were further analyzed with Fisher's t-tests.

Results and Discussion

[0215] To carry out CRISPR-mediated genome editing in mouse epidermal progenitor cells, DNA vectors were developed encoding the D10A mutant of Cas9 (CRISPR associated protein 9; Ran et al., 2013), two gRNAs (guide RNA) 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 the modified hBChE gene (FIG. **19**A). Primary

epidermal basal cells were isolated from 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 (FIG. **19**B). Engineered epidermal cells exhibited robust expression and secretion of hBChE as shown by immunoblots and ELISA (enzyme-linked immunosorbent assay) (FIGS. **19**C and **19**D). The secreted hBChE protein was functional as the conditioned medium collected from hBChE-expressing cells but not the control cells significantly induced degradation of cocaine in vitro (FIG. **19**E). Consistent with previous reports, similar mutations in mBChE (mouse BChE) led to only residual activity in cocaine hydrolysis (Chen, X. et al. Kinetic characterization of a cocaine hydrolase engineered from mouse butyrylcholinesterase. The Biochemical journal 466, 243-251 (2015); FIG. **19**E). Expression of hBChE in epidermal stem cells did not significantly change cell proliferation (FIG. **19**F and FIG. **20**A) or differentiation (FIG. **20**B) in vitro. To confirm that modified epidermal cells were not tumorigenic, the potential anchorage-independent growth of cells was examined. The results indicated that epidermal stem cells with hBChE targeting cannot grow in suspension. By contrast, cancer initiating cells (Schober, M. et al. Tumor-initiating stem cells of squamous cell carcinomas and their control by TGF-beta and integrin/focal adhesion kinase (FAK) signaling. *Proc Natl Acad Sci U S A* 108, 10544-10549 (2011)) isolated from mouse SCC (squamous cell carcinoma) exhibited robust colony forming efficiency in soft agar medium (FIG. **20**C).

[0216] To efficiently transplant mouse epidermal progenitor cells, a new organotypic culture model with mouse epidermal progenitor cells was developed in vitro by culturing the cells on top of acellularized mouse dermis. Exposure to the air/liquid interface can induce stratification of cultured cells to generate a skin-like organoid in vitro. Expression of hBChE did not change the ability of epidermal stem cells to stratify (FIG. **21**A). To investigate the potential therapeutic effect of hBChE expression in vivo, we transplanted the organoids to isogenic host animals (CD1 and C57BL/6) (FIGS. **22**A and **22**B). No significant rejection of the skin grafts was observed for at least 5 months after transplantation, suggesting that the targeted epidermal stem cells were well tolerated immunologically in vivo. Grafted skin exhibited normal epidermal stratification, proliferation, and cell death (FIGS. **21**B, **21**C, and **22**C). The mice that were grafted with hBChE-expressing cells displayed significantly elevated levels of human BChE in the blood (FIG. **22**D). Expression of hBChE in grafted animals was stable for more than 10 weeks (FIG. **22**D). Consistent with previous observations, our results confirm that a skin-derived therapeutic protein can cross the basement membrane barrier and enter circulation in vivo.

[0217] To first determine whether engrafting hBChE-expressing cells protects mice from acute systemic toxicity of cocaine, we delivered different doses of cocaine to grafted mice and calculated the lethality rates of cocaine. Doses of 40, 80, 120, 160 mg/kg of cocaine had nearly 0 lethality in mice grafted with hBChE-expressing cells (GhBChE), whereas 80 mg/kg of cocaine induced roughly 50% lethality and 120 and 160 mg/kg cocaine induced 100% lethality in control mice grafted with WT epidermal cells (GWT) (FIG. **22**E). Parallel testing was conducted to test the toxicity of a related stimulant METH (methamphetamine) in GhBChE and GWT mice. There was no difference in lethality induced by various doses of METH between GhBChE and GWT animals (FIG. **22**F). This finding shows that engraftment of hBChE-expressing cells can lead to significant release of functional hBChE in vivo and protect mice from the toxicity of an acute cocaine overdose.

[0218] Next, protection against development of cocaine-seeking was assessed using the conditioned place preference (CPP) paradigm, which is thought to model reward learning and seeking because experimental animals approach and remain in contact with cues that have been paired with the effects of the reward. hBChE-expressing cells were grafted to cocaine-naïve mice with GWT animals as controls. After 4 days of place conditioning, GWT mice spent significantly more time in environments previously associated with cocaine, whereas GhBChE mice showed no such preference (FIG. **18**A). As an additional control, ethanol CPP was measured after 4 days of conditioning in GhBChE and GWT mice. In contrast, both GhBChE and GWT mice spent significantly more time in ethanol-paired environments (FIG. **18**B). This finding indicates that engraftment of hBChE-expressing cells efficiently and specifically attenuated cocaine-induced rewarding effect.

[0219] To determine whether engrafting hBChE-expressing cells affect cocaine-induced reinstatement of drug-seeking, hBChE-expressing cells were grafted in mice that previously acquired cocaine CPP. Following 10 days of recovery, we performed extinction training and drug-elicited reinstatement. After a priming dose of cocaine injection, the preference for the previously cocaine-associated environment was restored in the GWT mice but not in the GhBChE mice (FIG. **18**C). Because hBChE-expression did not prevent CPP induction by ethanol (FIG. **18**B), these GhBChE and GWT mice were used to perform extinction training followed by reinstatement. In contrast to those induced by cocaine, ethanol CPP was similarly reinstated in both GhBChE and GWT mice (FIG. **18**D). These results suggest that skin-derived hBChE efficiently and specifically disrupts cocaine-elicited reinstatement.

[0220] 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. To perform CRISPR-mediated genome editing in human cells, we developed vectors encoding two gRNAs targeting human AAVS1 (adeno-associated virus integration site 1) locus, and an AAVS1-targeting vector (FIG. **23**A) that harbors the expression cassette encoding engineered hBChE. Human epidermal keratinocytes were electroporated with the targeting vector together with plasmids encoding Cas9 and the gRNAs. Clones were isolated and correct integration confirmed by southern blotting analysis (FIG. **23**B). Like mouse cells, engineered human epidermal cells exhibited strong hBChE production as determined by immunoblots and ELISA (FIGS. **23**C and **23**D). Expression of the hBChE protein in human cells did not significantly change cell proliferation (FIG. **24**A) or differentiation (FIG. **24**B) in vitro. The engineered cells stratified and formed skin organoids in vitro, which were transplanted to nude host (FIG. **23**E). Grafted skin exhibited normal epidermal stratification, proliferation, and apoptosis in vivo (FIG. **23**F and FIGS. **24**C-D). Together, these results indicate that CRISPR editing of human epidermal progenitor cells does not sig-

nificantly alter cellular dynamics and persistence in vivo. ELISA confirmed that the mice with engraftment of hBChE-expressing cells had significantly levels of human BChE in the blood, whose expression was stable for more than 8 weeks in vivo (FIG. **23**G). Our results suggest the potential clinical relevance of cutaneous gene delivery for treatment of cocaine abuse and overdose in the future.

[0221] This study demonstrates for the first time that grafted skin stem cells expressing hBChE can be highly drug-specific in addressing several key aspects of cocaine abuse including reducing development of cocaine-seeking, preventing cocaine-induced reinstatement of drug-seeking and protecting against acute cocaine overdose. Because of the high levels of hBChE present, this approach is very efficient with little individual variation. Cutaneous gene delivery with engineered epidermal stem cells may provide therapeutic opportunities for drug abuse or co-abuse beyond cocaine.

[0222] For instance, glucagon-like peptide 1 (GLP1) is a major physiological incretin that controls food intake and glucose homeostasis (Sandoval et al., 2015). Several GLP1 receptor agonists have been approved by the FDA to treat type II diabetes. Our recent study indicates that skin-derived expression of GLP1 can effectively correct diet-induced obesity and diabetes in mice (Yue et al., 2017). Interestingly, GLP1 receptor agonists can also attenuate the reinforcing properties of alcohol and nicotine in rodents (Skibicka, K. P., The central GLP-1: implications for food and drug reward. Front Neurosci. 7, 181 (2013); Egecioglu et al. The glucagon-like peptide 1 analogue Exendin-4 attenuates alcohol mediated behaviors in rodents. *Psychoneuroendocrinol.* 38, 1259-1270 (2013); Shirazi et al., Gut peptide GLP-1 and its analogue, Exendin-4, decrease alcohol intake and reward. PLoS One. 8, e61965 (2013); Vallöf et al. The glucagon-like peptide 1 receptor agonist liraglutide attenuates the reinforcing properties of alcohol in rodents. *Addict. Biol.* 21, 422-437 (2016)). Thus, future studies will determine whether a constitutive or inducible expression of GLP1 from skin transplants can reduce alcohol and nicotine use and relapse in patients with alcohol use disorder and nicotine dependence. Additionally, it will be important to investigate whether co-expression of hBChE and GLP1 in skin can be used for treatment of alcohol and cocaine and ethanol and nicotine co-abuse, which occurs with high frequency and significantly increases the risk of drug-related morbidity and mortality.

[0223] Epidermal stem cells of the skin provide an ideal platform for ex vivo gene therapy, allowing efficient genetic manipulation with minimal risk of tumorigenesis or other detrimental complications in vivo. Cultured human epidermal progenitor cells have been used to generate CEA (cultured epidermal autograft), which has been clinically used to treat massive burn wounds for decades. Engineered skin stem cells and CEA have also been used to treat other skin diseases, including vitiligo and skin genetic disorders, such as epidermolysis bullosa. The regenerated skin is stable in vivo and can last for long term in the clinical follow-up studies. As such, the cutaneous gene therapy is long-lasting, minimally invasive and safe.

[0224] Gene therapy-derived products can be recognized as foreign antigens by the host immune system, which may mount an immune response leading to the neutralization of the therapeutic molecules or the clearance of genetically modified cells (Collins and Thrasher, 2015). Our skin transplantation model built with WT isogenic animals provides a unique approach to examine this process in vivo. Skin epidermal keratinocytes have low immunogenicity. Within normal skin epidermis, the Langerhans cells function as the only cell type that expresses major histocompatibility complex (MHC) class II and presents antigen. Epidermal keratinocytes 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. Consequently, engineered skin grafts are generally well taken and immunologically tolerated in VVT isogenic animals. Indeed, skin-derived expression of hBChE in host mice with intact immune systems can be stable for more than 10 weeks without significant decrease in the serum level of engineered enzyme, strongly suggesting that the low immunogenicity of skin environment may help to reduce the antigenicity and immune reaction toward hBChE. Moreover, the oldest GhBChE mice are 6 months old and healthy with no tissue rejections lending further support for the long-lasting feasibility of cutaneous gene therapy targeting cocaine abuse. Taken together, these results show promise for cutaneous gene therapy as a safe and cost-effective therapeutic option for cocaine abuse in the future.

Example No. 4: Treatment of Alcohol Abuse

Introduction

[0225] Alcohol use disorder (AUD) is one of the foremost public health problems. AUD involves problems controlling drinking, continuing to use alcohol even when it causes problems, having to drink more to get the same effect, or having withdrawal symptoms when one decreases or stops drinking (Koob et al. Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* 3, 760-773 (2016); Koob, G. F. Neurocircuitry of alcohol addiction: synthesis from animal models. *Handb. Clin. Neurol.* 125, 33-54 (2014)). 7.2 percent or 17 million adults in the United States ages 18 and older had an AUD in 2012 (Grant et al. Epidemiology of DSM-5 alcohol use disorder: results from the national epidemiologic survey on alcohol and related conditions III. *JAMA Psychiatry* 72, 757-766 (2015)). There are three FDA-approved medications and behavioral counseling for stopping or reducing drinking and preventing relapse in humans (Johnson, B. A. Update on neuropharmacological treatments for alcoholism: scientific basis and clinical findings. *Biochem. Pharmacol.* 75, 34-56 (2008)). However, only 1.3 million receive treatment (NIAAA, 2015). Moreover, not all people respond to these medications and types of treatment, and not all people follow the treatment regimens offered (Cohen et al. Alcohol treatment utilization: findings from the national epidemiologic survey on alcohol and related conditions. *Drug Alcohol Depend.* 86, 214-221 (2007)). Consequently, additional approaches are needed for combating AUD. Indeed, if a treatment were available that prevented an individual from desiring to consume alcohol, such a treatment could ultimately lead to the individual stopping consumption of alcohol.

[0226] Glucagon-like peptide 1 (GLP1) is a gastrointestinal peptide and a major physiological incretin that controls food intake and glucose homeostasis (Sandoval et al. 2015). Several GLP1 receptor agonists have been approved by the FDA to treat type II diabetes. Interestingly, GLP1 receptor

agonists can also attenuate the reinforcing properties of alcohol in rodents (Skibicka, K. P., The central GLP-1: implications for food and drug reward. *Front Neurosci.* 7, 181 (2013); Egecioglu et al. The glucagon-like peptide 1 analogue Exendin-4 attenuates alcohol mediated behaviors in rodents. *Psychoneuroendocrinol.* 38, 1259-1270 (2013); Shirazi et al., Gut peptide GLP-1 and its analogue, Exendin-4, decrease alcohol intake and reward. *PLoS One.* 8, e61965 (2013); Vallöf et al. The glucagon-like peptide 1 receptor agonist liraglutide attenuates the reinforcing properties of alcohol in rodents. *Addict. Biol.* 21, 422-437 (2016); Sørensen et al. Effects of the GLP-1 agonist Exendin-4 on intravenous ethanol self-administration in mice. *Alcohol Clin. Exp. Res.* 40, 2247-2252 (2016); Suchankova et al. The glucagon-like peptide-1 receptor as a potential treatment target in alcohol use disorder: evidence from human genetic association studies and a mouse model of alcohol dependence. *Transl. Psychi.* 5, e583 (2015)).

[0227] While GLP1 may be potentially used in treating AUD (Suchankova et al. 2015), the native GLP1 must be delivered through a parenteral route to achieve its effect, and it has an extremely short circulating half-life (Sandoval and D'Alessio, 2015). Therefore, the somatic gene transfer approach used in Example No. 2 was used to determine the efficacy of GLP1 in treating AUD in mice.

Experimental Procedures

[0228] Unless indicated to the contrary, reagents and experimental procedures follow those described in Example No. 2 above.

[0229] Modified GLP1

[0230] The GLP1 gene (mGLP1 or DImGLP1) was modified to produce a novel protein with longer half-life in vivo (Kumar et al., Gene therapy of diabetes using a novel GLP-1/IgG1-Fc fusion construct normalizes glucose levels in db/db mice. *Gene therapy* 14, 162-172, (2007)). To be able to stably deliver mGLP1 in vivo and control its expression, we made a Rosa26-targeting vector encoding a Gly8-mutant mGLP1 and mouse IgG-Fc fragment fusion protein (SEQ ID NO: 45) driven by a dox-dependent promoter (FIG. **25**A; Yue et al. Treatment of diabetes and obesity with CRISPR-mediated genome editing in epidermal progenitor cells. Cell Stem Cell, In press (2017); Kumar et al., 2007). The glycine mutation renders the peptide resistant to DPP-IV-mediated cleavage (Mentlein et al., Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1 (7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur. J. Biochem.* 214, 829-835 (1993)).

[0231] Fusion with IgG-Fc further enhances mGLP1 stability and circulation half-life when expressed in epidermal cells (Kumar et al., 2007), and it can pass the blood-brain barrier to reach the brain (Pardridge, W. M., CSF, blood-brain barrier, and brain drug delivery. *Expert Opin. Drug Deliv.* 13, 963-975 (2016)). The Rosa26 allele has been widely used as a safe locus for gene targeting in mice (Soriano, P., Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genetics* 21, 70-71, (1999)). To use CRISPR-mediated genome editing, vectors encoding the D10A mutant Cas9 (CRISPR associated protein 9) (Ran et al., 2013) and two gRNA (guide RNA) targeting the mouse Rosa26 allele were developed. The D10A mutation in Cas9 and double nicking system reduce the undesired off-target mutagenesis in the genome to a minimum level (Ran et al., 2013).

[0232] Skin Organoid Culture and Transplantation

[0233] The epidermal progenitor cells were isolated from newborn pups of CD1 or C57BL/6J mice. Cells were transfected with the targeting vector and plasmids encoding Cas9 and Rosa26-specific gRNAs. Targeted clones were selected in the medium containing puromycin, and correct incorporation into the Rosa26 locus was confirmed by PCR and southern blots (FIG. **25**B). Mouse skin substitute was prepared by seeding the targeted cells to the acellularized newborn dermis and differentiation upon exposure to the air/liquid interphase and the resultant tissues were transplanted to CD1 or C57BL/6J mice (GLP1).

[0234] When fed with dox food, GGLP1 mice began to display significantly enhanced levels of mGLP1 in the blood within 3 days (FIG. **25**C; Yue et al., 2017). There was a dose-dependent release of mGLP1 in plasma (not shown). Expression of mGLP1 in GLP1 mice was stable for up to 4 months in the presence of dox (FIG. **25**D).

Results and Discussion

[0235] mGLP1 expression attenuated ethanol-induced CPP in GLP1 mice (FIG. **26**) compared to mock grafted (GWT).

[0236] GLP1 mice did not exhibit significant ethanol-induced CPP. Following 2 free explorations (Pre-test) on day 1, separate groups of GLP1 and GWT mice (n=9 each) received alternative ethanol (2 g/kg) and saline i.p. injections twice daily for the next 4 days, as previously described (Chen et al., Dopamine D1 and D3 receptors are differentially involved in cue-elicited cocaine seeking. *J. Neurochem.* 114, 530-541 (2010); Kong et al., Activation of dopamine D3 receptors inhibits reward-related learning induced by cocaine. *Neurosci.* 176, 152-161 (2011)). CPP expression was tested on day 6. Results represent mean±SEM time spent on the drug-paired side minus the saline-paired side. Repeated-measures ANOVA with test days as the within group factor and status of grafting as the between-subject factor were used (Chen et al., 2010; Kong et al., 2011). F value was calculated and Newman-Keuls post-hoc test was performed (Chen et al., 2010; Kong et al., 2011). GLP1 and GVVT mice were on dox food for the entire duration.

[0237] These results demonstrate that mGLP1 expression attenuated ethanol-induced CPP. Therefore, tissue organoids expressing mGLP1 have the potential for treating AUD. In addition, Egecioglu et al., also reported that GLP-1 receptor agonist, Exendin-4 (Ex4) attenuates nicotine-induced locomotor stimulation, accumbal dopamine release, and the expression of conditioned place preference in mice (Egecioglu et al., 2013). Therefore, it is believed that tissue organoids expressing mGLP1 can also be used to treat nicotine addiction. It follows that tissue organoids expressing mGLP1 can also be used to treat individuals with AUD who are addicted to nicotine at the same time. Moreover, it is believed that tissue organoids designed to express multiple therapeutic agents, such as mGLP1 and hBChE can be used to reduce incidents of cocaine and ethanol and/or nicotine co-abuse and potentially reduce abuse and co-abuse of other drugs, such as amphetamines (see Skibicka K. P. The central GLP-1: implications for food and drug reward,

Front Neurosci. 7:181 (2013)). It is also contemplated herein to that GLP-1 analogs (see above) can be employed in a similar fashion.

Example No. 5: Tissue Organoids for Treating Alcohol Abuse

[0238] Alcoholism is a debilitating disease characterized by dependence on alcohol consumption and is often associated with social and/or health problems. Medications are available to treat alcoholism that discourage alcohol consumption by causing nausea when consuming alcohol, by reducing the pleasure associated with drinking, or by reducing the craving for alcohol. However, as with all medications, treatment compliance is difficult. Treatment compliance is further complicated when the medications are for breaking an addiction and require self-administration. Therefore, automatic administration of a therapeutic agent for combatting alcoholism induced when alcohol is consumed would be desirable.

[0239] Therefore, a tissue organoid engineered to express one or more alcohol-inducible genes encoding a spider-derived pain peptides, such as DkTx (S2-DkTx; SEQ ID NO: 36) or VaTx (S2-VaTx3; SEQ ID NO: 35) is prepared as described above (see FIG. **27**). In addition, a GLP-1 and/or a GLP-1 analog can be further added to the tissue organoid for simultaneous or on-demand expression along with DkTx or VaTx. It is envisioned that any of these therapeutic agents can be expressed, and/or administered, individually or in any combination.

[0240] After engineering an alcohol-inducible therapeutic tissue organoid, the organoid is transplanted into mice before and after training mice in a two-bottle choice paradigm. This paradigm tests how well the system works for protecting mice from acquiring or relapsing into alcohol drinking. In the mouse housing cages, there are two liquid bottles with one containing regular drinking water, and the other a certain percentage of an alcohol solution. The amount of alcohol or water consumption is measured daily. Previous work has established that mice prefer drinking alcohol over water over a period, reflecting the rewarding effects of alcohol.

[0241] Therefore, with an alcohol-inducible therapeutic tissue organoid implanted, mice are expected to never develop the preference (acquisition) or relapse into alcohol drinking (relapse).

[0242] Similarly, a human patient with a biointegrated tissue organoid, according to this example, experiences 1) pain or discomfort from the expressed toxin and 2) lack of reward from the expressed GLP-1/GLP-1 analog when alcohol metabolites activate the expression of the therapeutic agent (e.g., a spider-derived toxin and GLP-1 or GLP-1 analog). In this way, the therapeutic agents expressed in response to alcohol metabolites work synergistically, and the patient is disinclined to drink.

Example No. 6: Tissue Organoids for Monitoring Silent Heart Attack

[0243] Silent heart attacks have few or no overt "classical" symptoms, such as chest pressure, chest heaviness, arm pain, neck pain, jaw pain, shortness of breath, sweating, extreme fatigue, dizziness, and nausea. However, though nearly half of heart attacks are silent, they are correlated with similar risk of death as "overt" heart attacks, and if successfully diagnosed, they may treated with similar medications and lifestyle changes. Therefore, the ability to monitor factors indicative of silent heart attacks would be beneficial, such as Heart-type Fatty Acid-Binding Protein (H-FABP) and myocardial myoglobin.

[0244] A tissue organoid engineered to report occurrence of a silent heart attack is prepared as described above using a targeting vector harboring genes encoding reporter molecules specific for H-FABP (SEQ ID NO: 62) and myocardial myoglobin (SEQ ID NO: 63).

Example No. 7: Tissue Organoids for Monitoring Stroke

[0245] Cerebrovascular accidents or "strokes" are the sudden death of brain cells due to lack of oxygen when the blood flow to the brain is impaired by blockage or blood vessel rupture. Glial fibrillary acidic protein (GFAP) (SEQ ID NO: 64) is a biomarker associated with symptoms of acute stroke. S100B (SEQ ID NO: 65) is a marker associated with astrocyte damage and increased BBB permeability associated with stroke. The ability to monitor such markers of strokes would permit earlier intervention when an individual experiences a stroke.

[0246] Therefore, a tissue organoid engineered to report occurrence of a stroke is prepared as described herein using a targeting vector harboring genes encoding reporter molecules specific for GFAP (SEQ ID NO: 64) and S100B (SEQ ID NO: 65).

Example No. 8: Tissue Organoids for Treating Phenylketonuria (PKU)

Introduction

[0247] PKU is the most prevalent inherited disease involving the metabolism of amino acids. It results from mutations in the gene encoding a key hepatic enzyme, phenylalanine hydroxylase (PAH), which catalyzes the hydroxylation reaction that converts phenylalanine to tyrosine. If untreated, PKU will lead to a dramatic increase of plasma phenylalanine levels, causing profound and irreversible mental disability, epilepsy, and other behavioral problems. The current treatment of PKU is stringent dietary restriction of natural protein intake and supplementation of amino acids other than phenylalanine by a chemically manufactured protein substitute, which can prevent most of the complications of the disease after birth. However, it has been well documented that neuropsychological deficits still exist for patients with dietary restriction, and maintaining the dietary control has been proven difficult, especially in adolescents, young adults, and pregnant women. Somatic gene therapy holds the potential for development of more effective treatment for PKU.

[0248] Although PAH is naturally a hepatic enzyme, it has been speculated that circulating phenylalanine could be adequately cleared by ectopic expression of PAH in tissues other than liver, such as T cells, muscle cells, and skin epidermal cells. However, a potential issue is that catalytic activity of PAH requires a non-protein cofactor, BH4 (tetrahydrobiopterin), which is synthesized de novo from guanosine triphosphate in hepatocytes by a three-enzyme pathway involving GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydrobiopterin synthase (PTPS), and sepiapterin reductase (SR). Without sufficient supplement of BH4 or

reconstitution of the BH4 synthetic pathway, ectopic expression of PAH cannot function properly. Transgenic mice that express both PAH and GTPCH, the rate limiting enzyme for BH4 synthesis, under various skin specific promoters cannot sufficiently rescue the phenylalanine defects in vivo, suggesting that alternative approaches must be pursued to convert skin epidermis to an effective "phenylalanine sink."

[0249] Enzyme replacement therapy has been proposed for treatment of PKU. Phenylalanine ammonia-lyase (PAL) is an attractive alternative for clearance of phenylalanine. PAL can carry out non-oxidative deamination of L-phenylalanine to form ammonia and trans-cinnamic acid (cinnamate), which can be excreted as hippurate in urine, along with small amounts of cinnamic and benzoic acid. Unlike PAH, PAL is an autocatalytic enzyme that requires no cofactors, making it a simple and effective way to remove plasma phenylalanine.

[0250] Preliminary results in mouse epidermal keratinocytes show efficient expression of PAL upon infection with lentivirus encoding PAL and degradation of phenylalanine in culture media much faster than cells expressing PAH. Exogenous expression of PAL in epidermal progenitor cells does not significantly alter cell proliferation or differentiation in vitro, and the cells readily generate skin organoids when cultured on top of acellularized dermis. Therefore, using CRISPR-mediated genome editing with epidermal progenitor cell technology the possibility for treatment of PKU by cutaneous expression of PAL is investigated.

[0251] The mouse model of PKU has been developed by ENU (N-ethyl-N-nitrosourea)-mediated mutagenesis in BTBR strain (Jax strain 002232).

[0252] With establishment of skin engraftment in PAH mutant mice, plasma phenylalanine level are monitored. Three weeks post skin grafting with PAL expressing cells or control cells (BTBR epidermal progenitor cells treated with control vectors), blood samples are collected from the tail clipping biweekly for up to 30 weeks. Plasma is separated by centrifugation. Phenylalanine concentration is determined by established fluorometric protocol. Lowering of phenylalanine level restores melanin biosynthesis, and leads to a change of fur color. Potential alteration in fur coat color is recorded weekly for all the grafted animals. It has been demonstrated before that treatment of PKU has gender-dependent response, for both viral vector-mediated therapy and enzyme replacement therapy with modified PAL. Thus, two different cohorts of animals (male and female) are established to test the potential gender-dependent effect of cutaneous gene therapy in vivo.

[0253] At the endpoint of the experiment (30 weeks), grafted animals are euthanized and tissue samples (grafted skin, host skin, and liver) are collected. Presence of enzyme activity in the tissues is determined by PAH and PAL activity assay.

[0254] In vitro analysis demonstrated that exogenous expression of PAL in mouse epidermal cells can clear ~120 nmol of phenylalanine/hour/10^6 cells. Thus, two 1 cm^2 epidermal grafts that contain 2×10^7 metabolically active cells is likely to remove 57 μmol of phenylalanine per day, which would be sufficient for a complete reversal of hyperphenylalaninemia (PAH mutant mice usually have ~2 mM of plasma phenylalanine). A complete phenotypic change from brown hair coat to dark black fur is also expected. For tissue enzymatic analysis, PAL activity detection in grafted skin is expected but not in other tissues. PAH activity is absent in the liver of the mutant mice. Female mice are more resistant to PKU therapy. If this is the case for cutaneous gene therapy, female PAH mutant mice are tested to determine whether they require larger skin grafts or more grafts than male mice for correction of hyperphenylalaninemia.

[0255] One potential issue is that overexpression of PAL can lead to excessive removal of phenylalanine in mice and cause hypophenylalaninemia, which has been reported for human PKU patients receiving enzyme replacement therapy with recombinant PAL. If this occurs, a Tet (tetracycline)-inducible system to drive PAL expression as described above can be used. Expression level of PAL can be controlled by administration of different dose of Doxycycline.

[0256] Approach 1: Express PAL Gene in Grafted Cells

[0257] Although exogenous expression of PAL does not alter cell proliferation or differentiation program in mouse epidermal keratinocytes, expression of PAL could impair human keratinocyte growth or skin regeneration. If this occurs, a Tet-inducible system to drive PAL gene expression is used (see FIG. **28**).

[0258] Approach 2: Immune Intolerance of PAL

[0259] If exogenous expression of PAL indeed leads to a severe immune response that cannot be suppressed, expression of PAH, the natural phenylalanine processing enzyme, is tested for use in treatment of PKU. Although PAH requires BH4 as cofactor, it has been shown that co-expression of GTPCH and PTPS together with PAH in muscle can result in stable and long-term reduction of plasma phenylalanine in mice model. Therefore, a Rosa26 targeting vector harboring a triple-cistronic expression cassette encoding PAH (SEQ ID NO: 54), GTPCH (SEQ ID NO: 55), and PTPS (SEQ ID NO: 56) is developed.

[0260] Targeted epidermal progenitor cells are examined both in vitro and in vivo for phenylalanine clearance and potential therapeutic effect for PKU. If co-expression of PAH, GTPCH, and PTPS is not sufficient, a Rosa26 targeting vector harboring a quadruple-cistronic expression cassette encoding PAH, GTPCH, PTPS, and SR (SEQ ID NO: 57) is developed to reconstitute the entire BH4 de novo synthesis pathway in a tissue organoid.

Example No. 9: Tissue Organoids for Treating Hemophilia

Introduction

[0261] Hemophilia is an inherited blood clotting disorder caused by a deficiency of clotting Factor VIII (type A) or IX (type B) in the blood plasma. The unstoppable bleeding itself can be life-threatening, but hemophiliacs usually also suffer from recurrent bleeding into soft tissues, joints and muscles, leading to chronic synovitis, crippling arthropathy and physical disability. Hemophilia is an excellent candidate for cutaneous gene therapy because both Factor VIII and IX are secreted proteins and, therefore, their exogenous expression in epidermal cells could correct the deficiency. In addition, particularly for hemophilia A, the coding sequence of Factor VIII is more than 7 kb (kilobase), far beyond the packaging limitation of typical viral vectors used for gene therapy, such as AAV (adeno-associated virus) vectors. Somatic gene therapy in skin provides a more sustained and affordable treatment for hemophilia A than the current standard therapy by intravenous infusions with purified Factor VIII.

[0262] Previous work has demonstrated that Factor VIII and IX can be expressed in human or mouse epidermal keratinocytes, and exogenously expressed clotting factors can pass the epidermal/dermal barrier to reach the circulation. Particularly, when transgenic skin that expresses Factor VIII under the involucrin promoter is grafted onto immunocompromised hosts (Factor VIII and Rag1 double knockout), epidermal expression of Factor VIII significantly restores the plasma Factor VIII level, strongly supporting the feasibility of treatment of hemophilia A with cutaneous gene therapy approach.

[0263] A Factor VIII expression gene with B domain deletion (SEQ ID NO: 58) is envisioned, where the B domain, which is a long internal domain and functionally disposable for blood coagulation is removed. The transfection targeting vector is envisioned to further incorporate a Tet-inducible system to control excessive expression of Factor VIII.

[0264] Development of an inhibitory antibody against Factor VIII is the most severe complication of current enzyme replacement therapy. If expression of mouse Factor VIII in the mutant mice (Factor VIII-deficient) induces antibody production, three approaches are employed:

[0265] 1) Instead of B domain-deleted Factor VIII, a full length Factor VIII (SEQ ID NO: 59) for cutaneous gene therapy is used. Although B domain is not essential for blood coagulation, meta-analysis of prospective clinical results suggests that deletion of B domain can shorten the half-life of recombinant Factor VIII, increase bleeding incidence in patients, and significantly increase the risk for development of neutralizing antibody.

[0266] 2) The Fc domain of immunoglobulin (mouse IgG1 Fc (SEQ ID NO: 34) or human IgG1 Fc (SEQ ID NO: 39)) is conjugated with Factor VIII. The Fc domain of IgG can interact with neonatal Fc receptor, which can protect IgG from catabolism and elongate its half-life in circulation. It has been shown that fusion of human Factor VIII with IgG1 Fc domain can significantly increase its half-life in patients. In addition, current clinical results suggest that Fc conjugation of Factor VIII will not induce neutralizing antibody production in patients. Consistently, it has been shown that the Fc-Factor VIII conjugate elicits much lower immune reaction compared to non-conjugated Factor VIII in hemophilia A mouse model.

[0267] 3) Factor VIII is coupled with albumin (SEQ ID NO: 60). Albumin is the most abundant plasma protein and a natural carrier for a variety of molecules in circulation. Albumin has very long half-life in blood and low immunogenicity. Hence, conjugation with albumin will likely enhance the effectiveness of cutaneous gene therapy for hemophilia A. It has been demonstrated that albumin conjugation with Factor IX (SEQ ID NO: 61) can significantly protect Factor IX in circulation and reduce its immunogenicity. Although fusion of Factor VIII with albumin has not been successfully pursued before, different strategies of fusion are employed (e.g., conjugation to amino- or carboxyl-terminal of Factor VIII and/or using different linker peptide for fusion) and effects in vitro and in vivo are examined.

[0268] For Hemophilia B, similar approaches are taken as those described above using Factor IX.

Example No. 10: Evaluating Immune Response on Skin Grafting

[0269] Development of skin engraftment procedures with immunocompetent mice provides a unique opportunity to evaluate potential immune responses after cutaneous gene therapy. To examine potential inflammatory infiltration in vivo upon tissue organoid engraftment, grafted skin tissues are collected at different time points post engraftment. Potential infiltration of immune cells are assessed by immunofluorescence staining with α-CD3, α-CD4, α-CD8 (T cell), α-B220 (B cell), α-CD11c (dendritic cell), α-Mac-1 (macrophage), α-CD335 (NK cells), and α-Gr1 (granulocytes) antibodies. The presence of Langerhans cells in the skin grafts is determined by staining with an α-CD1a antibody. In addition, skin samples are stained for MHC class I and II antigens, including HLA-ABC and HLA-DR, as indicators for potential graft rejection, tissue antigenicity, and epidermal reactivity.

[0270] As a secondary approach, immune responses are assessed by flow cytometry (fluorecence-activated cell sorting), which allows for a more comprehensive and quantitative analysis of immune cell populations. For this purpose, specific panels of antibodies are developed to more closely examine the phenotypes of immune cells that are changing in response to engraftment. These experiments are guided by results from immunofluorescence staining of sections. For example, if differences in the number and/or location of macrophages in stainings are seen, then a 'macrophage panel' is set up that allows determination of whether the recruited macrophages adopt a pro-inflammatory M1 (CD38, CD274, CD319) or an anti-inflammatory M2 (CD206, CD163, TFRC) phenotype using surface markers. Similarly, if the staining experiments indicate a T-cell response, then a 'T cell panel' comprised of CD3 (all T cells), CD4 (helper T cell), CD8 (killer T cell), FoxP3/ CD25 (regulatory T cell), and CD44/CD62L (activated T cell) is implemented. Understanding the precise nature of the immune response (i.e., immunogenic vs. tolerogenic) is required to determine whether the host response to engraftment requires modulation.

[0271] To determine whether engraftment of modified-epidermis can increase the host white blood cells specific for grafted keratinocytes, in vitro cellular proliferation assay are carried out. Peripheral blood mononuclear cells (PBMC) are isolated before and after skin transplantation, and assessed for cellular proliferation assay with either irradiated autologous PBMC or irradiated epidermal progenitor cells with modified expression. Phytohemagglutinin is used as a positive control.

[0272] To examine NK (natural killer) cells activity toward epidermal progenitor cells, PBMC isolated from grafted animals are used. An in vitro cytotoxicity assay is performed to determine the activity of NK cells at different effector: target ratios. A NK-sensitive cell line is used as a positive control.

[0273] To examine the immunogenicity of the modified protein, blood samples from grafted animals at different time points (up to 6 months) are collected. Serum antibody levels are determined by ELISA (enzyme-linked immunosorbent assay) analysis. As a positive control, an intravenous injection of the recombinant modified protein is injected into the mice.

Example No. 11: Removal of Skin Grafts Non-Surgically

[0274] Biointegrated tissue organoids can be for temporary use and removed surgically. However, skin stem cell technology permits another non-invasive and effective way to achieve clearance of grafted cells.

[0275] To test this possibility, a Rosa26 targeting vector encoding phenylalanine ammonia lyase (PAL) (SEQ ID NO: 51) together with inducible "suicide" genes HSV-TK (Herpes Simplex Virus Thymidine Kinase) (SEQ ID NO: 52) and yCD (yeast cytosine deaminase) (SEQ ID NO: 53) was used for epidermal progenitor cell transfection. Results (data not shown) indicate that both suicide genes were expressed safely in epidermal progenitor cells, and efficiently induced cell death upon treatment with prodrugs (ganciclovir and 5-fluorocytosine, respectively).

[0276] A potential issue is that high level of suicide gene expression (TK or yCD) may lead to bystander effect that causes cell death in the surrounding tissue. To prevent this, a Tet promoter can be used to drive the suicide gene expression resulting in suicide gene expression levels being controlled by administration of doxycycline.

[0277] Therefore, tissue organoids are contemplated that encode a reporter molecule and/or a therapeutic agent along with an inducible suicide gene to remove the biointegrated tissue organoid by non-surgical means.

Example No. 12: Tissue Organoids for Treating Obesity

[0278] Recent reports have shown that nearly 40% of American adults and approximately 20% of children are obese. Peptide YY (PYY) (SEQ ID NO: 66) is associated with hunger and satiation and is released from cells in the ileum and colon in response to feeding. Increased levels of PYY are believed to be associated with satiation. The administration of PYY, in accordance with the present disclosure, may allow those that suffer from obesity to decrease their food intake, thereby making it possible for them to not only lose weight, but maintain weight loss.

[0279] Therefore, a tissue organoid engineered to treat and/or prevent obesity is prepared as described herein using a targeting vector encoding PYY (SEQ ID NO: 66). It is anticipated that increased PYY levels will decrease craving in individuals receiving the PYY-expressing tissue organoid. As a result, it is anticipated that the individuals will experience decreased food seeking and consumption thereby treating and/or preventing obesity.

Example No. 13: Tissue Organoids for Anti-Aging Effects

[0280] The global anti-aging market is estimated to be $250 Billion. Effects of aging include, but are not limited to, vascular and heart diseases and decreases in immune response. Tissue inhibitor of metalloproteinases 2 (TIMP2) (SEQ ID NO: 67) encodes a protein that is a natural inhibitor of matrix metalloproteinases (MMP). TIMP2 is found in high concentrations very early in age but declines with age. Memory studies with mice have shown that older mice perform comparably with younger mice following injections with TIMP2 (Castellano et al., Nature, 544, 488-492 (2017)). Controlled administration of TIMP2 may have anti-aging effects such as memory improvement.

[0281] Therefore, a tissue organoid engineered to treat and/or prevent the effects of aging is prepared as described herein using a targeting vector encoding TIMP2 (SEQ ID NO: 67). It is anticipated that increased TIMP2 levels will exhibit anti-aging effects in individuals receiving the TIMP2-expressing tissue organoid. As a result, it is anticipated that treated individuals can experience a decrease in the negative effects of aging, which can include memory loss, reduced vascular response, and/or reduced immune response.

Example No. 14: Tissue Organoids for Nicotine Abuse

[0282] Similarly, tobacco use is a major cause of death from cardiovascular disease, pulmonary disease and cancer (Nature, 362, 2295-2303 (2010)). The Center for Disease Control claims that nearly 480,000 people die each year from tobacco-related deaths. It has been long determined that nicotine is responsible for the additive properties of tobacco. Nicotine replacement therapies which include the transdermal nicotine patch and nicotine gum were the first FDA approved treatments for use in smoking cessation. While nicotine replacement is commonly used, other forms of treatment are available which include antibdepressant bupropion (National Institute on Drug Abuse, Tobacco/Nicotine, Research Report Series (2012). Despite the numerous treatments available, nicotine abuse still remains prevalent. Egecioglu et al., reported that GLP-1 receptor agonist, Exendin-4 (Ex4) attenuates nicotine-induced locomotor stimulation, accumbal dopamine release, and the expression of conditioned place preference in mice (Egecioglu et al., 2013). Therefore, it is believed that tissue organoids expressing mGLP1 can also be used to treat nicotine addiction.

Experimental Procedures

[0283] Unless indicated to the contrary, reagents and experimental procedures follow those described in Example No. 2 above.

[0284] Modified GLP1

[0285] The GLP1 gene (mGLP1) was modified to produce a novel protein with longer half-life in vivo (Kumar et al., Gene therapy of diabetes using a novel GLP-1/IgG1-Fc fusion construct normalizes glucose levels in db/db mice. Gene therapy 14, 162-172, (2007)). To be able to stably deliver mGLP1 in vivo and control its expression, we made a Rosa26-targeting vector encoding a Gly8-mutant mGLP1 and mouse IgG-Fc fragment fusion protein (SEQ ID NO: 45) driven by a dox-dependent promoter (FIG. **25**A; Yue et al. Treatment of diabetes and obesity with CRISPR-mediated genome editing in epidermal progenitor cells. Cell Stem Cell, In press (2017); Kumar et al., 2007). The glycine mutation renders the peptide resistant to DPP-IV-mediated cleavage (Mentlein et al., Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1 (7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur. J. Biochem. 214, 829-835 (1993)).

[0286] Fusion with IgG-Fc further enhances mGLP1 stability and circulation half-life when expressed in epidermal cells (Kumar et al., 2007), and it can pass the blood-brain barrier to reach the brain (Pardridge, W. M., CSF, blood-brain barrier, and brain drug delivery. Expert Opin. Drug Deliv. 13, 963-975 (2016)). The Rosa26 allele has been

widely used as a safe locus for gene targeting in mice (Soriano, P., Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genetics 21, 70-71, (1999)). To use CRISPR-mediated genome editing, vectors encoding the D10A mutant Cas9 (CRISPR associated protein 9) (Ran et al., 2013) and two gRNA (guide RNA) targeting the mouse Rosa26 allele were developed. The D10A mutation in Cas9 and double nicking system reduce the undesired off-target mutagenesis in the genome to a minimum level (Ran et al., 2013).

[0287] Skin Organoid Culture and Transplantation

[0288] The epidermal progenitor cells were isolated from newborn pups of CD1 or C57BL/6J mice. Cells were transfected with the targeting vector and plasmids encoding Cas9 and Rosa26-specific gRNAs. Targeted clones were selected in the medium containing puromycin, and correct incorporation into the Rosa26 locus was confirmed by PCR and southern blots (FIG. **25**B). Mouse skin substitute was prepared by seeding the targeted cells to the acellularized newborn dermis and differentiation upon exposure to the air/liquid interphase and the resultant tissues were transplanted to CD1 or C57BL/6J mice (GLP1).

[0289] When fed with dox food, mGLP1 mice began to display significantly enhanced levels of mGLP1 in the blood within 3 days (FIG. **25**C; Yue et al., 2017). There was a dose-dependent release of GLP1 in plasma (not shown). Expression of mGLP1 in mGLP1 mice was stable for up to 4 months in the presence of dox (FIG. **25**D).

Results and Discussion

[0290] GLP1 expression attenuated nicotine-induced CPP in GLP1 mice (FIG. **29**) compared to mock grafted (GWT).

[0291] GLP1 mice did not exhibit significant nicotine-induced CPP. Following 2 free explorations (Pre-test) on day 1, separate groups of mGLP1 and GWT (n=7 each) received alternative nicotine (0.4 mg/kg) and saline i.p. injections twice daily for the next 4 days, as previously described (Chen et al., Dopamine D1 and D3 receptors are differentially involved in cue-elicited cocaine seeking. J. Neurochem. 114, 530-541 (2010); Kong et al., Activation of dopamine D3 receptors inhibits reward-related learning induced by cocaine. Neurosci. 176, 152-161 (2011)). CPP expression was tested on day 6. Results represent mean ±SEM time spent on the drug-paired side minus the saline-paired side. Repeated-measures ANOVA with test days as the within group factor and status of grafting as the between-subject factor were used (Chen et al., 2010; Kong et al., 2011). F value was calculated and Newman-Keuls post-hoc test was performed (Chen et al., 2010; Kong et al., 2011). GLP1 and GVVT mice were on dox food for the entire duration.

[0292] These results demonstrate that mGLP1 expression attenuated nicotine-induced CPP. Therefore, tissue organoids expressing mGLP1 have the potential for treating nicotine abuse. Moreover, it is believed that tissue organoids designed to express multiple therapeutic agents, such as mGLP1 and hBChE can be used to reduce incidents of cocaine and ethanol and/or nicotine co-abuse and potentially reduce abuse and co-abuse of other drugs, such as amphetamines (see Skibicka K. P. The central GLP-1: implications for food and drug reward, Front Neurosci. 7:181 (2013)). It is also contemplated herein to that GLP-1 analogs (see above) can be employed in a similar fashion.

Sequences

[0293]

SEQ ID NO: 1	Luciferase
SEQ ID NO: 2	H2B-RFP
SEQ ID NO: 3	gRNA expression cassette forward primer
SEQ ID NO: 4	gRNA expression cassette reverse primer
SEQ ID NO: 5	Rosa26 - targeting gRNA forward primer 1
SEQ ID NO: 6	Rosa26 - targeting gRNA reverse primer 1
SEQ ID NO: 7	Rosa26 - targeting gRNA forward primer 2
SEQ ID NO: 8	Rosa26 - targeting gRNA reverse primer 2
SEQ ID NO: 9	AAVS1 - targeting gRNA forward primer 1
SEQ ID NO: 10	AAVS1 - targeting gRNA reverse primer 1
SEQ ID NO: 11	AAVS1 - targeting gRNA forward primer 2
SEQ ID NO: 12	AAVS1 - targeting gRNA reverse primer 2
SEQ ID NO: 13	Rosa26 targeting vector forward primer 1
SEQ ID NO: 14	Rosa26 targeting vector reverse primer 1
SEQ ID NO: 15	Rosa26 targeting vector forward primer 2
SEQ ID NO: 16	Rosa26 targeting vector reverse primer 2
SEQ ID NO: 17	Rosa26 targeting vector forward primer 3
SEQ ID NO: 18	Rosa26 targeting vector reverse primer 3
SEQ ID NO: 19	AAVS1 targeting vector constructed with AAVS1 hPGK-PuroR-pA donor forward primer 1
SEQ ID NO: 20	AAVS1 targeting vector constructed with AAVS1 hPGK-PuroR-pA donor reverse primer 1
SEQ ID NO: 21	AAVS1 targeting vector constructed with AAVS1 hPGK-PuroR-pA donor forward primer 2
SEQ ID NO: 22	AAVS1 targeting vector constructed with AAVS1 hPGK-PuroR-pA donor reverse primer 2
SEQ ID NO: 23	AAVS1 targeting vector constructed with AAVS1 hPGK-PuroR-pA donor forward primer 3
SEQ ID NO: 24	AAVS1 targeting vector constructed with AAVS1 hPGK-PuroR-pA donor reverse primer 3
SEQ ID NO: 25	Genotyping primer for CRISPR mediated knockin #1
SEQ ID NO: 26	Genotyping primer for CRISPR mediated knockin #2
SEQ ID NO: 27	Genotyping primer for CRISPR mediated knockin #3
SEQ ID NO: 28	CFP/YFP FRET sensor with A213R/L238S double mutant of GGBP
SEQ ID NO: 29	Cas9 D10A
SEQ ID NO: 30	Rosa26 gRNA1
SEQ ID NO: 31	Rosa26 gRNA2
SEQ ID NO: 32	Rosa26 targeting vector
SEQ ID NO: 33	Rosa26 targeting vector with GGBP
SEQ ID NO: 34	IgG Fc (mouse)
SEQ ID NO: 35	S2-VaTx3 with signal
SEQ ID NO: 36	S2-DkTx with signal
SEQ ID NO: 37	VatX3 target vector cassette: signal->VaTx3->Furin->IgGFc
SEQ ID NO: 38	DkTx target vector cassette: signal->DkTx->Furin->IgGFc
SEQ ID NO: 39	IgG Fc (human)
SEQ ID NO: 40	AAVSI targeting vector
SEQ ID NO: 41	Human mutant BChE
SEQ ID NO: 42	Nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 41
SEQ ID NO: 43	Mouse mutant BChE
SEQ ID NO: 44	Nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 43
SEQ ID NO: 45	mGLP-1 (GLY8 mutant with IgG-Fc fusion)
SEQ ID NO: 46	Nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 45
SEQ ID NO: 47	Primer A
SEQ ID NO: 48	Primer B
SEQ ID NO: 49	Primer C
SEQ ID NO: 50	Primer D
SEQ ID NO: 51	PAL
SEQ ID NO: 52	HSV-TK
SEQ ID NO: 53	yCD

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SEQ ID NO: 54	PAH
SEQ ID NO: 55	GTPCH
SEQ ID NO: 56	PTPS
SEQ ID NO: 57	SR (sepiapterin reductase)
SEQ ID NO: 58	Factor VIII minus B
SEQ ID NO: 59	Factor VIII
SEQ ID NO: 60	Albumin
SEQ ID NO: 61	Factor IX

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SEQ ID NO: 62	H-FABP
SEQ ID NO: 63	Myocardial myoglobin
SEQ ID NO: 64	GFAP
SEQ ID NO: 65	S100B
SEQ ID NO: 66	PYY (*Homo sapiens*)
SEQ ID NO: 67	TIMP2 (*Homo sapiens*)

Name	Sequence
SEQ ID NO: 1	ATGCCAGAGCCAGCGAAGTCTGCTCCCGCCCCGAAAAAGGGCTCC AAGAAGGCGGTGACTAAGGCGCAGAAGAAAGGCGGCAAGAAGCGC AAGCGCAGCCGCAAGGAGAGCTATTCCATCTATGTGTACAAGGTTC TGAAGCAGGTCCACCCTGACACCGGCATTTCGTCCAAGGCCATGGG CATCATGAACTCGTTTGTGAACGACATTTTCGAGCGCATCGCAGGTG AGGCTTCCCGCCTGGCGCATTACAACAAGCGCTCGACCATCACCTC CAGGGAGATCCAGACGGCCGTGCGCCTGCTGCTGCCTGGGGAGTT GGCCAAGCACGCCGTGTCCGAGGGTACTAAGGCCATCACCAAGTAC ACCAGCGCTAAGGATCCACCGGTCGCCACCATGGCCTCCTCCGAG GACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCT CCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGC CGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAG GGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCC AGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCG ACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGT GATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTC CTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGG CACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATG GGCTGGGAGGCCTCCACCGAGCGGATGTACCCCGAGGACGGCGC CCTGAAGGGCGAGATCAAGATGAGGCTGAAGCTGAAGGACGGCGG CCACTACGACGCCGAGGTCAAGACCACCTACATGGCCAAGAAGCCC GTGCAGCTGCCCGGCGCCTACAAGACCGACATCAAGCTGGACATCA CCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAGCGCGC CGAGGGCCGCCACTCCACCGGCGCC
SEQ ID NO: 2	ATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATC CGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAA GAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATA TCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTCG GTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAA TCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTATGCCGGTGTTG GGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTT ATAATGAACGTGAATTGCTCAACAGTATGGGCATTTCGCAGCCTACC GTGGTGTTCGTTTCCAAAAAGGGGTTGCAAAAAATTTTGAACGTGCA AAAAAAGCTCCCAATCATCCAAAAAATTATTATCATGGATTCTAAAAC GGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATC TACCTCCCGGTTTTAATGAATACGATTTTGTGCCAGAGTCCTTCGAT AGGGACAAGACAATTGCACTGATCATGAACTCCTCTGGATCTACTGG TCTGCCTAAAGGTGTCGCTCTGCCTCATAGAACTGCCTGCGTGAGA TTCTCGCATGCCAGAGATCCTATTTTTGGCAATCAAATCATTCCGGA TACTGCGATTTTAAGTGTTGTTCCATTCCATCACGGTTTTGGAATGTT TACTACACTCGGATATTTGATATGTGGATTTCGAGTCGTCTTAATGTA TAGATTTGAAGAAGAGCTGTTTCTGAGGAGCCTTCAGGATTACAAGA TTCAAAGTGCGCTGCTGGTGCCAACCCTATTCTCCTTCTTCGCCAAA AGCACTCTGATTGACAAATACGATTTATCTAATTTACACGAAATTGCT TCTGGTGGCGCTCCCCTCTCTAAGGAAGTCGGGGAAGCGGTTGCCA AGAGGTTCCATCTGCCAGGTATCAGGCAAGGATATGGGCTCACTGA GACTACATCAGCTATTCTGATTACACCCGAGGGGGATGATAAACCG GGCGCGGTCGGTAAAGTTGTTCCATTTTTTGAAGCGAAGGTTGTGG ATCTGGATACCGGGAAAACGCTGGGCGTTAATCAAAGAGGCGAACT GTGTGTGAGAGGTCCTATGATTATGTCCGGTTATGTAAACAATCCGG AAGCGACCAACGCCTTGATTGACAAGGATGGATGGCTACATTCTGG AGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATCGTTGACC GCCTGAAGTCTCTGATTAAGTACAAAGGCTATCAGGTGGCTCCCGC TGAATTGGAATCCATCTTGCTCCAACACCCCAACATCTTCGACGCAG GTGTCGCAGGTCTTCCCGACGATGACGCCGGTGAACTTCCCGCCG CCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAAGAGAT CGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTGCGC GGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAA AACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAA GGGCGGAAAGATCGCCG

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Name	Sequence
SEQ ID NO: 3	AAG GAA AAA AGC CCC CCC TGT ACA AAA AAG CAG G
SEQ ID NO: 4	gGA ATT CTA ATG CCA ACT TTG TAC
SEQ ID NO: 5	ACA CCG GCA GGC TTA AAG GCT AAC CG
SEQ ID NO: 6	AAA ACG GTT AGC CTT TAA GCC TGC CG
SEQ ID NO: 7	ACA CCG AGG ACA ACG CCC ACA CAC Cg
SEQ ID NO: 8	AAA ACG CTC TGT GGG CGT TGT CCT CG
SEQ ID NO: 9	ACA CCG TCA CCA ATC CTG TCC CTA GG
SEQ ID NO: 10	AAA ACC TAG GGA CAG GAT TGG TGA CG
SEQ ID NO: 11	ACA CCG CCC CAC ACT GGG GCC ACT AG
SEQ ID NO: 12	AAA ACT ACT CCC CCC ACT CTC GGG CG
SEQ ID NO: 13	GAC TAG TGA ATT CCC ATC CTT AAT TAA CCC CTC CCC GCC GGG TTT TGG CG
SEQ ID NO: 14	GAC TAG TCC CCC CCC ATC CAC CCC TCA GGA ACA GGT GGT CCC CCC CC
SEQ ID NO: 15	CCC GAT CCA CCG CTC ACC GCA GAG GAA GCC TTC TAA C
SEQ ID NO: 16	TCC CCC GGG TAC AAA ATC AGA ACC ACA GGG AAG
SEQ ID NO: 17	GGA ATT CAA TAA AAT ATC TTT ATT TTC ATT ACA TC
SEQ ID NO: 18	CCT TAA TTA ACC ATC CAC CCC TGT TTA AAC ACC GGT TTT ACG AGG GTA GGA AGT GGT AC
SEQ ID NO: 19	CCC AAG CTT CTC GAG TTG GGG TTG CCC CTT TTC CAA G
SEQ ID NO: 20	CCC AAG CTT CCA TAG AGC CCA CCG CAT CCC C
SEQ ID NO: 21	CAG GGT CTA GAC GCC GGA TCC GGT ACC CTG TGC CTT CTA GTT GC
SEQ ID NO: 22	GGA TCC CCC CTC TAG ACC CTG GGG AGA GAG CTC GGT G
SEQ ID NO: 23	CCG CTC GAG AAT AAA ATA TCT TTA TTT TCA TTA CAT C
SEQ ID NO: 24	GCT CTA GAC CAA CTC ACG ATC ACA CCC ATC
SEQ ID NO: 25	GAG CTG GGA CCA CCT TAT ATT C
SEQ ID NO: 26	GGT GCA TGA CCC GCA AG
SEQ ID NO: 27	GAG AGA TGG CTC CAG GAA ATG
SEQ ID NO: 28	ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATC CTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTG TCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG AAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC TCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCTACCC CGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAA GGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACT ACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGA ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACAT CCTGGGGCACAAGCTGGAGTACAACTACATCAGCCACAACGTCTAT ATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGA TCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACT ACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCG ACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAA CGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGC CGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGTGGTACCGG AGGCGCCGCTGATACTCGCATTGGTGTAACAATCTATAAGTACGAC GATAACTTTATGTCTGTAGTGCGCAAGGCTATTGAGCAAGATGCGAA AGCCGCGCCAGATGTTCAGCTGCTGATGAATGATTCTCAGAATGAC

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Name	Sequence
	CAGTCCAAGCAGAACGATCAGATCGACGTATTGCTGGCGAAAGGGG TGAAGGCACTGGCAATCAACCTGGTTGACCCGGCAGCTGCGGGTAC GGTGATTGAGAAAGCGCGTGGGCAAAACGTGCCGGTGGTTTTCTTC AACAAAGAACCGTCTCGTAAGGCGCTGGATAGCTACGACAAAGCCT ACTACGTTGGCACTGACTCCAAAGAGTCCGGCATTATTCAAGGCGAT TTGATTGCTAAACACTGGGCGGCGAATCAGGGTTGGGATCTGAACA AAGACGGTCAGATTCAGTTCGTACTGCTGAAAGGTGAACCGGGCCA TCCGGATGCAGAAGCACGTACCACTTACGTGATTAAAGAATTGAACG ATAAAGGCATCAAAACTGAACAGTTACAGTTAGATACCGCAATGTGG GACACCGCTCAGGCGAAAGATAAGATGGACGCCTGGCTGTCTGGC CCGAACGCCAACAAAATCGAAGTGGTTATCGCCAACAACGATcgGAT GGCAATGGGCGCGGTTGAAGCGCTGAAAGCACACAACAAGTCCAG CATTCCGGTGTTTGGCGTCGATGCGtcGCCAGAAGCGCTGGCGCTG GTGAAATCCGGTGCACTGGCGGGCACCGTACTGAACGATGCTAACA ACCAGGCGAAAGCGACCTTTGATCTGGCGAAAAACCTGGCCGATGG TAAAGGTGCGGCTGATGGCACCAACTGGAAAATCGACAACAAAGTG GTCCGCGTACCTTATGTTGGCGTAGATAAAGACAACCTGGCTGAATT CAGCAAGAAAGGCGCCGGTACCGGTGGAATGGTGAGCAAGGGCGA GGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGG CGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGG CGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGC TACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGC ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCG CACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAG GTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAG GGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTG GAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCA GAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAG GACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGC TACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCAT GGACGAGCTG
SEQ ID NO: 29	GCCACCATGGACAAGAAGTACTCCATTGGGCTCGCTATCGGCACAA ACAGCGTCGGCTGGGCCGTCATTACGGACGAGTACAAGGTGCCGA GCAAAAAATTCAAAGTTCTGGGCAATACCGATCGCCACAGCATAAAG AAGAACCTCATTGGCGCCCTCCTGTTCGACTCCGGGGAGACGGCC GAAGCCACGCGGCTCAAAAGAACAGCACGGCGCAGATATACCCGC AGAAAGAATCGGATCTGCTACCTGCAGGAGATCTTTAGTAATGAGAT GGCTAAGGTGGATGACTCTTTCTTCCATAGGCTGGAGGAGTCCTTTT TGGTGGAGGAGGATAAAAAGCACGAGCGCCACCCAATCTTTGGCAA TATCGTGGACGAGGTGGCGTACCATGAAAAGTACCCAACCATATATC ATCTGAGGAAGAAGCTTGTAGACAGTACTGATAAGGCTGACTTGCG GTTGATCTATCTCGCGCTGGCGCATATGATCAAATTTCGGGGACACT TCCTCATCGAGGGGGACCTGAACCCAGACAACAGCGATGTCGACAA ACTCTTTATCCAACTGGTTCAGACTTACAATCAGCTTTTCGAAGAGAA CCCGATCAACGCATCCGGAGTTGACGCCAAAGCAATCCTGAGCGCT AGGCTGTCCAAATCCCGGCGGCTCGAAAACCTCATCGCACAGCTCC CTGGGGAGAAGAAGAACGGCCTGTTTGGTAATCTTATCGCCCTGTC ACTCGGGCTGACCCCCAACTTTAAATCTAACTTCGACCTGGCCGAA GATGCCAAGCTTCAACTGAGCAAAGACACCTACGATGATGATCTCGA CAATCTGCTGGCCCAGATCGGCGACCAGTACGCAGACCTTTTTTTG GCGGCAAAGAACCTGTCAGACGCCATTCTGCTGAGTGATATTCTGC GAGTGAACACGGAGATCACCAAAGCTCCGCTGAGCGCTAGTATGAT CAAGCGCTATGATGAGCACCACCAAGACTTGACTTTGCTGAAGGCC CTTGTCAGACAGCAACTGCCTGAGAAGTACAAGGAAATTTTCTTCGA TCAGTCTAAAAATGGCTACGCCGGATACATTGACGGCGGAGCAAGC CAGGAGGAATTTTACAAATTTATTAAGCCCATCTTGGAAAAAATGGA CGGCACCGAGGAGCTGCTGGTAAAGCTTAACAGAGAAGATCTGTTG CGCAAACAGCGCACTTTCGACAATGGAAGCATCCCCCACCAGATTC ACCTGGGCGAACTGCACGCTATCCTCAGGCGGCAAGAGGATTTCTA CCCCTTTTTGAAAGATAACAGGGAAAAGATTGAGAAAATCCTCACAT TTCGGATACCCTACTATGTAGGCCCCCTCGCCCGGGGAAATTCCAG ATTCGCGTGGATGACTCGCAAATCAGAAGAGACCATCACTCCCTGG AACTTCGAGGAAGTCGTGGATAAGGGGGCCTCTGCCCAGTCCTTCA TCGAAAGGATGACTAACTTTGATAAAAATCTGCCTAACGAAAAGGTG CTTCCTAAACACTCTCTGCTGTACGAGTACTTCACAGTTTATAACGA GCTCACCAAGGTCAAATACGTCACAGAAGGGATGAGAAAGCCAGCA TTCCTGTCTGGAGAGCAGAAGAAAGCTATCGTGGACCTCCTCTTCAA GACGAACCGGAAAGTTACCGTGAAACAGCTCAAAGAAGACTATTTCA AAAAGATTGAATGTTTCGACTCTGTTGAAATCAGCGGAGTGGAGGAT CGCTTCAACGCATCCCTGGGAACGTATCACGATCTCCTGAAAATCAT

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Name	Sequence
	TAAAGACAAGGACTTCCTGGACAATGAGGAGAACGAGGACATTCTT GAGGACATTGTCCTCACCCTTACGTTGTTTGAAGATAGGGAGATGAT TGAAGAACGCTTGAAAACTTACGCTCATCTCTTCGACGACAAAGTCA TGAAACAGCTCAAGAGGCGCCGATATACAGGATGGGGGCGGCTGT CAAGAAAACTGATCAATGGGATCCGAGACAAGCAGAGTGGAAAGAC AATCCTGGATTTTCTTAAGTCCGATGGATTTGCCAACCGGAACTTCA TGCAGTTGATCCATGATGACTCTCTCACCTTTAAGGAGGACATCCAG AAAGCACAAGTTTCTGGCCAGGGGGACAGTCTTCACGAGCACATCG CTAATCTTGCAGGTAGCCCAGCTATCAAAAAGGGAATACTGCAGACC GTTAAGGTCGTGGATGAACTCGTCAAAGTAATGGGAAGGCATAAGC CCGAGAATATCGTTATCGAGATGGCCCGAGAGAACCAAACTACCCA GAAGGGACAGAAGAACAGTAGGGAAAGGATGAAGAGGATTGAAGA GGGTATAAAAGAACTGGGGTCCCAAATCCTTAAGGAACACCCAGTT GAAAACACCCAGCTTCAGAATGAGAAGCTCTACCTGTACTACCTGCA GAACGGCAGGGACATGTACGTGGATCAGGAACTGGACATCAATCGG CTCTCCGACTACGACGTGGATCATATCGTGCCCCAGTCTTTTCTCAA AGATGATTCTATTGATAATAAAGTGTTGACAAGATCCGATAAAAATAG AGGGAAGAGTGATAACGTCCCCTCAGAAGAAGTTGTCAAGAAAATG AAAAATTATTGGCGGCAGCTGCTGAACGCCAAACTGATCACACAAC GGAAGTTCGATAATCTGACTAAGGCTGAACGAGGTGGCCTGTCTGA GTTGGATAAAGCCGGCTTCATCAAAAGGCAGCTTGTTGAGACACGC CAGATCACCAAGCACGTGGCCCAAATTCTCGATTCACGCATGAACA CCAAGTACGATGAAAATGACAAACTGATTCGAGAGGTGAAAGTTATT ACTCTGAAGTCTAAGCTGGTCTCAGATTTCAGAAAGGACTTTCAGTT TTATAAGGTGAGAGAGATCAACAATTACCACCATGCGCATGATGCCT ACCTGAATGCAGTGGTAGGCACTGCACTTATCAAAAAATATCCCAAG CTTGAATCTGAATTTGTTTACGGAGACTATAAAGTGTACGATGTTAG GAAAATGATCGCAAAGTCTGAGCAGGAAATAGGCAAGGCCACCGCT AAGTACTTCTTTTACAGCAATATTATGAATTTTTTCAAGACCGAGATT ACACTGGCCAATGGAGAGATTCGGAAGCGACCACTTATCGAAACAA ACGGAGAAACAGGAGAAATCGTGTGGGACAAGGGTAGGGATTTCGC GACAGTCCGGAAGGTCCTGTCCATGCCGCAGGTGAACATCGTTAAA AAGACCGAAGTACAGACCGGAGGCTTCTCCAAGGAAAGTATCCTCC CGAAAAGGAACAGCGACAAGCTGATCGCACGCAAAAAAGATTGGGA CCCCAAGAAATACGGCGGATTCGATTCTCCTACAGTCGCTTACAGTG TACTGGTTGTGGCCAAAGTGGAGAAAGGGAAGTCTAAAAAACTCAAA AGCGTCAAGGAACTGCTGGGCATCACAATCATGGAGCGATCAAGCT TCGAAAAAAACCCCATCGACTTTCTCGAGGCGAAAGGATATAAAGAG GTCAAAAAAGACCTCATCATTAAGCTTCCCAAGTACTCTCTCTTTGAG CTTGAAAACGGCCGGAAACGAATGCTCGCTAGTGCGGGCGAGCTG CAGAAAGGTAACGAGCTGGCACTGCCCTCTAAATACGTTAATTTCTT GTATCTGGCCAGCCACTATGAAAAGCTCAAAGGGTCTCCCGAAGAT AATGAGCAGAAGCAGCTGTTCGTGGAACAACACAAACACTACCTTGA TGAGATCATCGAGCAAATAAGCGAATTCTCCAAAAGAGTGATCCTCG CCGACGCTAACCTCGATAAGGTGCTTTCTGCTTACAATAAGCACAGG GATAAGCCCATCAGGGAGCAGGCAGAAAACATTATCCACTTGTTTAC TCTGACCAACTTGGGCGCGCCTGCAGCCTTCAAGTACTTCGACACC ACCATAGACAGAAAGCGGTACACCTCTACAAAGGAGGTCCTGGACG CCACACTGATTCATCAGTCAATTACGGGGCTCTATGAAACAAGAATC GACCTCTCTCAGCTCGGTGGAGACAGCAGGGCTGACCCCAAGAAG AAGAGGAAGGTGTGA
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SEQ ID NO: 31	GACTGGAGTTGCAGATCACGAGG
SEQ ID NO: 32	CCGCGGCAGGCCCTCCGAGCGTGGTGGAGCCGTTCTGTGAGACAG CCGGGTACGAGTCGTGACGCTGGAAGGGGCAAGCGGGTGGTGGG CAGGAATGCGGTCCGCCCTGCAGCAACCGGAGGGGGAGGGAGAA GGGAGCGGAAAAGTCTCCACCGGACGCGGCCATGGCTCGGGGGG GGGGGGGCAGCGGAGGAnCGCTTCCGGCCGACGTCTCGTCGCTGA TTGGCTTnTTTTCCTCCCGCCGTGTGTGAAAACACAAATGGCGTGTT TTGGTTGGCGTAAGGCGCCTGTCAGTTAACGGCAGCCGGAGTGCG CAGCCGCCGGCAGCCTCGCTCTGCCCACTGGGTGGGGCGGGAGG TAGGTGGGGTGAGGCGAGCTGNACGTGCGGGCGCGGTCGGCCTCT GGCGGGGCGGGGGAGGGGAGGGAGGGTCAGCGAAAGTAGCTCGC GCGCGAGCGGCCGCCCACCCTCCCCTTCCTCTGGGGGAGTCGTTT TACCCGCCGCCGGCCGGGCCTCGTCGTCTGATTGGCTCTCGGGGC CCAGAAAACTGGCCCTTGCCATTGGCTCGTGTTCGTGCAAGTTGAG TCCATCCGCCGGCCAGCGGGGGCGGCGAGGAGGCGCTCCCAGGT TCCGGCCCTCCCCTCGGCCCCGCGCCGCAGAGTCTGGCCGCGCGC CCCTGCGCAACGTGGCAGGAAGCGCGCGCTGGGGGCGGGGACGG GCAGTAGGGCTGAGCGGCTGCGGGGCGGGTGCAAGCACGTTTCCG ACTTGAGTTGCCTCAAGAGGGGCGTGCTGAGCCAGACCTCCATCGC

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Name	Sequence
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Name	Sequence
	CAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGAAGAGC AACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGG GCCTGCACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGG TAAA
SEQ ID NO: 35	ATGCGCCGCGGCCGCCTGCTGGAGATCGCCCTGGGCTTCACCGTG CTGCTGGCCTCCTACACCTCCCACGGCGCCGACGCCGCTGAATTCG AGTGCCGCTGGTACCTGGGCGGCTGCAAGGAGGACTCCGAGTGCT GCGAGCACCTGCAGTGCCACTCCTACTGGGAGTGGTGCCTGTGGG ACGGCTCCTTCTAA SIGNAL PEPTIDE-> VATX3 CODING
SEQ ID NO: 36	ATGCGCCGCGGCCGCCTGCTGGAGATCGCCCTGGGCTTCACCGTG CTGCTGGCCTCCTACACCTCCCACGGCGCCGACGCCGCTGAATTCG GATCTGACTGCGCCAAGGAGGGCGAGGTGTGCTCCTGGGGCAAGA AGTGCTGCGACCTGGACAACTTCTACTGCCCCATGGAGTTCATCCC CCACTGCAAGAAGTACAAGCCCTACGTGCCCGTGACCACCAACTGC GCCAAGGAGGGCGAGGTGTGCGGCTGGGGCTCCAAGTGCTGCCAC GGCCTGGACTGCCCCCTGGCCTTCATCCCCTACTGCGAGAAGTACC GCTAA SIGNAL PEPTIDE-> DKTX CODING
SEQ ID NO: 37	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACT TGTCACGAATTCGGGATCTGAGTGCCGCTGGTACCTGGGCGGCTGC AAGGAGGACTCCGAGTGCTGCGAGCACCTGCAGTGCCACTCCTACT GGGAGTGGTGCCTGTGGGACGGCTCCTTC**CGACGGAAGCGAGGG** **AT**ATCGGCCATGGTTAGATCTGGTTGTAAGCCTTGCATATGTACAGT CCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAAAGCCCAAGGATG TGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGAC ATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATG ATGTGGAGGTGCACACAGCTCAGACGCAACCCCGGGAGGAGCAGT TCAACAGCACTTTCCGCTCAGTCAGTGAACTTCCCATCATGCACCAG GACTGGCTCAATGGCAAGGAGTTCAAATGCAGGGTCAACAGTGCAG CTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAACCAAAGGCAGA CCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGA TGGCCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTC CCTGAAGACATTACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCG GAGAACTACAAGAACACTCAGCCCATCATGGACACAGATGGCTCTTA CTTCGTCTACAGCAAGCTCAATGTGCAGAAGAGCAACTGGGAGGCA GGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTGCACAACCA CCATACTGAGAAGAGCCTCTCCCACTCTCCTGGTAAAtaa SIGNAL PEPTIDE-> VATX3 CODING-> **FURIN SITE**-> IGG-FC
SEQ ID NO: 38	ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACT TGTCACGAATTCGGGATCTGACTGCGCCAAGGAGGGCGAGGTGTG CTCCTGGGGCAAGAAGTGCTGCGACCTGGACAACTTCTACTGCCCC ATGGAGTTCATCCCCCACTGCAAGAAGTACAAGCCCTACGTGCCCG TGACCACCAACTGCGCCAAGGAGGGCGAGGTGTGCGGCTGGGGCT CCAAGTGCTGCCACGGCCTGGACTGCCCCCTGGCCTTCATCCCCTA CTGCGAGAAGTACCGCCGGAAGCGAGGGATATCGGCCATGGTTAG ATCTGGTTGTAAGCCTTGCATATGTACAGTCCCAGAAGTATCATCTG TCTTCATCTTCCCCCCAAAGCCCAAGGATGTGCTCACCATTACTCTG ACTCCTAAGGTCACGTGTGTTGTGGTAGACATCAGCAAGGATGATC CCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGTGCACAC AGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGC TCAGTCAGTGAACTTCCCATCATGCACCAGGACTGGCTCAATGGCA AGGAGTTCAAATGCAGGGTCAACAGTGCAGCTTTCCCTGCCCCCAT CGAGAAAACCATCTCCAAAACCAAAGGCAGACCGAAGGCTCCACAG GTGTACACCATTCCACCTCCCAAGGAGCAGATGGCCAAGGATAAAG TCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGACATTACT GTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAAC ACTCAGCCCATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAA GCTCAATGTGCAGAAGAGCAACTGGGAGGCAGGAAATACTTTCACC TGCTCTGTGTTACATGAGGGCCTGCACAACCACCATACTGAGAAGA GCCTCTCCCACTCTCCTGGTAAATAA SIGNAL PEPTIDE-> DKTX CODING-> **FURIN SITE**-> IGG-FC
SEQ ID NO: 39	GACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGG GGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCT CATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTG AGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCG TGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACA ACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGA CTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCC

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Name	Sequence
	CTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGC
	CCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGA
	TGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTA
	TCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGA
	GAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCC
	TTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGC
	AGGGGAACGTCTTCTCATGCTCCGTGATGCACGAGGCTCTGCACAA
	CCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA
SEQ ID NO: 40	TGCTTTCTCTGACCAGCATTCTCTCCCCTGGGCCTGTGCCGCTTTCT
	GTCTGCAGCTTGTGGCCTGGGTCACCTCTACGGCTGGCCCAGATCC
	TTCCCTGCCGCCTCCTTCAGGTTCCGTCTTCCTCCACTCCCTCTTCC
	CCTTGCTCTCTGCTGTGTTGCTGCCCAAGGATGCTCTTTCCGGAGC
	ACTTCCTTCTCGGCGCTGCACCACGTGATGTCCTCTGAGCGGATCC
	TCCCCGTGTCTGGGTCCTCTCCGGGCATCTCTCCTCCCTCACCCAA
	CCCCATGCCGTCTTCACTCGCTGGGTTCCCTTTTCCTTCTCCTTCTG
	GGGCCTGTGCCATCTCTCGTTTCTTAGGATGGCCTTCTCCGACGGA
	TGTCTCCCTTGCGTCCCGCCTCCCCTTCTTGTAGGCCTGCATCATCA
	CCGTTTTTCTGGACAACCCCAAAGTACCCCGTCTCCCTGGCTTTAGC
	CACCTCTCCATCCTCTTGCTTTCTTTGCCTGGACACCCCGTTCTCCT
	GTGGATTCGGGTCACCTCTCACTCCTTTCATTTGGGCAGCTCCCCTA
	CCCCCCTTACCTCTCTAGTCTGTGCTAGCTCTTCCAGCCCCCTGTCA
	TGGCATCTTCCAGGGGTCCGAGAGCTCAGCTAGTCTTCTTCCTCCA
	ACCCGGGCCCCTATGTCCACTTCAGGACAGCATGTTTGCTGCCTCC
	AGGGATCCTGTGTCCCCGAGCTGGGACCACCTTATATTCCCAGGGC
	CGGTTAATGTGGCTCTGGTTCTGGGTACTTTTATCTGTCCCCTCCAC
	CCCACAGTGGGGCAAGCTTCTCGAGTTGGGGTTGCGCCTTTTCCAA
	GGCAGCCCTGGGTTTGCGCAGGGACGCGGCTGCTCTGGGCGTGGT
	TCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGCACATTCTTCA
	CGTCCGTTCGCAGCGTCACCCGGATCTTCGCCGCTACCCTTGTGGG
	CCCCCCGGCGACGCTTCCTGCTCCGCCCCTAAGTCGGGAAGGTTC
	CTTGCGGTTCGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACG
	TCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCA
	GCGCGCCGACCGCGATGGGCTGTGGCCAATAGCGGCTGCTCAGCA
	GGGCGCGCCGAGAGCAGCGGCCGGGAAGGGGCGGTGCGGGAGG
	CGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCTGCCCGCGCGGT
	GTTCCGCATTCTGCAAGCCTCCGGAGCGCACGTCGGCAGTCGGCT
	CCCTCGTTGACCGAATCACCGACCTCTCTCCCCAGGGTCTAGACGC
	CACCATGGTGTCCAAGGGCGAGGAGGTGATCAAGGAGTTCATGCG
	CTTCAAGGTGCGCATGGAGGGCTCCATGAACGGCCACGAGTTCGA
	GATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGA
	CCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCT
	GGGACATCCTGTCCCCCCAGTTCATGTACGGCTCCAAGGCCTACGT
	GAAGCACCCCGCCGACATCCCCGACTACAAGAAGCTGTCCTTCCCC
	GAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGC
	CTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCACCCTG
	ATCTACAAGGTGAAGATGCGCGGCACCAACTTCCCCCCCGACGGCC
	CCGTGATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGC
	GCCTGTACCCCCGCGACGGCGTGCTGAAGGGCGAGATCCACCAGG
	CCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGA
	CCATCTACATGGCCAAGAAGCCCGTGCAGCTGCCCGGCTACTACTA
	CGTGGACACCAAGCTGGACATCACCTCCCACAACGAGGACTACACC
	ATCGTGGAGCAGTACGAGCGCTCCGAGGGCCGCCACCACCTGTTC
	CTGGGATCCGAGGGCAGAGGAAGCCTTCTAACATGCGGTGACGTG
	GAGGAGAATCCCGGCCCTTCCGGGATGACCGAGTACAAGCCCACG
	GTGCGCCTCGCCACCCGCGACGACGTCCCCAGGGCCGTACGCACC
	CTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCGTC
	GATCCGGACCGCCACATCGAGCGGGTCACCGAGCTGCAAGAACTC
	TTCCTCACGCGCGTCGGGCTCGACATCGGCAAGGTGTGGGTCGCG
	GACGACGGCGCCGCGGTGGCGGTCTGGACCACGCCGGAGAGCGT
	CGAAGCGGGGGCGGTGTTCGCCGAGATCGGCCCGCGCATGGCCG
	AGTTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCC
	TCCTGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTGGCCA
	CCGTCGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGCAGCG
	CCGTCGTGCTCCCCGGAGTGGAGGCGGCCGAGCGCGCCGGGGTG
	CCCGCCTTCCTGGAGACCTCCGCGCCCCGCAACCTCCCCTTCTACG
	AGCGGCTCGGCTTCACCGTCACCGCCGACGTCGAGGTGCCCGAAG
	GACCGCGCACCTGGTGCATGACCCGCAAGCCCGGTGCCTGAATCT
	AGGTCGACATTCTACTTGGTACCCTGTGCCTTCTAGTTGCCAGCCAT
	CTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGC
	CACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATT
	GTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGG
	ACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGG
	ATGCGGTGGGCTCTATGGAAGCTTTACTAGGGACAGGATTGGTGAC

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Name	Sequence
	AGAAAAGCCCCATCCTTAGGCCTCCTCCTTCCTAGTCTCCTGATATT GGGTCTAACCCCCACCTCCTGTTAGGCAGATTCCTTATCTGGTGACA CACCCCCATTTCCTGGAGCCATCTCTCTCCTTGCCAGAACCTCTAAG GTTTGCTTACGATGGAGCCAGAGAGGATCCTGGGAGGGAGAGCTTG GCAGGGGGTGGGAGGGAAGGGGGGGATGCGTGACCTGCCCGGTT CTCAGTGGCCACCCTGCGCTACCCTCTCCCAGAACCTGAGCTGCTC TGACGCGGCTGTCTGGTGCGTTTCACTGATCCTGGTGCTGCAGCTT CCTTACACTTCCCAAGAGGAGAAGCAGTTTGGAAAAACAAAATCAGA ATAAGTTGGTCCTGAGTTCTAACTTTGGCTCTTCACCTTTCTAGTCCC CAATTTATATTGTTCCTCCGTGCGTCAGTTTTACCTGTGAGATAAGG CCAGTAGCCAGCCCCGTCCTGGCAGGGCTGTGGTGAGGAGGGGG GTGTCCGTGTGGAAAACTCCCTTTGTGAGAATGGTGCGTCCTAGGT GTTCACCAGGTCGTGGCCGCCTCTACTCCCTTTCTCTTTCTCCATCC TTCTTTCCTTAAAGAGTCCCCAGTGCTATCTGGGACATATTCCTCCG CCCAGAGCAGGGTCCCGCTTCCCTAAGGCCCTGCTCTGGGCTTCTG GGTTTGAGTCCTTGGCAAGCCCAGGAGAGGCGCTCAGGCTTCCCTG TCCCCCTTCCTCGTCCACCATCTCATGCCCCTGGCTCTCCTGCCCCT TCCCTACAGGGGTTCCTGGCTCTGCTCT
SEQ ID NO: 41	MHSKVTIICIRFLFWFLLLCMLIGKSHTEDDIIIATKNGKVRGMNLTVFGG TVTAFLGIPYAQPPLGRLRFKKPQSLTKWSDIWNATKYANSCCQNIDQS FPGFHGSEMWNPNTDLSEDCLYLNVWIPAPKPKNATVLIWIYGGGFQT GTSSLHVYDGKFLARVERVIVVSMNYRVGALGFLALPGNPEAPGNMGL FDQQLALQWVQKNIAAFGGNPKSVTLFGESSGAASVSLHLLSPGSHSL FTRAILQSGSANAPWAVTSLYEARNRTLNLAKLTGCSRENETEIIKCLRN KDPQEILLNEAFVVPYGTALGVNFGPTVDGDFLTDMPDILLELGQFKKT QILVGVNKDEGTWFLVGGAPGFSKDNNSIITRKEFQEGLKIFFPGVSEF GKESILFHYTDWVDDQRPENYREALGDVVGDYNFICPALEFTKKFSEW GNNAFFYYFEHRSSKLPWPEWMGVMHGYEIEFVFGLPLERRDNYTKA EEILSRSIVKRWANFAKYGNPNETQNNSTSWPVFKSTEQKYLTLNTEST RIMTKLRAQQCRFWTSFFPKVLEMTGNIDEAEWEWKAGFHRWNNYM MDWKNQFNDYTSKKESCVGL
SEQ ID NO: 42	ATGCACAGCAAGGTGACCATCATCTGCATCAGGTTCCTGTTCTGGTT CCTGCTGCTGTGCATGCTGATCGGCAAGAGCCACACCGAGGACGA CATCATCATCGCCACCAAGAACGGCAAGGTGAGGGGCATGAACCTG ACCGTGTTCGGCGGCACCGTGACCGCCTTCCTGGGCATCCCCTAC GCCCAGCCCCCCCTGGGCAGGCTGAGGTTCAAGAAGCCCCAGAGC CTGACCAAGTGGAGCGACATCTGGAACGCCACCAAGTACGCCAACA GCTGCTGCCAGAACATCGACCAGAGCTTCCCCGGCTTCCACGGCAG CGAGATGTGGAACCCCAACACCGACCTGAGCGAGGACTGCCTGTAC CTGAACGTGTGGATCCCCGCCCCCAAGCCCAAGAACGCCACCGTG CTGATCTGGATCTACGGCGGCGGCTTCCAGACCGGCACCAGCAGC CTGCACGTGTACGACGGCAAGTTCCTGGCCAGGGTGGAGAGGGTG ATCGTGGTGAGCATGAACTACAGGGTGGGCGCCCTGGGCTTCCTG GCCCTGCCCGGCAACCCCGAGGCCCCCGGCAACATGGGCCTGTTC GACCAGCAGCTGGCCCTGCAGTGGGTGCAGAAGAACATCGCCGCC TTCGGCGGCAACCCCAAGAGCGTGACCCTGTTCGGCGAGAGCAGC GGCGCCGCCAGCGTGAGCCTGCACCTGCTGAGCCCCGGCAGCCAC AGCCTGTTCACCAGGGCCATCCTGCAGAGCGGCAGCGCCAACGCC CCCTGGGCCGTGACCAGCCTGTACGAGGCCAGGAACAGGACCCTG AACCTGGCCAAGCTGACCGGCTGCAGCAGGGAGAACGAGACCGAG ATCATCAAGTGCCTGAGGAACAAGGACCCCCAGGAGATCCTGCTGA ACGAGGCCTTCGTGGTGCCCTACGGCACCGCCCTGGGCGTGAACT TCGGCCCCACCGTGGACGGCGACTTCCTGACCGACATGCCCGACA TCCTGCTGGAGCTGGGCCAGTTCAAGAAGACCCAGATCCTGGTGGG CGTGAACAAGGACGAGGGCACCTGGTTCCTGGTGGGCGGCGCCCC CGGCTTCAGCAAGGACAACAACAGCATCATCACCAGGAAGGAGTTC CAGGAGGGCCTGAAGATCTTCTTCCCCGGCGTGAGCGAGTTCGGC AAGGAGAGCATCCTGTTCCACTACACCGACTGGGTGGACGACCAGA GGCCCGAGAACTACAGGGAGGCCCTGGGCGACGTGGTGGGCGACT ACAACTTCATCTGCCCCGCCCTGGAGTTCACCAAGAAGTTCAGCGA GTGGGGCAACAACGCCTTCTTCTACTACTTCGAGCACAGGAGCAGC AAGCTGCCCTGGCCCGAGTGGATGGGCGTGATGCACGGCTACGAG ATCGAGTTCGTGTTCGGCCTGCCCCTGGAGAGGAGGGACAACTACA CCAAGGCCGAGGAGATCCTGAGCAGGAGCATCGTGAAGAGGTGGG CCAACTTCGCCAAGTACGGCAACCCCAACGAGACCCAGAACAACAG CACCAGCTGGCCCGTGTTCAAGAGCACCGAGCAGAAGTACCTGACC CTGAACACCGAGAGCACCAGGATCATGACCAAGCTGAGGGCCCAG CAGTGCAGGTTCTGGACCAGCTTCTTCCCCAAGGTGCTGGAGATGA CCGGCAACATCGACGAGGCCGAGTGGGAGTGGAAGGCCGGCTTCC ACAGGTGGAACAACTACATGATGGACTGGAAGAACCAGTTCAACGA CTACACCAGCAAGAAGGAGAGCTGCGTGGGCCTG

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Name	Sequence
SEQ ID NO: 43	MQTQHTKVTQTHFLLWILLLCMPFGKSHTEEDFIITTKTGRVRGLSMPVL GGTVTAFLGIPYAQPPLGSLRFKKPQPLNKWPDIHNATQYANSCYQNID QAFPGFQGSEMWNPNTNLSEDCLYLNVWIPVPKPKNATVMVWIYGGG FQTGTSSLPVYDGKFLARVERVIVVSMNYRVGALGFLAFPGNPDAPGN MGLFDQQLALQWVQRNIAAFGGNPKSITIFGESSGAASVSLHLLCPQSY PLFTRAILESGSANAPWAVKHPEEARNRTLTLAKFTGCSKENEMEMIKC LRSKDPQEILRNERFVLPSDSALGINFGPTVDGDFLTDMPHTLLQLGKV KKAQILVGVNKDEGTWFLVGGAPGFSKDNDSLITRKEFQEGLNMYFPG VSRLGKEAVLFYYVDWLGEQSPEVYRDALDDVIGDYNIICPALEFTKKFA ELENNAFFYFFEHRSSKLPWPEWMGVMHGYEIEFVFGLPLGRRVNYTR AEEIFSRSIMKTWANFAKYGHPNGTQGNSTMWPVFTSTEQKYLTLNTE KSKIYSKLRAPQCQFWRLFFPKVLEMTGDIDETEQEWKAGFHRWSNY MMDWQNQFNDYTSKKESCTAL
SEQ ID NO: 44	ATGCAGACCCAGCACACCAAGGTGACCCAGACCCACTTCCTGCTGT GGATCCTGCTGCTGTGCATGCCCTTCGGCAAGAGCCACACCGAGGA GGACTTCATCATCACCACCAAGACCGGCAGGGTGAGGGGCCTGAG CATGCCCGTGCTGGGCGGCACCGTGACCGCCTTCCTGGGCATCCC CTACGCCCAGCCCCCCCTGGGCAGCCTGAGGTTCAAGAAGCCCCA GCCCCTGAACAAGTGGCCCGACATCCACAACGCCACCCAGTACGCC AACAGCTGCTACCAGAACATCGACCAGGCCTTCCCCGGCTTCCAGG GCAGCGAGATGTGGAACCCCAACACCAACCTGAGCGAGGACTGCC TGTACCTGAACGTGTGGATCCCCGTGCCCAAGCCCAAGAACGCCAC CGTGATGGTGTGGATCTACGGCGGCGGCTTCCAGACCGGCACCAG CAGCCTGCCCGTGTACGACGGCAAGTTCCTGGCCAGGGTGGAGAG GGTGATCGTGGTGAGCATGAACTACAGGGTGGGCGCCCTGGGCTT CCTGGCCTTCCCCGGCAACCCCGACGCCCCCGGCAACATGGGCCT GTTCGACCAGCAGCTGGCCCTGCAGTGGGTGCAGAGGAACATCGC CGCCTTCGGCGGCAACCCCAAGAGCATCACCATCTTCGGCGAGAG CAGCGGCGCCGCCAGCGTGAGCCTGCACCTGCTGTGCCCCCAGAG CTACCCCCTGTTCACCAGGGCCATCCTGGAGAGCGGCAGCGCCAA CGCCCCCTGGGCCGTGAAGCACCCCGAGGAGGCCAGGAACAGGAC CCTGACCCTGGCCAAGTTCACCGGCTGCAGCAAGGAGAACGAGAT GGAGATGATCAAGTGCCTGAGGAGCAAGGACCCCCAGGAGATCCT GAGGAACGAGAGGTTCGTGCTGCCCAGCGACAGCGCCCTGGGCAT CAACTTCGGCCCCACCGTGGACGGCGACTTCCTGACCGACATGCCC CACACCCTGCTGCAGCTGGGCAAGGTGAAGAAGGCCCAGATCCTG GTGGGCGTGAACAAGGACGAGGGCACCTGGTTCCTGGTGGGCGGC GCCCCCGGCTTCAGCAAGGACAACGACAGCCTGATCACCAGGAAG GAGTTCCAGGAGGGCCTGAACATGTACTTCCCCGGCGTGAGCAGG CTGGGCAAGGAGGCCGTGCTGTTCTACTACGTGGACTGGCTGGGC GAGCAGAGCCCCGAGGTGTACAGGGACGCCCTGGACGACGTGATC GGCGACTACAACATCATCTGCCCCGCCCTGGAGTTCACCAAGAAGT TCGCCGAGCTGGAGAACAACGCCTTCTTCTACTTCTTCGAGCACAG GAGCAGCAAGCTGCCCTGGCCCGAGTGGATGGGCGTGATGCACGG CTACGAGATCGAGTTCGTGTTCGGCCTGCCCCTGGGCAGGAGGGT GAACTACACCAGGGCCGAGGAGATCTTCAGCAGGAGCATCATGAAG ACCTGGGCCAACTTCGCCAAGTACGGCCACCCCAACGGCACCCAG GGCAACAGCACCATGTGGCCCGTGTTCACCAGCACCGAGCAGAAGT ACCTGACCCTGAACACCGAGAAGAGCAAGATCTACAGCAAGCTGAG GGCCCCCCAGTGCCAGTTCTGGAGGCTGTTCTTCCCCAAGGTGCTG GAGATGACCGGCGACATCGACGAGACCGAGCAGGAGTGGAAGGCC GGCTTCCACAGGTGGAGCAACTACATGATGGACTGGCAGAACCAGT TCAACGACTACACCAGCAAGAAGGAGAGCTGCACCGCCCTG
SEQ ID NO: 45	MYRMQLLSCIALSLALVTNSHGEGTFTSDVSSYLEGQAAKEFIAWLVKG RGRSGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPE VQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEF KCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMI TDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSN WEAGNTFTCSVLHEGLHNHHTEKSLSHSPGK
SEQ ID NO: 46	ATGTACAGGATGCAGCTGCTGAGCTGCATCGCCCTGAGCCTGGCCC TGGTGACCAACAGCCACGGCGAGGGCACCTTCACCAGCGACGTGA GCAGCTACCTGGAGGGCCAGGCCGCCAAGGAGTTCATCGCCTGGC TGGTGAAGGGCAGGGGCAGGAGCGGCTGCAAGCCCTGCATCTGCA CCGTGCCCGAGGTGAGCAGCGTGTTCATCTTCCCCCCCAAGCCCAA GGACGTGCTGACCATCACCCTGACCCCCAAGGTGACCTGCGTGGT GGTGGACATCAGCAAGGACGACCCCGAGGTGCAGTTCAGCTGGTT CGTGGACGACGTGGAGGTGCACACCGCCCAGACCCAGCCCAGGGA GGAGCAGTTCAACAGCACCTTCAGGAGCGTGAGCGAGCTGCCCATC ATGCACCAGGACTGGCTGAACGGCAAGGAGTTCAAGTGCAGGGTG AACAGCGCCGCCTTCCCCGCCCCCATCGAGAAGACCATCAGCAAGA CCAAGGGCAGGCCCAAGGCCCCCCAGGTGTACACCATCCCCCCCC

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Name	Sequence
	CCAAGGAGCAGATGGCCAAGGACAAGGTGAGCCTGACCTGCATGAT CACCGACTTCTTCCCCGAGGACATCACCGTGGAGTGGCAGTGGAAC GGCCAGCCCGCCGAGAACTACAAGAACACCCAGCCCATCATGGACA CCGACGGCAGCTACTTCGTGTACAGCAAGCTGAACGTGCAGAAGAG CAACTGGGAGGCCGGCAACACCTTCACCTGCAGCGTGCTGCACGA GGGCCTGCACAACCACCACACCGAGAAGAGCCTGAGCCACAGCCC CGGCAAG
SEQ ID NO: 47	GCT CTA GAG CCA CCA TGC AGA CTC AGC ATA CCA AGG
SEQ ID NO: 48	CGG GAT CCA CCG GTT TAG AGA GCT GTA CAA GAT TCT TTC TTG
SEQ ID NO: 49	CCC AAG CTT GCC ACC ATG CAT AGC AAA GTC ACA ATC
SEQ ID NO: 50	ACG CGT CGA CTT AGA GAC CCA CAC AAC TTT CTT TCT TG
SEQ ID NO: 51	ATGAAGACGCTGTCACAGGCCCAGTCCAAGACCTCCTCCCAGCAGT TCTCCTTCACCGGCAACTCCTCCGCCAACGTGATCATCGGCAACCA GAAGCTGACCATCAACGACGTGGCCCGCGTGGCCCGCAACGGCAC CCTGGTGTCCCTGACCAACAACACCGACATCCTGCAGGGCATCCAG GCCTCCTGCGACTACATCAACAACGCCGTGGAGTCCGGCGAGCCC ATCTACGGCGTGACCTCCGGCTTCGGCGGCATGGCCAACGTGGCC ATCTCCCGCGAGCAGGCCTCCGAGCTGCAGACCAACCTGGTGTGG TTCCTGAAGACCGGCGCCGGCAACAAGCTGCCCCTGGCCGACGTG CGCGCCGCCATGCTGCTGCGCGCCAACTCCCACATGCGCGGCGCC TCCGGCATCCGCCTcGAGCTGATCAAGCGCATGGAGATCTTCCTGA ACGCCGGCGTGACCCCCTACGTGTACGAGTTCGGCTCCATCGGCG CCTCCGGCGACCTGGTGCCCCTGTCCTACATCACCGGCTCCCTGAT CGGCCTGGACCCCTCCTTCAAGGTGGACTTCAACGGCAAGGAGATG GACGCCCCCACCGCCCTGCGCCAGCTGAACCTGTCCCCCCTGACC CTGCTGCCCAAGGAGGGCCTGGCCATGATGAACGGCACCTCCGTG ATGACCGGCATCGCCGCCAACTGCGTGTACGACACCCAGATCCTGA CCGCCATCGCCATGGGTGTACACGCTCTGGACATCCAGGCCCTGAA CGGCACCAACCAGTCCTTCCACCCCTTCATCCACAACTCCAAGCCC CACCCCGGCCAGCTGTGGGCCGCCGACCAGATGATCTCCCTCCTC GCCAACTCCCAGCTGGTGCGCGACGAGCTGGACGGCAAGCACGAC TACCGCGACCACGAGCTGATCCAGGACCGCTACTCCCTGCGCTGCC TGCCCCAGTACCTGGGCCCCATCGTGGACGGCATCTCCCAGATCGC CAAGCAGATCGAGATCGAGATCAACTCCGTGACCGACAACCCCCTG ATCGACGTGGACAACCAGGCCTCCTACCACGGCGGCAACTTCCTGG GCCAGTACGTGGGCATGGGCATGGACCACCTGCGCTACTACATCG GCCTGCTGGCCAAGCACCTGGACGTGCAGATCGCCCTGCTGGCCT CCCCCGAGTTCTCCAACGGCCTGCCCCCCTCCCTGCTGGGCAACC GCGAGCGCAAGGTGAACATGGGCCTGAAGGGCCTGCAGATCTGCG GTAACTCGATAATGCCCCTGCTGACCTTCTACGGCAACTCCATCGCC GACCGCTTCCCCACCCACGCCGAGCAGTTCAACCAGAACATCAACT CCCAGGGCTACACCTCCGCCACCCTGGCCCGCCGCTCCGTGGACA TCTTCCAGAACTACGTGGCCATCGCCCTGATGTTCGGCGTTCAAGC TGTAGACCTGCGCACCTACAAGAAGACCGGCCACTACGACGCCCGC GCCTCCCTGTCCCCCGCCACCGAGCGCCTGTACTCCGCCGTGCGC CACGTGGTGGGCCAGAAGCCCACCTCCGACCGCCCCTACATCTGG AACGACAACGAGCAGGGCCTGGACGAGCACATCGCCCGCATCTCC GCCGACATCGCAGCAGGTGGCGTGATCGTGCAGGCCGTGCAGGAC ATCCTGCCCTCCCTGCAC
SEQ ID NO: 52	ATGGCTTCGTACCCCGGCCATCAGCACGCGTCTGCGTTCGACCAGG CTGCGCGTTCTCGCGGCCATAGCAACCGACGTACGGCGTTGCGCC CTCGCCGGCAGCAAGAAGCCACGGAAGTCCGCCCGGAGCAGAAAA TGCCCACGCTACTGCGGGTTTATATAGACGGTCCCCACGGGATGGG GAAAACCACCACCACGCAACTGCTGGTGGCCCTGGGTTCGCGCGA CGATATCGTCTACGTACCCGAGCCGATGACTTACTGGCGGGTGCTG GGGGCTTCCGAGACAATCGCGAACATCTACACCACACAACACCGCC TTGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGA CAAGCGCCCAGATAACAATGGGCATGCCTTATGCCGTGACCGACGC CGTTCTGGCTCCTCATATCGGGGGGGAGGCTGGGAGCTCACATGC CCCGCCCCCGGCCCTCACCCTCATCTTCGACCGCCATCCCATCGCC GCCCTCCTGTGCTACCCGGCCGCGCGATACCTTATGGGCAGCATGA CCCCCCAGGCCGTGCTGGCGTTCGTGGCCCTCATCCCGCCGACCT TGCCCGGCACAAACATCGTGTTGGGGGCCCTTCCGGAGGACAGAC ACATCGACCGCCTGGCCAAACGCCAGCGCCCCGGCGAGCGGCTTG ACCTGGCTATGCTGGCCGCGATTCGCCGCGTTTACGGGCTGCTTGC CAATACGGTGCGGTATCTGCAGGGCGGCGGGTCGTGGCGGGAGGA TTGGGGACAGCTTTCGGGGACGGCCGTGCCGCCCCAGGGTGCCGA GCCCCAGAGCAACGCGGGCCCACGACCCCATATCGGGGACACGTT

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Name	Sequence
	ATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACGGCGAC CTGTACAACGTGTTTGCCTGGGCCTTGGACGTCTTGGCCAAACGCC TCCGTCCCATGCACGTCTTTATCCTGGATTACGACCAATCGCCCGCC GGCTGCCGGGACGCCCTGCTGCAACTTACCTCCGGGATGATCCAG ACCCACGTCACCACCCCAGGCTCCATACCGACGATCTGCGACCTGG CGCGCACGTTTGCCCGGGAGATGGGGGAGGCTAAC
SEQ ID NO: 53	ATGGTGACCGGCGGCATGGCCTCCAAGTGGGACCAGAAGGGCATG GACATCGCCTACGAGGAGGCCGCCCTGGGCTACAAGGAGGGCGGC GTGCCCATCGGCGGCTGCCTGATCAACAACAAGGACGGCTCCGTG CTGGGCCGCGGCCACAACATGCGCTTCCAGAAGGGCTCCGCCACC CTGCACGGCGAGATCTCCACCCTGGAGAACTGCGGCCGCCTGGAG GGCAAGGTGTACAAGGACACCACCCTGTACACCACCCTGTCCCCCT GCGACATGTGCACCGGCGCCATCATCATGTACGGCATCCCCCGCTG CGTGGTGGGCGAGAACGTGAACTTCAAGTCCAAGGGCGAGAAGTA CCTGCAGACCCGCGGCCACGAGGTGGTGGTGGTGGACGACGAGC GCTGCAAGAAGATCATGAAGCAGTTCATCGACGAGCGCCCCCAGGA CTGGTTCGAGGACATCGGCGAG
SEQ ID NO: 54	ATGTCCACTGCGGTCCTGGAAAACCCAGGCTTGGGCAGGAAACTCT CTGACTTTGGACAGGAAACAAGCTATATTGAAGACAACTGCAATCAA AATGGTGCCATATCACTGATCTTCTCACTCAAAGAAGAAGTTGGTGC ATTGGCCAAAGTATTGCGCTTATTTGAGGAGAATGATGTAAACCTGA CCCACATTGAATCTAGACCTTCTCGTTTAAAGAAAGATGAGTATGAAT TTTTCACCCATTTGGATAAACGTAGCCTGCCTGCTCTGACAAACATC ATCAAGATCTTGAGGCATGACATTGGTGCCACTGTCCATGAGCTTTC ACGAGATAAGAAGAAAGACACAGTGCCCTGGTTCCCAAGAACCATT CAAGAGCTGGACAGATTTGCCAATCAGATTCTCAGCTATGGAGCGG AACTGGATGCTGACCACCCTGGTTTTAAAGATCCTGTGTACCGTGCA AGACGGAAGCAGTTTGCTGACATTGCCTACAACTACCGCCATGGGC AGCCCATCCCTCGAGTGGAATACATGGAGGAAGAAAAGAAAACATG GGGCACAGTGTTCAAGACTCTGAAGTCCTTGTATAAAACCCATGCTT GCTATGAGTACAATCACATTTTTCCACTTCTTGAAAAGTACTGTGGCT TCCATGAAGATAACATTCCCCAGCTGGAAGACGTTTCTCAATTCCTG CAGACTTGCACTGGTTTCCGCCTCCGACCTGTGGCTGGCCTGCTTT CCTCTCGGGATTTCTTGGGTGGCCTGGCCTTCCGAGTCTTCCACTG CACACAGTACATCAGACATGGATCCAAGCCCATGTATACCCCCGAA CCTGACATCTGCCATGAGCTGTTGGGACATGTGCCCTTGTTTTCAGA TCGCAGCTTTGCCCAGTTTTCCCAGGAAATTGGCCTTGCCTCTCTGG GTGCACCTGATGAATACATTGAAAAGCTCGCCACAATTTACTGGTTT ACTGTGGAGTTTGGGCTCTGCAAACAAGGAGACTCCATAAAGGCAT ATGGTGCTGGGCTCCTGTCATCCTTTGGTGAATTACAGTACTGCTTA TCAGAGAAGCCAAAGCTTCTCCCCCTGGAGCTGGAGAAGACAGCCA TCCAAAATTACACTGTCACGGAGTTCCAGCCCCTGTATTACGTGGCA GAGAGTTTTAATGATGCCAAGGAGAAAGTAAGGAACTTTGCTGCCAC AATACCTCGGCCCTTCTCAGTTCGCTACGACCCATACACCCAAAGGA TTGAGGTCTTGGACAATACCCAGCAGCTTAAGATTTTGGCTGATTCC ATTAACAGTGAAATTGGAATCCTTTGCAGTGCCCTCCAGAAAATAAA G
SEQ ID NO: 55	ATGGAGAAGCCGCGGGGAGTCAGGTGCACCAATGGGTTCTCCGAG CGTGAACTACCTCGTCCTGGAGCAAGCCCACCTGCAGAGAAGTCCC GACCTCCTGAAGCAAAGGGCGCACAGCCGGCCGACGCCTGGAAGG CAGGGCGGCACCGCAGCGAGGAGGAAAACCAGGTGAACCTCCCCA AACTGGCGGCCGCTTACTCGTCCATTCTGCTCTCGCTGGGCGAGGA CCCCCAGCGGCAGGGGCTGCTCAAGACGCCCTGGAGGGCGGCCA CCGCCATGCAGTACTTCACCAAGGGATACCAGGAGACCATCTCAGA TGTCCTGAATGATGCTATATTTGATGAAGATCATGACGAGATGGTGA TTGTGAAGGACATAGATATGTTCTCCATGTGTGAGCATCACCTTGTT CCATTTGTAGGAAGGGTCCATATTGGCTATCTTCCTAACAAGCAAGT CCTTGGTCTCAGTAAACTTGCCAGGATTGTAGAAATCTACAGTAGAC GACTACAAGTTCAAGAGCGCCTCACCAAACAGATTGCGGTGGCCAT CACAGAAGCCTTGCAGCCTGCTGGCGTTGGAGTAGTGATTGAAGCG ACACACATGTGCATGGTAATGCGAGGCGTGCAGAAAATGAACAGCA AGACTGTCACTAGCACCATGCTGGGCGTGTTCCGGGAAGACCCCAA GACTCGGGAGGAGTTCCTCACACTAATCAGGAGC
SEQ ID NO: 56	ATGAGCGCTGCTGGTGATCTTCGTCGTCGTGCGCGACTGTCGCGCC TCGTGTCCTTCAGCGCGAGCCACCGGCTGCACAGCCCATCTCTGAG CGATGAAGAGAACTTAAGAGTGTTTGGGAAATGCAACAATCCGAATG GCCACGGGCACAACTATAAAGTTGTGGTGACAGTCCATGGAGAGAT TGATCCTGTTACAGGAATGGTTATGAATTTGACCGACCTCAAAGAAT ACATGGAGGAGGCCATCATGAAGCCTCTTGATCACAAGAACCTGGA CCTGGATGTGCCGTACTTTGCGGATGCTGTGAGCACGACAGAAAAT

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Name	Sequence
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SEQ ID NO: 57	ATGGAGGGCGGGCTGGGGCGTGCTGTGTGCTTGCTGACCGGGGCC TCCCGCGGCTTCGGCCGGACGCTGGCCCCGCTCCTGGCCTCGCTG CTGTCGCCCGGCTCCGTGCTTGTCCTTAGCGCCCGCAACGACGAG GCACTGCGCCAGCTGGAGGCCGAGCTGGGCGCCGAGCGGTCTGG CCTGCGCGTGGTGCGGGTGCCCGCCGACCTGGGCGCCGAGGCCG GCTTGCAGCAGCTGCTCGGCGCCCTGCGCGAGCTCCCCCGGCCCA AGGGGCTGCAGCGACTGCTGCTTATCAACAACGCGGGCTCTCTTGG GGATGTGTCCAAAGGCTTCGTGGACCTGAGTGACTCCACTCAAGTG AACAACTACTGGGCACTGAACTTGACCTCCATGCTCTGCCTGACTTC CAGCGTCCTGAAGGCCTTCCCGGACAGTCCTGGCCTCAACAGAACC GTGGTTAACATCTCGTCCCTCTGTGCCCTGCAACCTTTCAAAGGCTG GGCGCTGTACTGTGCAGGAAAGGCTGCTCGTGATATGCTGTTCCAG GTCCTGGCGCTGGAGGAACCTAATGTGAGGGTGCTGAACTATGCCC CAGGTCCTCTGGACACAGACATGCAGCAGTTGGCCCGGGAGACCT CCGTGGACCCAGACATGCGAAAAGGGCTGCAGGAGCTGAAGGCAA AGGGGAAGCTGGTGGATTGCAAGGTGTCAGCCCAGAAACTGCTGA GCTTACTGGAAAAGGACGAGTTCAAGTCTGGAGCCCACGTGGACTT CTATGACAAA
SEQ ID NO: 58	ATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGTGCCTTTTGCGATT CTGCTTTAGTGCCACCAGAAGATACTACCTGGGTGCAGTGGAACTG TCATGGGACTATATGCAAAGTGATCTCGGTGAGCTGCCTGTGGACG CAAGATTTCCTCCTAGAGTGCCAAAATCTTTTCCATTCAACACCTCAG TCGTGTACAAAAAGACTCTGTTTGTAGAATTCACGGATCACCTTTTCA ACATCGCTAAGCCAAGGCCACCCTGGATGGGTCTGCTAGGTCCTAC CATCCAGGCTGAGGTTTATGATACAGTGGTCATTACACTTAAGAACA TGGCTTCCCATCCTGTCAGTCTTCATGCTGTTGGTGTATCCTACTGG AAAGCTTCTGAGGGAGCTGAATATGATGATCAGACCAGTCAAAGGG AGAAAGAAGATGATAAAGTCTTCCCTGGTGGAAGCCATACATATGTC TGGCAGGTCCTGAAAGAGAATGGTCCAATGGCCTCTGACCCACTGT GCCTTACCTACTCATATCTTTCTCATGTGGACCTGGTAAAAGACTTG AATTCAGGCCTCATTGGAGCCCTACTAGTATGTAGAGAAGGGAGTCT GGCCAAGGAAAAGACACAGACCTTGCACAAATTTATACTACTTTTTG CTGTATTTGATGAAGGGAAAAGTTGGCACTCAGAAACAAAGAACTCC TTGATGCAGGATAGGGATGCTGCATCTGCTCGGGCCTGGCCTAAAA TGCACACAGTCAATGGTTATGTAAACAGGTCTCTGCCAGGTCTGATT GGATGCCACAGGAAATCAGTCTATTGGCATGTGATTGGAATGGGCA CCACTCCTGAAGTGCACTCAATATTCCTCGAAGGTCACACATTTCTT GTGAGGAACCATCGCCAGGCGTCCTTGGAAATCTCGCCAATAACTT TCCTTACTGCTCAAACACTCTTGATGGACCTTGGACAGTTTCTACTG TTTTGTCATATCTCTTCCCACCAACATGATGGCATGGAAGCTTATGTC AAAGTAGACAGCTGTCCAGAGGAACCCCAACTACGAATGAAAAATAA TGAAGAAGCGGAAGACTATGATGATGATCTTACTGATTCTGAAATGG ATGTGGTCAGGTTTGATGATGACAACTCTCCTTCCTTTATCCAAATTC GCTCAGTTGCCAAGAAGCATCCTAAAACTTGGGTACATTACATTGCT GCTGAAGAGGAGGACTGGGACTATGCTCCCTTAGTCCTCGCCCCCG ATGACAGAAGTTATAAAAGTCAATATTTGAACAATGGCCCTCAGCGG ATTGGTAGGAAGTACAAAAAAGTCCGATTTATGGCATACACAGATGA AACCTTTAAGACTCGTGAAGCTATTCAGCATGAATCAGGAATCTTGG GACCTTTACTTTATGGGGAAGTTGGAGACACACTGTTGATTATATTTA AGAATCAAGCAAGCAGACCATATAACATCTACCCTCACGGAATCACT GATGTCCGTCCTTTGTATTCAAGGAGATTACCAAAAGGTGTAAAACA TTTGAAGGATTTTCCAATTCTGCCAGGAGAAATATTCAAATATAAATG GACAGTGACTGTAGAAGATGGGCCAACTAAATCAGATCCTCGGTGC CTGACCCGCTATTACTCTAGTTTCGTTAATATGGAGAGAGATCTAGC TTCAGGACTCATTGGCCCTCTCCTCATCTGCTACAAAGAATCTGTAG ATCAAAGAGGAAACCAGATAATGTCAGACAAGAGGAATGTCATCCTG TTTTCTGTATTTGATGAGAACCGAAGCTGGTACCTCACAGAGAATAT ACAACGCTTTCTCCCCAATCCAGCTGGAGTGCAGCTTGAGGATCCA GAGTTCCAAGCCTCCAACATCATGCACAGCATCAATGGCTATGTTTT TGATAGTTTGCAGTTGTCAGTTTGTTTGCATGAGGTGGCATACTGGT ACATTCTAAGCATTGGAGCACAGACTGACTTCCTTTCTGTCTTCTTCT CTGGATATACCTTCAAACACAAAATGGTCTATGAAGACACACTCACC CTATTCCCATTCTCAGGAGAAACTGTCTTCATGTCGATGGAAAACCC AGGTCTATGGATTCTGGGGTGCCACAACTCAGACTTTCGGAACAGA GGCATGACCGCCTTACTGAAGGTTTCTAGTTGTGACAAGAACACTG GTGATTATTACGAGGACAGTTATGAAGATATTTCAGCATACTTGCTG AGTAAAAACAATGCCATTGAACCAAGAAGCTTCTCCCAGAATTCAAG ACACCCTAGCACTAGGCAAAAGCAATTTAATGCCACCCCACCAGTCT TGAAACGCCATCAACGGGAAATAACTCGTACTACTCTTCAGTCAGAT

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Name	Sequence
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SEQ ID NO: 59	ATGCAAATAGAGCTCTCCACCTGCTTCTTTCTGTGCCTTTTGCGATT CTGCTTTAGTGCCACCAGAAGATACTACCTGGGTGCAGTGGAACTG TCATGGGACTATATGCAAAGTGATCTCGGTGAGCTGCCTGTGGACG CAAGATTTCCTCCTAGAGTGCCAAAATCTTTTCCATTCAACACCTCAG TCGTGTACAAAAAGACTCTGTTTGTAGAATTCACGGATCACCTTTTCA ACATCGCTAAGCCAAGGCCACCCTGGATGGGTCTGCTAGGTCCTAC CATCCAGGCTGAGGTTTATGATACAGTGGTCATTACACTTAAGAACA TGGCTTCCCATCCTGTCAGTCTTCATGCTGTTGGTGTATCCTACTGG AAAGCTTCTGAGGGAGCTGAATATGATGATCAGACCAGTCAAAGGG AGAAAGAAGATGATAAAGTCTTCCCTGGTGGAAGCCATACATATGTC TGGCAGGTCCTGAAAGAGAATGGTCCAATGGCCTCTGACCCACTGT GCCTTACCTACTCATATCTTTCTCATGTGGACCTGGTAAAAGACTTG AATTCAGGCCTCATTGGAGCCCTACTAGTATGTAGAGAAGGGAGTCT GGCCAAGGAAAAGACACAGACCTTGCACAAATTTATACTACTTTTTG CTGTATTTGATGAAGGGAAAAGTTGGCACTCAGAAACAAAGAACTCC TTGATGCAGGATAGGGATGCTGCATCTGCTCGGGCCTGGCCTAAAA TGCACACAGTCAATGGTTATGTAAACAGGTCTCTGCCAGGTCTGATT GGATGCCACAGGAAATCAGTCTATTGGCATGTGATTGGAATGGGCA CCACTCCTGAAGTGCACTCAATATTCCTCGAAGGTCACACATTTCTT GTGAGGAACCATCGCCAGGCGTCCTTGGAAATCTCGCCAATAACTT TCCTTACTGCTCAAACACTCTTGATGGACCTTGGACAGTTTCTACTG TTTTGTCATATCTCTTCCCACCAACATGATGGCATGGAAGCTTATGTC AAAGTAGACAGCTGTCCAGAGGAACCCCAACTACGAATGAAAAATAA TGAAGAAGCGGAAGACTATGATGATGATCTTACTGATTCTGAAATGG ATGTGGTCAGGTTTGATGATGACAACTCTCCTTCCTTTATCCAAATTC GCTCAGTTGCCAAGAAGCATCCTAAAACTTGGGTACATTACATTGCT GCTGAAGAGGAGGACTGGGACTATGCTCCCTTAGTCCTCGCCCCCG ATGACAGAAGTTATAAAAGTCAATATTTGAACAATGGCCCTCAGCGG ATTGGTAGGAAGTACAAAAAAGTCCGATTTATGGCATACACAGATGA

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Name	Sequence
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	TGATAGTTTGCAGTTGTCAGTTTGTTTGCATGAGGTGGCATACTGGT
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Name	Sequence
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SEQ ID NO: 60	ATGAAGTGGGTAACCTTTATTTCCCTTCTTTTTCTCTTTAGCTCGGCT TATTCCAGGGGTGTGTTTCGTCGAGATGCACACAAGAGTGAGGTTG CTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCAAAGCCTTGGTG TTGATTGCCTTTGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCAT GTAAAATTAGTGAATGAAGTAACTGAATTTGCAAAAACATGTGTTGCT GATGAGTCAGCTGAAAATTGTGACAAATCACTTCATACCCTTTTTGG AGACAAATTATGCACAGTTGCAACTCTTCGTGAAACCTATGGTGAAA TGGCTGACTGCTGTGCAAAACAAGAACCTGAGAGAAATGAATGCTTC TTGCAACACAAAGATGACAACCCAAACCTCCCCCGATTGGTGAGAC CAGAGGTTGATGTGATGTGCACTGCTTTTCATGACAATGAAGAGACA TTTTTGAAAAAATACTTATATGAAATTGCCAGAAGACATCCTTACTTTT ATGCCCCGGAACTCCTTTTCTTTGCTAAAAGGTATAAAGCTGCTTTTA CAGAATGTTGCCAAGCTGCTGATAAAGCTGCCTGCCTGTTGCCAAA GCTCGATGAACTTCGGGATGAAGGGAAGGCTTCGTCTGCCAAACAG AGACTCAAGTGTGCCAGTCTCCAAAAATTTGGAGAAAGAGCTTTCAA AGCATGGGCAGTAGCTCGCCTGAGCCAGAGATTTCCCAAAGCTGAG TTTGCAGAAGTTTCCAAGTTAGTGACAGATCTTACCAAAGTCCACAC GGAATGCTGCCATGGAGATCTGCTTGAATGTGCTGATGACAGGGCG GACCTTGCCAAGTATATCTGTGAAAATCAAGATTCGATCTCCAGTAA ACTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAATCCCACTGCA TTGCCGAAGTGGAAAATGATGAGATGCCTGCTGACTTGCCTTCATTA GCTGCTGATTTTGTTGAAAGTAAGGATGTTTGCAAAAACTATGCTGA GGCAAAGGATGTCTTCCTGGGCATGTTTTTGTATGAATATGCAAGAA GGCATCCTGATTACTCTGTCGTGCTGCTGCTGAGACTTGCCAAGAC ATATGAAACCACTCTAGAGAAGTGCTGTGCCGCTGCAGATCCTCATG

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Name	Sequence
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SEQ ID NO: 62	ATGGTGGACGCTTTCCTGGGCACCTGGAAGCTAGTGGACAGCAAGA ATTTCGATGACTACATGAAGTCACTCGCTCATATACTCATAACCTTCC CCCTACCCTCAGGTGTGGGTTTTGCTACCAGGCAGGTGGCCAGCAT GACCAAGCCTACCACAATCATCGAAAAGAATGGGGACATTCTCACC CTAAAAACACACAGCACCTTCAAGAACACAGAGATCAGCTTTAAGTT GGGGGTGGAGTTCGATGAGACAACAGCAGATGACAGGAAGGTCAA GTCCATTGTGACACTGGATGGAGGGAAACTTGTTCACCTGCAGAAAT GGGACGGGCAAGAGACCACACTTGTGCGGGAGCTAATTGATGGAAA ACTCATCCTGACACTCACCCACGGCACTGCAGTTTGCACTCGCACTT ATGAGAAAGAGGCA
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[0294] Having described the invention in detail and by reference to specific aspects and/or embodiments thereof, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims. More specifically, although some aspects of the present invention may be identified herein as particularly advantageous, it is contemplated that the present invention is not limited to these particular aspects of the invention.

SEQUENCE LISTING

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<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: AAVS1 targeting vector constructed with AAVS1
hPGK-PuroR-pA donor forward primer 2

<400> SEQUENCE: 21

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<210> SEQ ID NO 22
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<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: AAVS1 targeting vector constructed with AAVS1
hPGK-PuroR-pA donor reverse primer 2

<400> SEQUENCE: 22

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<210> SEQ ID NO 23
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<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: AAVS1 targeting vector constructed with AAVS1
hPGK-PuroR-pA donor forward primer 3

<400> SEQUENCE: 23

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<210> SEQ ID NO 24
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<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
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hPGK-PuroR-pA donor reverse primer 3

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<400> SEQUENCE: 24

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<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Genotyping primer for CRISPR mediated knockin
#1

<400> SEQUENCE: 25

gagctgggac caccttatat tc 22

<210> SEQ ID NO 26
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Genotyping primer for CRISPR mediated knockin
#2

<400> SEQUENCE: 26

ggtgcatgac ccgcaag 17

<210> SEQ ID NO 27
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Genotyping primer for CRISPR mediated knockin
#3

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
<211> LENGTH: 2391
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: CFP/YFP FRET sensor with A213R/L238S double
mutant of GGBP

<400> SEQUENCE: 28

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ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc 180

ctcgtgacca ccctgacctg gggcgtgcag tgcttcagcc gctaccccga ccacatgaag 240

cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc 300

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gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac 420

aagctggagt acaactacat cagccacaac gtctatatca ccgccgacaa gcagaagaac 480

ggcatcaagg ccaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc 540

gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac 600

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tttatgtctg tagtgcgcaa ggctattgag caagatgcga aagccgcgcc agatgttcag    840

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gtgattgaga aagcgcgtgg gcaaaacgtg ccggtggttt tcttcaacaa agaaccgtct   1020

cgtaaggcgc tggatagcta cgacaaagcc tactacgttg gcactgactc caaagagtcc   1080

ggcattattc aaggcgattt gattgctaaa cactgggcgg cgaatcaggg ttgggatctg   1140

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ttacagttag ataccgcaat gtgggacacc gctcaggcga aagataagat ggacgcctgg   1320

ctgtctggcc cgaacgccaa caaaatcgaa gtggttatcg ccaacaacga tcggatggca   1380

atgggcgcgg ttgaagcgct gaaagcacac aacaagtcca gcattccggt gtttggcgtc   1440

gatgcgtcgc cagaagcgct ggcgctggtg aaatccggtg cactggcggg caccgtactg   1500

aacgatgcta acaaccaggc gaaagcgacc tttgatctgg cgaaaaacct ggccgatggt   1560

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aagctggagt acaactacaa cagccacaac gtctatatca tggccgacaa gcagaagaac   2160

ggcatcaagg tgaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc   2220

gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac   2280

tacctgagct accagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc   2340

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<210> SEQ ID NO 29
<211> LENGTH: 4146
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Cas9 D10A

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gatcgccaca gcataaagaa gaacctcatt ggcgccctcc tgttcgactc cggggagacg    180

gccgaagcca cgcggctcaa aagaacagca cggcgcagat atacccgcag aaagaatcgg    240
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aggaagaagc ttgtagacag tactgataag gctgacttgc ggttgatcta tctcgcgctg    480
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atgaaaaatt attggcggca gctgctgaac gccaaactga tcacacaacg gaagttcgat 2700

aatctgacta aggctgaacg aggtggcctg tctgagttgg ataaagccgg cttcatcaaa 2760

aggcagcttg ttgagacacg ccagatcacc aagcacgtgg cccaaattct cgattcacgc 2820

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aagtctaagc tggtctcaga tttcagaaag gactttcagt tttataaggt gagagagatc 2940

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gtgtga 4146

<210> SEQ ID NO 30
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Rosa26 gRNA1

<400> SEQUENCE: 30

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<210> SEQ ID NO 31
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Rosa26 gRNA2

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<400> SEQUENCE: 31

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<210> SEQ ID NO 32
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<223> OTHER INFORMATION: Rosa26 targeting vector
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<223> OTHER INFORMATION: n is a, c, g, or t
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<223> OTHER INFORMATION: n is a, c, g, or t
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<223> OTHER INFORMATION: n is a, c, g, or t
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<221> NAME/KEY: misc_feature
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<223> OTHER INFORMATION: n is a, c, g, or t
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<221> NAME/KEY: misc_feature
<222> LOCATION: (7562)..(7562)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 32

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tgaggtggac taatattaat gagtccctcc cctataaaag agctattaag gctttttgtc   7560
ttatactaac ttttttttta aatgtggtat ctttagaacc aagggtctta gagttttagt   7620
atacagaaac tgttgcatcg cttaatcaga ttttctagtt tcaaatccag agaatccaaa   7680
ttcttcacag ccaaagtcaa attaagaatt tctgacttta atgttatttg ctactgtgaa   7740
tataaaatga tagcttttcc tgaggcaggg tctcactatg tatctctgcc tgatctgcaa   7800
caagatatgt agactaaagt tctgcctgct tttgtctcct gaatactaag gttaaaatgt   7860
agtaatactt ttggaacttg caggtcagat tcttttatag gggacacact aagggagctt   7920
gggtgatagt tggtaaatgt gtttaagtga tgaaaacttg aattattatc accgcaacct   7980
actttttaaa aaaaaaagcc aggcctgtta gagcatgcta agggatccct aggacttgct   8040
gagcacacaa gagtagtact tggcaggctc ctggtgagag catatttcaa aaaacaaggc   8100
agacaaccaa gaaactacag taaggttacc tgtctttaac catctgcata tacacaggga   8160
tattaaaata ttccaaataa tatttcattc aagttttccc ccatcaaatt gggacatgga   8220
tttctccggt gaataggcag agttggaaac taaacaaatg ttggttttgt gatttgtgaa   8280
attgttttca agtgatagtt aaagcccatg agatacagaa caaagctgct atttcgaggt   8340
ctcttggtta tactcagaag cacttctttg ggtttccctg cactatcctg atcatgtgct   8400
aggcctncct taggctgatt gttgttcaaa taacttaagt ttcctgtcag gtgatgtcat   8460
```

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```
atgatttcat atatcaaggc aaaacatgtt atatatgtta aacatttgna cttaatgtga   8520

aagttaggtc tttgtgggtt ttgattttaa tttcaaaacc tgagctaaat aagtcatttt   8580

acatgtctta catttggtga attgtatatt gtggtttgca ggcaagactc tctgacctag   8640

taaccctcct atagagcact ttgctgggtc acaagtctag gagtcaagca tttcaccttg   8700

aagttgagac gttttgttag tgtatactag ttatatgttg gaggacatgt ttatccagaa   8760

gatattcagg actatttttg actgggctaa ggaattgatt ctgattagca ctgttagtga   8820

gcattgagtg gcctttaggc ttgaattgga gtcacttgta tatctcaaat aatgctggcc   8880

ttttttnaaa agcccttgtt ctttatcacc ctgttttcta cataattttt gttcaaagaa   8940

atacttgttt ggatctcctt ttgacaacaa tagcatgttt tcaagccata ttttttttcc   9000

tttttttttt tttttttggt ttttcgagac agggtttctc tgtatagccc tggctgtcct   9060

ggaactcact ttgtagacca ggctggcctc gaactcagaa atccgcctgc ctctgcctcc   9120

tgagtgccgg gattaaaggc gtgcaccacc acgcctggct aagttggata ttttgtatat   9180

aactataacc aatactaact ccactgggtg gatttttaat tcagtcagta gtcttaagtg   9240

gtctttattg gcccttatta aaatctactg ttcactctaa cagaggctgt tggactagtg   9300

gnactaagca acttcctacg gatatactag cagataaggg tcagggatag aaactagtct   9360

agcgttttgt atacctacca gcttatacta ccttgttctg atagaaatat ttaggacatc   9420

tagcttatcg atccg                                                    9435
```

<210> SEQ ID NO 34
<211> LENGTH: 747
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: IgGFc (mouse)

<400> SEQUENCE: 34

```
atgtacagga tgcaactcct gtcttgcatt gcactaagtc ttgcacttgt cacgaattcg     60

atatcggcca tggttagatc tggttgtaag ccttgcatat gtacagtccc agaagtatca    120

tctgtcttca tcttcccccc aaagcccaag gatgtgctca ccattactct gactcctaag    180

gtcacgtgtg ttgtggtaga catcagcaag gatgatcccg aggtccagtt cagctggttt    240

gtagatgatg tggaggtgca cacagctcag acgcaacccc gggaggagca gttcaacagc    300

actttccgct cagtcagtga acttcccatc atgcaccagg actggctcaa tggcaaggag    360

ttcaaatgca gggtcaacag tgcagctttc cctgccccca tcgagaaaac catctccaaa    420

accaaaggca gaccgaaggc tccacaggtg tacaccattc cacctcccaa ggagcagatg    480

gccaaggata aagtcagtct gacctgcatg ataacagact tcttccctga agacattact    540

gtggagtggc agtggaatgg gcagccagcg gagaactaca agaacactca gcccatcatg    600

gacacagatg gctcttactt cgtctacagc aagctcaatg tgcagaagag caactgggag    660

gcaggaaata ctttcacctg ctctgtgtta catgagggcc tgcacaacca ccatactgag    720

aagagcctct cccactctcc tggtaaa                                        747
```

<210> SEQ ID NO 35
<211> LENGTH: 195
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:

-continued

<223> OTHER INFORMATION: S2-VaTx3 with signal

<400> SEQUENCE: 35

atgcgccgcg gccgcctgct ggagatcgcc ctgggcttca ccgtgctgct ggcctcctac 60

acctcccacg gcgccgacgc cgctgaattc gagtgccgct ggtacctggg cggctgcaag 120

gaggactccg agtgctgcga gcacctgcag tgccactcct actgggagtg gtgcctgtgg 180

gacggctcct tctaa 195

<210> SEQ ID NO 36
<211> LENGTH: 324
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: S2-DkTx with signal

<400> SEQUENCE: 36

atgcgccgcg gccgcctgct ggagatcgcc ctgggcttca ccgtgctgct ggcctcctac 60

acctcccacg gcgccgacgc cgctgaattc ggatctgact gcgccaagga gggcgaggtg 120

tgctcctggg gcaagaagtg ctgcgacctg gacaacttct actgccccat ggagttcatc 180

ccccactgca agaagtacaa gccctacgtg cccgtgacca ccaactgcgc caaggagggc 240

gaggtgtgcg gctggggctc caagtgctgc cacggcctgg actgcccccct ggccttcatc 300

ccctactgcg agaagtaccg ctaa 324

<210> SEQ ID NO 37
<211> LENGTH: 873
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: VatX3 target vector cassette: signal->VaTx3->
Furin->IgGFc

<400> SEQUENCE: 37

atgtacagga tgcaactcct gtcttgcatt gcactaagtc ttgcacttgt cacgaattcg 60

ggatctgagt gccgctggta cctgggcggc tgcaaggagg actccgagtg ctgcgagcac 120

ctgcagtgcc actcctactg ggagtggtgc ctgtgggacg gctccttccg acggaagcga 180

gggatatcgg ccatggttag atctggttgt aagccttgca tatgtacagt cccagaagta 240

tcatctgtct tcatcttccc cccaaagccc aaggatgtgc tcaccattac tctgactcct 300

aaggtcacgt gtgttgtggt agacatcagc aaggatgatc ccgaggtcca gttcagctgg 360

tttgtagatg atgtggaggt gcacacagct cagacgcaac ccgggaggа gcagttcaac 420

agcactttcc gctcagtcag tgaacttccc atcatgcacc aggactggct caatggcaag 480

gagttcaaat gcagggtcaa cagtgcagct ttccctgccc ccatcgagaa aaccatctcc 540

aaaaccaaag gcagaccgaa ggctccacag gtgtacacca ttccacctcc caaggagcag 600

atggccaagg ataaagtcag tctgacctgc atgataacag acttcttccc tgaagacatt 660

actgtggagt ggcagtggaa tgggcagcca gcggagaact acaagaacac tcagcccatc 720

atggacacag atggctctta cttcgtctac agcaagctca atgtgcagaa gagcaactgg 780

gaggcaggaa atactttcac ctgctctgtg ttacatgagg gcctgcacaa ccaccatact 840

gagaagagcc tctcccactc tcctggtaaa taa 873

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<210> SEQ ID NO 38
<211> LENGTH: 993
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: DkTx target vector cassette: signal->DkTx->
Furin->IgGFc

<400> SEQUENCE: 38

atgtacagga tgcaactcct gtcttgcatt gcactaagtc ttgcacttgt cacgaattcg 60

ggatctgact gcgccaagga gggcgaggtg tgctcctggg gcaagaagtg ctgcgacctg 120

gacaacttct actgccccat ggagttcatc ccccactgca agaagtacaa gccctacgtg 180

cccgtgacca ccaactgcgc caaggagggc gaggtgtgcg gctggggctc caagtgctgc 240

cacggcctgg actgcccct ggccttcatc ccctactgcg agaagtaccg ccggaagcga 300

gggatatcgg ccatggttag atctggttgt aagccttgca tatgtacagt cccagaagta 360

tcatctgtct tcatcttccc cccaaagccc aaggatgtgc tcaccattac tctgactcct 420

aaggtcacgt gtgttgtggt agacatcagc aaggatgatc ccgaggtcca gttcagctgg 480

tttgtagatg atgtggaggt gcacacagct cagacgcaac ccgggagga gcagttcaac 540

agcactttcc gctcagtcag tgaacttccc atcatgcacc aggactggct caatggcaag 600

gagttcaaat gcagggtcaa cagtgcagct ttccctgccc ccatcgagaa aaccatctcc 660

aaaaccaaag gcagaccgaa ggctccacag gtgtacacca ttccacctcc caaggagcag 720

atggccaagg ataaagtcag tctgacctgc atgataacag acttcttccc tgaagacatt 780

actgtggagt ggcagtggaa tgggcagcca gcggagaact acaagaacac tcagcccatc 840

atggacacag atggctctta cttcgtctac agcaagctca atgtgcagaa gagcaactgg 900

gaggcaggaa atactttcac ctgctctgtg ttacatgagg gcctgcacaa ccaccatact 960

gagaagagcc tctcccactc tcctggtaaa taa 993

<210> SEQ ID NO 39
<211> LENGTH: 681
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: IgGFc (human)

<400> SEQUENCE: 39

gacaaaactc acacatgccc accgtgccca gcacctgaac tcctgggggg accgtcagtc 60

ttcctcttcc ccccaaaacc caaggacacc ctcatgatct cccggacccc tgaggtcaca 120

tgcgtggtgg tggacgtgag ccacgaagac cctgaggtca agttcaactg gtacgtggac 180

ggcgtggagg tgcataatgc caagacaaag ccgcgggagg agcagtacaa cagcacgtac 240

cgtgtggtca gcgtcctcac cgtcctgcac caggactggc tgaatggcaa ggagtacaag 300

tgcaaggtct ccaacaaagc cctcccagcc cccatcgaga aaaccatctc caaagccaaa 360

gggcagcccc gagaaccaca ggtgtacacc ctgcccccat cccgggagga gatgaccaag 420

aaccaggtca gcctgacctg cctggtcaaa ggcttctatc ccagcgacat cgccgtggag 480

tgggagagca atgggcagcc ggagaacaac tacaagacca cgcctcccgt gctggactcc 540

gacggctcct tcttcctcta cagcaagctc accgtggaca agagcaggtg gcagcagggg 600

aacgtcttct catgctccgt gatgcacgag gctctgcaca accactacac gcagaagagc 660

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ctctccctgt ctccgggtaa a 681

<210> SEQ ID NO 40
<211> LENGTH: 3778
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: AAVSI targeting vector

<400> SEQUENCE: 40

tgctttctct gaccagcatt ctctcccctg ggcctgtgcc gctttctgtc tgcagcttgt 60

ggcctgggtc acctctacgg ctggcccaga tccttccctg ccgcctcctt caggttccgt 120

cttcctccac tccctcttcc ccttgctctc tgctgtgttg ctgcccaagg atgctctttc 180

cggagcactt ccttctcggc gctgcaccac gtgatgtcct ctgagcggat cctccccgtg 240

tctgggtcct ctccgggcat ctctcctccc tcacccaacc ccatgccgtc ttcactcgct 300

gggttccctt ttccttctcc ttctggggcc tgtgccatct ctcgtttctt aggatggcct 360

tctccgacgg atgtctccct tgcgtcccgc ctccccttct tgtaggcctg catcatcacc 420

gtttttctgg acaaccccaa agtaccccgt ctccctggct ttagccacct ctccatcctc 480

ttgctttctt tgcctggaca ccccgttctc ctgtggattc gggtcacctc tcactccttt 540

catttgggca gctcccctac cccccttacc tctctagtct gtgctagctc ttccagcccc 600

ctgtcatggc atcttccagg ggtccgagag ctcagctagt cttcttcctc caacccgggc 660

ccctatgtcc acttcaggac agcatgtttg ctgcctccag ggatcctgtg tccccgagct 720

gggaccacct tatattccca gggccggtta atgtggctct ggttctgggt acttttatct 780

gtcccctcca ccccacagtg gggcaagctt ctcgagttgg ggttgcgcct tttccaaggc 840

agccctgggt ttgcgcaggg acgcggctgc tctgggcgtg gttccgggaa acgcagcggc 900

gccgaccctg ggtctcgcac attcttcacg tccgttcgca gcgtcacccg gatcttcgcc 960

gctacccttg tgggcccccc ggcgacgctt cctgctccgc ccctaagtcg ggaaggttcc 1020

ttgcggttcg cggcgtgccg gacgtgacaa acggaagccg cacgtctcac tagtaccctc 1080

gcagacggac agcgccaggg agcaatggca gcgcgccgac cgcgatgggc tgtggccaat 1140

agcggctgct cagcagggcg cgccgagagc agcggccggg aaggggcggt gcgggaggcg 1200

gggtgtgggg cggtagtgtg ggccctgttc ctgcccgcgc ggtgttccgc attctgcaag 1260

cctccggagc gcacgtcggc agtcggctcc ctcgttgacc gaatcaccga cctctctccc 1320

cagggtctag acgccaccat ggtgtccaag ggcgaggagg tgatcaagga gttcatgcgc 1380

ttcaaggtgc gcatggaggg ctccatgaac ggccacgagt tcgagatcga gggcgagggc 1440

gagggccgcc cctacgaggg cacccagacc gccaagctga aggtgaccaa gggcggcccc 1500

ctgcccttcg cctgggacat cctgtccccc cagttcatgt acggctccaa ggcctacgtg 1560

aagcaccccg ccgacatccc cgactacaag aagctgtcct tccccgaggg cttcaagtgg 1620

gagcgcgtga tgaacttcga ggacggcggc ctggtgaccg tgacccagga ctcctccctg 1680

caggacggca ccctgatcta caaggtgaag atgcgcggca ccaacttccc ccccgacggc 1740

cccgtgatgc agaagaagac catgggctgg gaggcctcca ccgagcgcct gtaccccccgc 1800

gacggcgtgc tgaagggcga gatccaccag gccctgaagc tgaaggacgg cggccactac 1860

ctggtggagt tcaagaccat ctacatggcc aagaagcccg tgcagctgcc cggctactac 1920

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tacgtggaca ccaagctgga catcacctcc cacaacgagg actacaccat cgtggagcag   1980
tacgagcgct ccgagggccg ccaccacctg ttcctgggat ccgagggcag aggaagcctt   2040
ctaacatgcg gtgacgtgga ggagaatccc ggcccttccg ggatgaccga gtacaagccc   2100
acggtgcgcc tcgccacccg cgacgacgtc cccagggccg tacgcaccct cgccgccgcg   2160
ttcgccgact accccgccac gcgccacacc gtcgatccgg accgccacat cgagcgggtc   2220
accgagctgc aagaactctt cctcacgcgc gtcgggctcg acatcggcaa ggtgtgggtc   2280
gcggacgacg gcgccgcggt ggcggtctgg accacgccgg agagcgtcga agcgggggcg   2340
gtgttcgccg agatcggccc gcgcatggcc gagttgagcg gttcccggct ggccgcgcag   2400
caacagatgg aaggcctcct ggcgccgcac cggcccaagg agcccgcgtg gttcctggcc   2460
accgtcggcg tctcgcccga ccaccagggc aagggtctgg gcagcgccgt cgtgctcccc   2520
ggagtggagg cggccgagcg cgccggggtg cccgccttcc tggagacctc cgcgccccgc   2580
aacctcccct tctacgagcg gctcggcttc accgtcaccg ccgacgtcga ggtgcccgaa   2640
ggaccgcgca cctggtgcat gacccgcaag cccggtgcct gaatctaggt cgacattcta   2700
cttggtaccc tgtgccttct agttgccagc catctgttgt ttgcccctcc cccgtgcctt   2760
ccttgaccct ggaaggtgcc actcccactg tcctttccta ataaaatgag gaaattgcat   2820
cgcattgtct gagtaggtgt cattctattc tggggggtgg ggtggggcag gacagcaagg   2880
gggaggattg ggaagacaat agcaggcatg ctggggatgc ggtgggctct atggaagctt   2940
tactagggac aggattggtg acagaaaagc cccatcctta ggcctcctcc ttcctagtct   3000
cctgatattg ggtctaaccc ccacctcctg ttaggcagat tccttatctg gtgacacacc   3060
cccatttcct ggagccatct ctctccttgc cagaacctct aaggtttgct tacgatggag   3120
ccagagagga tcctgggagg gagagcttgg caggggggtgg gagggaaggg ggggatgcgt   3180
gacctgcccg gttctcagtg gccaccctgc gctaccctct cccagaacct gagctgctct   3240
gacgcggctg tctggtgcgt ttcactgatc ctggtgctgc agcttcctta cacttcccaa   3300
gaggagaagc agtttggaaa aacaaaatca gaataagttg gtcctgagtt ctaactttgg   3360
ctcttcacct ttctagtccc caatttatat tgttcctccg tgcgtcagtt ttacctgtga   3420
gataaggcca gtagccagcc ccgtcctggc agggctgtgg tgaggagggg ggtgtccgtg   3480
tggaaaactc cctttgtgag aatggtgcgt cctaggtgtt caccaggtcg tggccgcctc   3540
tactcccttt ctctttctcc atccttcttt ccttaaagag tccccagtgc tatctgggac   3600
atattcctcc gcccagagca gggtcccgct tccctaaggc cctgctctgg gcttctgggt   3660
ttgagtcctt ggcaagccca ggagaggcgc tcaggcttcc ctgtcccccт tcctcgtcca   3720
ccatctcatg cccctggctc tcctgcccct tccctacagg ggttcctggc tctgctct     3778
```

<210> SEQ ID NO 41
<211> LENGTH: 602
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Human mutant BChE

<400> SEQUENCE: 41

```
Met His Ser Lys Val Thr Ile Ile Cys Ile Arg Phe Leu Phe Trp Phe
1               5                   10                  15

Leu Leu Leu Cys Met Leu Ile Gly Lys Ser His Thr Glu Asp Asp Ile
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            20                  25                  30

Ile Ile Ala Thr Lys Asn Gly Lys Val Arg Gly Met Asn Leu Thr Val
        35                  40                  45

Phe Gly Gly Thr Val Thr Ala Phe Leu Gly Ile Pro Tyr Ala Gln Pro
    50                  55                  60

Pro Leu Gly Arg Leu Arg Phe Lys Lys Pro Gln Ser Leu Thr Lys Trp
65                  70                  75                  80

Ser Asp Ile Trp Asn Ala Thr Lys Tyr Ala Asn Ser Cys Cys Gln Asn
                85                  90                  95

Ile Asp Gln Ser Phe Pro Gly Phe His Gly Ser Glu Met Trp Asn Pro
            100                 105                 110

Asn Thr Asp Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile Pro
        115                 120                 125

Ala Pro Lys Pro Lys Asn Ala Thr Val Leu Ile Trp Ile Tyr Gly Gly
    130                 135                 140

Gly Phe Gln Thr Gly Thr Ser Ser Leu His Val Tyr Asp Gly Lys Phe
145                 150                 155                 160

Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg Val
                165                 170                 175

Gly Ala Leu Gly Phe Leu Ala Leu Pro Gly Asn Pro Glu Ala Pro Gly
            180                 185                 190

Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln Lys
        195                 200                 205

Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Val Thr Leu Phe Gly
    210                 215                 220

Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Ser Pro Gly
225                 230                 235                 240

Ser His Ser Leu Phe Thr Arg Ala Ile Leu Gln Ser Gly Ser Ala Asn
                245                 250                 255

Ala Pro Trp Ala Val Thr Ser Leu Tyr Glu Ala Arg Asn Arg Thr Leu
            260                 265                 270

Asn Leu Ala Lys Leu Thr Gly Cys Ser Arg Glu Asn Glu Thr Glu Ile
        275                 280                 285

Ile Lys Cys Leu Arg Asn Lys Asp Pro Gln Glu Ile Leu Leu Asn Glu
    290                 295                 300

Ala Phe Val Val Pro Tyr Gly Thr Ala Leu Gly Val Asn Phe Gly Pro
305                 310                 315                 320

Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro Asp Ile Leu Leu Glu
                325                 330                 335

Leu Gly Gln Phe Lys Lys Thr Gln Ile Leu Val Gly Val Asn Lys Asp
            340                 345                 350

Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly Phe Ser Lys Asp
        355                 360                 365

Asn Asn Ser Ile Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Lys Ile
    370                 375                 380

Phe Phe Pro Gly Val Ser Glu Phe Gly Lys Glu Ser Ile Leu Phe His
385                 390                 395                 400

Tyr Thr Asp Trp Val Asp Asp Gln Arg Pro Glu Asn Tyr Arg Glu Ala
                405                 410                 415

Leu Gly Asp Val Val Gly Asp Tyr Asn Phe Ile Cys Pro Ala Leu Glu
            420                 425                 430
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```
Phe Thr Lys Lys Phe Ser Glu Trp Gly Asn Asn Ala Phe Phe Tyr Tyr
        435                 440                 445

Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly Val
    450                 455                 460

Met His Gly Tyr Glu Ile Glu Phe Val Phe Gly Leu Pro Leu Glu Arg
465                 470                 475                 480

Arg Asp Asn Tyr Thr Lys Ala Glu Glu Ile Leu Ser Arg Ser Ile Val
                485                 490                 495

Lys Arg Trp Ala Asn Phe Ala Lys Tyr Gly Asn Pro Asn Glu Thr Gln
            500                 505                 510

Asn Asn Ser Thr Ser Trp Pro Val Phe Lys Ser Thr Glu Gln Lys Tyr
        515                 520                 525

Leu Thr Leu Asn Thr Glu Ser Thr Arg Ile Met Thr Lys Leu Arg Ala
    530                 535                 540

Gln Gln Cys Arg Phe Trp Thr Ser Phe Phe Pro Lys Val Leu Glu Met
545                 550                 555                 560

Thr Gly Asn Ile Asp Glu Ala Glu Trp Glu Trp Lys Ala Gly Phe His
                565                 570                 575

Arg Trp Asn Asn Tyr Met Met Asp Trp Lys Asn Gln Phe Asn Asp Tyr
            580                 585                 590

Thr Ser Lys Lys Glu Ser Cys Val Gly Leu
        595                 600


<210> SEQ ID NO 42
<211> LENGTH: 1806
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Nucleic acid sequence encoding the amino acid
      sequence of SEQ ID NO: 41

<400> SEQUENCE: 42

atgcacagca aggtgaccat catctgcatc aggttcctgt tctggttcct gctgctgtgc     60

atgctgatcg gcaagagcca caccgaggac gacatcatca tcgccaccaa gaacggcaag    120

gtgaggggca tgaacctgac cgtgttcggc ggcaccgtga ccgccttcct gggcatcccc    180

tacgcccagc ccccccctggg caggctgagg ttcaagaagc cccagagcct gaccaagtgg    240

agcgacatct ggaacgccac caagtacgcc aacagctgct gccagaacat cgaccagagc    300

ttccccggct tccacggcag cgagatgtgg aaccccaaca ccgacctgag cgaggactgc    360

ctgtacctga acgtgtggat ccccgccccc aagcccaaga acgccaccgt gctgatctgg    420

atctacggcg gcggcttcca gaccggcacc agcagcctgc acgtgtacga cggcaagttc    480

ctggccaggg tggagagggt gatcgtggtg agcatgaact acagggtggg cgccctgggc    540

ttcctggccc tgcccggcaa ccccgaggcc cccggcaaca tgggcctgtt cgaccagcag    600

ctggccctgc agtgggtgca gaagaacatc gccgccttcg gcggcaaccc caagagcgtg    660

accctgttcg gcgagagcag cggcgccgcc agcgtgagcc tgcacctgct gagccccggc    720

agccacagcc tgttcaccag ggccatcctg cagagcggca gcgccaacgc cccctgggcc    780

gtgaccagcc tgtacgaggc caggaacagg accctgaacc tggccaagct gaccggctgc    840

agcagggaga acgagaccga gatcatcaag tgcctgagga acaaggaccc ccaggagatc    900

ctgctgaacg aggccttcgt ggtgccctac ggcaccgccc tgggcgtgaa cttcggcccc    960
```

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```
accgtggacg gcgacttcct gaccgacatg cccgacatcc tgctggagct gggccagttc   1020

aagaagaccc agatcctggt gggcgtgaac aaggacgagg gcacctggtt cctggtgggc   1080

ggcgcccccg gcttcagcaa ggacaacaac agcatcatca ccaggaagga gttccaggag   1140

ggcctgaaga tcttcttccc cggcgtgagc gagttcggca aggagagcat cctgttccac   1200

tacaccgact gggtggacga ccagaggccc gagaactaca gggaggccct gggcgacgtg   1260

gtgggcgact acaacttcat ctgccccgcc ctggagttca ccaagaagtt cagcgagtgg   1320

ggcaacaacg ccttcttcta ctacttcgag cacaggagca gcaagctgcc ctggcccgag   1380

tggatgggcg tgatgcacgg ctacgagatc gagttcgtgt tcggcctgcc cctggagagg   1440

agggacaact acaccaaggc cgaggagatc ctgagcagga gcatcgtgaa gaggtgggcc   1500

aacttcgcca agtacggcaa ccccaacgag acccagaaca acagcaccag ctggcccgtg   1560

ttcaagagca ccgagcagaa gtacctgacc ctgaacaccg agagcaccag gatcatgacc   1620

aagctgaggg cccagcagtg caggttctgg accagcttct tccccaaggt gctggagatg   1680

accggcaaca tcgacgaggc cgagtgggag tggaaggccg gcttccacag gtggaacaac   1740

tacatgatgg actggaagaa ccagttcaac gactacacca gcaagaagga gagctgcgtg   1800

ggcctg                                                              1806


<210> SEQ ID NO 43
<211> LENGTH: 603
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Mouse mutant BChE

<400> SEQUENCE: 43

Met Gln Thr Gln His Thr Lys Val Thr Gln Thr His Phe Leu Leu Trp
1               5                   10                  15

Ile Leu Leu Leu Cys Met Pro Phe Gly Lys Ser His Thr Glu Glu Asp
            20                  25                  30

Phe Ile Ile Thr Thr Lys Thr Gly Arg Val Arg Gly Leu Ser Met Pro
        35                  40                  45

Val Leu Gly Gly Thr Val Thr Ala Phe Leu Gly Ile Pro Tyr Ala Gln
    50                  55                  60

Pro Pro Leu Gly Ser Leu Arg Phe Lys Lys Pro Gln Pro Leu Asn Lys
65                  70                  75                  80

Trp Pro Asp Ile His Asn Ala Thr Gln Tyr Ala Asn Ser Cys Tyr Gln
                85                  90                  95

Asn Ile Asp Gln Ala Phe Pro Gly Phe Gln Gly Ser Glu Met Trp Asn
            100                 105                 110

Pro Asn Thr Asn Leu Ser Glu Asp Cys Leu Tyr Leu Asn Val Trp Ile
        115                 120                 125

Pro Val Pro Lys Pro Lys Asn Ala Thr Val Met Val Trp Ile Tyr Gly
    130                 135                 140

Gly Gly Phe Gln Thr Gly Thr Ser Ser Leu Pro Val Tyr Asp Gly Lys
145                 150                 155                 160

Phe Leu Ala Arg Val Glu Arg Val Ile Val Val Ser Met Asn Tyr Arg
                165                 170                 175

Val Gly Ala Leu Gly Phe Leu Ala Phe Pro Gly Asn Pro Asp Ala Pro
            180                 185                 190
```

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Gly Asn Met Gly Leu Phe Asp Gln Gln Leu Ala Leu Gln Trp Val Gln
        195                 200                 205

Arg Asn Ile Ala Ala Phe Gly Gly Asn Pro Lys Ser Ile Thr Ile Phe
    210                 215                 220

Gly Glu Ser Ser Gly Ala Ala Ser Val Ser Leu His Leu Leu Cys Pro
225                 230                 235                 240

Gln Ser Tyr Pro Leu Phe Thr Arg Ala Ile Leu Glu Ser Gly Ser Ala
                245                 250                 255

Asn Ala Pro Trp Ala Val Lys His Pro Glu Glu Ala Arg Asn Arg Thr
            260                 265                 270

Leu Thr Leu Ala Lys Phe Thr Gly Cys Ser Lys Glu Asn Glu Met Glu
        275                 280                 285

Met Ile Lys Cys Leu Arg Ser Lys Asp Pro Gln Glu Ile Leu Arg Asn
    290                 295                 300

Glu Arg Phe Val Leu Pro Ser Asp Ser Ala Leu Gly Ile Asn Phe Gly
305                 310                 315                 320

Pro Thr Val Asp Gly Asp Phe Leu Thr Asp Met Pro His Thr Leu Leu
                325                 330                 335

Gln Leu Gly Lys Val Lys Lys Ala Gln Ile Leu Val Gly Val Asn Lys
            340                 345                 350

Asp Glu Gly Thr Trp Phe Leu Val Gly Gly Ala Pro Gly Phe Ser Lys
        355                 360                 365

Asp Asn Asp Ser Leu Ile Thr Arg Lys Glu Phe Gln Glu Gly Leu Asn
    370                 375                 380

Met Tyr Phe Pro Gly Val Ser Arg Leu Gly Lys Glu Ala Val Leu Phe
385                 390                 395                 400

Tyr Tyr Val Asp Trp Leu Gly Glu Gln Ser Pro Glu Val Tyr Arg Asp
                405                 410                 415

Ala Leu Asp Asp Val Ile Gly Asp Tyr Asn Ile Ile Cys Pro Ala Leu
            420                 425                 430

Glu Phe Thr Lys Lys Phe Ala Glu Leu Glu Asn Asn Ala Phe Phe Tyr
        435                 440                 445

Phe Phe Glu His Arg Ser Ser Lys Leu Pro Trp Pro Glu Trp Met Gly
    450                 455                 460

Val Met His Gly Tyr Glu Ile Glu Phe Val Phe Gly Leu Pro Leu Gly
465                 470                 475                 480

Arg Arg Val Asn Tyr Thr Arg Ala Glu Glu Ile Phe Ser Arg Ser Ile
                485                 490                 495

Met Lys Thr Trp Ala Asn Phe Ala Lys Tyr Gly His Pro Asn Gly Thr
            500                 505                 510

Gln Gly Asn Ser Thr Met Trp Pro Val Phe Thr Ser Thr Glu Gln Lys
        515                 520                 525

Tyr Leu Thr Leu Asn Thr Glu Lys Ser Lys Ile Tyr Ser Lys Leu Arg
    530                 535                 540

Ala Pro Gln Cys Gln Phe Trp Arg Leu Phe Phe Pro Lys Val Leu Glu
545                 550                 555                 560

Met Thr Gly Asp Ile Asp Glu Thr Glu Gln Glu Trp Lys Ala Gly Phe
                565                 570                 575

His Arg Trp Ser Asn Tyr Met Met Asp Trp Gln Asn Gln Phe Asn Asp
            580                 585                 590
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```
Tyr Thr Ser Lys Lys Glu Ser Cys Thr Ala Leu
        595                 600


<210> SEQ ID NO 44
<211> LENGTH: 1809
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Nucleic acid sequence encoding the amino acid
      sequence of SEQ ID NO: 43

<400> SEQUENCE: 44

atgcagaccc agcacaccaa ggtgacccag acccacttcc tgctgtggat cctgctgctg     60

tgcatgccct tcggcaagag ccacaccgag gaggacttca tcatcaccac caagaccggc    120

agggtgaggg gcctgagcat gcccgtgctg ggcggcaccg tgaccgcctt cctgggcatc    180

ccctacgccc agcccccct gggcagcctg aggttcaaga agccccagcc cctgaacaag    240

tggcccgaca tccacaacgc cacccagtac gccaacagct gctaccagaa catcgaccag    300

gccttccccg gcttccaggg cagcgagatg tggaacccca acaccaacct gagcgaggac    360

tgcctgtacc tgaacgtgtg gatccccgtg cccaagccca agaacgccac cgtgatggtg    420

tggatctacg gcggcggctt ccagaccggc accagcagcc tgcccgtgta cgacggcaag    480

ttcctggcca gggtggagag ggtgatcgtg gtgagcatga actacagggt gggcgccctg    540

ggcttcctgg ccttccccgg caaccccgac gcccccggca acatgggcct gttcgaccag    600

cagctggccc tgcagtgggt gcagaggaac atcgccgcct tcggcggcaa ccccaagagc    660

atcaccatct tcggcgagag cagcggcgcc gccagcgtga gcctgcacct gctgtgcccc    720

cagagctacc ccctgttcac cagggccatc ctggagagcg gcagcgccaa cgccccctgg    780

gccgtgaagc accccgagga ggccaggaac aggaccctga ccctggccaa gttcaccggc    840

tgcagcaagg agaacgagat ggagatgatc aagtgcctga ggagcaagga cccccaggag    900

atcctgagga acgagaggtt cgtgctgccc agcgacagcg ccctgggcat caacttcggc    960

cccaccgtgg acggcgactt cctgaccgac atgccccaca ccctgctgca gctgggcaag   1020

gtgaagaagg cccagatcct ggtgggcgtg aacaaggacg agggcacctg gttcctggtg   1080

ggcggcgccc ccggcttcag caaggacaac gacagcctga tcaccaggaa ggagttccag   1140

gagggcctga acatgtactt ccccggcgtg agcaggctgg gcaaggaggc cgtgctgttc   1200

tactacgtgg actggctggg cgagcagagc cccgaggtgt acagggacgc cctggacgac   1260

gtgatcggcg actacaacat catctgcccc gccctggagt tcaccaagaa gttcgccgag   1320

ctggagaaca acgccttctt ctacttcttc gagcacagga gcagcaagct gccctggccc   1380

gagtggatgg gcgtgatgca cggctacgag atcgagttcg tgttcggcct gcccctgggc   1440

aggagggtga actacaccag ggccgaggag atcttcagca ggagcatcat gaagacctgg   1500

gccaacttcg ccaagtacgg ccaccccaac ggcacccagg gcaacagcac catgtggccc   1560

gtgttcacca gcaccgagca gaagtacctg accctgaaca ccgagaagag caagatctac   1620

agcaagctga gggcccccca gtgccagttc tggaggctgt tcttccccaa ggtgctggag   1680

atgaccggcg acatcgacga gaccgagcag gagtggaagg ccggcttcca caggtggagc   1740

aactacatga tggactggca gaaccagttc aacgactaca ccagcaagaa ggagagctgc   1800

accgccctg                                                           1809
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<210> SEQ ID NO 45
<211> LENGTH: 275
<212> TYPE: PRT
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: mGLP-1 (GLY8 mutant with IgG-Fc fusion)

<400> SEQUENCE: 45

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu
1               5                   10                  15

Val Thr Asn Ser His Gly Glu Gly Thr Phe Thr Ser Asp Val Ser Ser
            20                  25                  30

Tyr Leu Glu Gly Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys
        35                  40                  45

Gly Arg Gly Arg Ser Gly Cys Lys Pro Cys Ile Cys Thr Val Pro Glu
    50                  55                  60

Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Val Leu Thr
65                  70                  75                  80

Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val Asp Ile Ser Lys
                85                  90                  95

Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp Asp Val Glu Val
            100                 105                 110

His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe Asn Ser Thr Phe
        115                 120                 125

Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp Trp Leu Asn Gly
    130                 135                 140

Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe Pro Ala Pro Ile
145                 150                 155                 160

Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys Ala Pro Gln Val
                165                 170                 175

Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys Asp Lys Val Ser
            180                 185                 190

Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp Ile Thr Val Glu
        195                 200                 205

Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys Asn Thr Gln Pro
    210                 215                 220

Ile Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser Lys Leu Asn Val
225                 230                 235                 240

Gln Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr Cys Ser Val Leu
                245                 250                 255

His Glu Gly Leu His Asn His His Thr Glu Lys Ser Leu Ser His Ser
            260                 265                 270

Pro Gly Lys
        275


<210> SEQ ID NO 46
<211> LENGTH: 825
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Nucleic acid sequence encoding the amino acid
      sequence of SEQ ID NO: 45

<400> SEQUENCE: 46

atgtacagga tgcagctgct gagctgcatc gccctgagcc tggccctggt gaccaacagc     60
```

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```
cacggcgagg gcaccttcac cagcgacgtg agcagctacc tggagggcca ggccgccaag    120

gagttcatcg cctggctggt gaagggcagg ggcaggagcg gctgcaagcc ctgcatctgc    180

accgtgcccg aggtgagcag cgtgttcatc ttccccccca agcccaagga cgtgctgacc    240

atcaccctga cccccaaggt gacctgcgtg gtggtggaca tcagcaagga cgaccccgag    300

gtgcagttca gctggttcgt ggacgacgtg gaggtgcaca ccgcccagac ccagcccagg    360

gaggagcagt tcaacagcac cttcaggagc gtgagcgagc tgcccatcat gcaccaggac    420

tggctgaacg gcaaggagtt caagtgcagg gtgaacagcg ccgccttccc cgcccccatc    480

gagaagacca tcagcaagac caagggcagg cccaaggccc cccaggtgta caccatcccc    540

ccccccaagg agcagatggc caaggacaag gtgagcctga cctgcatgat caccgacttc    600

ttccccgagg acatcaccgt ggagtggcag tggaacggcc agcccgccga gaactacaag    660

aacacccagc ccatcatgga caccgacggc agctacttcg tgtacagcaa gctgaacgtg    720

cagaagagca actgggaggc cggcaacacc ttcacctgca gcgtgctgca cgagggcctg    780

cacaaccacc acaccgagaa gagcctgagc cacagccccg gcaag                    825
```

```
<210> SEQ ID NO 47
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Primer A

<400> SEQUENCE: 47

gctctagagc caccatgcag actcagcata ccaagg                              36


<210> SEQ ID NO 48
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Primer B

<400> SEQUENCE: 48

cgggatccac cggtttagag agctgtacaa gattctttct tg                       42


<210> SEQ ID NO 49
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Primer C

<400> SEQUENCE: 49

cccaagcttg ccaccatgca tagcaaagtc acaatc                              36


<210> SEQ ID NO 50
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Primer D

<400> SEQUENCE: 50

acgcgtcgac ttagagaccc acacaacttt ctttcttg                            38
```

-continued

<210> SEQ ID NO 51
<211> LENGTH: 1701
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: PAL

<400> SEQUENCE: 51

atgaagacgc tgtcacaggc ccagtccaag acctcctccc agcagttctc cttcaccggc 60

aactcctccg ccaacgtgat catcggcaac cagaagctga ccatcaacga cgtggcccgc 120

gtggcccgca acggcaccct ggtgtccctg accaacaaca ccgacatcct gcagggcatc 180

caggcctcct gcgactacat caacaacgcc gtggagtccg gcgagcccat ctacggcgtg 240

acctccggct tcggcggcat ggccaacgtg gccatctccc gcgagcaggc ctccgagctg 300

cagaccaacc tggtgtggtt cctgaagacc ggcgccggca acaagctgcc cctggccgac 360

gtgcgcgccg ccatgctgct gcgcgccaac tcccacatgc gcggcgcctc cggcatccgc 420

ctcgagctga tcaagcgcat ggagatcttc ctgaacgccg gcgtgacccc ctacgtgtac 480

gagttcggct ccatcggcgc ctccggcgac ctggtgcccc tgtcctacat caccggctcc 540

ctgatcggcc tggacccctc cttcaaggtg gacttcaacg gcaaggagat ggacgccccc 600

accgccctgc gccagctgaa cctgtccccc ctgaccctgc tgcccaagga gggcctggcc 660

atgatgaacg gcacctccgt gatgaccggc atcgccgcca actgcgtgta cgacacccag 720

atcctgaccg ccatcgccat gggtgtacac gctctggaca tccaggccct gaacggcacc 780

aaccagtcct tccacccctt catccacaac tccaagcccc accccggcca gctgtgggcc 840

gccgaccaga tgatctccct cctcgccaac tcccagctgg tgcgcgacga gctggacggc 900

aagcacgact accgcgacca cgagctgatc caggaccgct actccctgcg ctgcctgccc 960

cagtacctgg gccccatcgt ggacggcatc tcccagatcg ccaagcagat cgagatcgag 1020

atcaactccg tgaccgacaa ccccctgatc gacgtggaca accaggcctc ctaccacggc 1080

ggcaacttcc tgggccagta cgtgggcatg ggcatggacc acctgcgcta ctacatcggc 1140

ctgctggcca agcacctgga cgtgcagatc gccctgctgg cctcccccga gttctccaac 1200

ggcctgcccc cctccctgct gggcaaccgc gagcgcaagg tgaacatggg cctgaagggc 1260

ctgcagatct gcggtaactc gataatgccc ctgctgacct tctacggcaa ctccatcgcc 1320

gaccgcttcc ccacccacgc cgagcagttc aaccagaaca tcaactccca gggctacacc 1380

tccgccaccc tggcccgccg ctccgtggac atcttccaga actacgtggc catcgccctg 1440

atgttcggcg ttcaagctgt agacctgcgc acctacaaga agaccggcca ctacgacgcc 1500

cgcgcctccc tgtcccccgc caccgagcgc ctgtactccg ccgtgcgcca cgtggtgggc 1560

cagaagccca cctccgaccg cccctacatc tggaacgaca acgagcaggg cctggacgag 1620

cacatcgccc gcatctccgc cgacatcgca gcaggtggcg tgatcgtgca ggccgtgcag 1680

gacatcctgc cctccctgca c 1701

<210> SEQ ID NO 52
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: HSV-TK

<400> SEQUENCE: 52

-continued

```
atggcttcgt accccggcca tcagcacgcg tctgcgttcg accaggctgc gcgttctcgc     60
ggccatagca accgacgtac ggcgttgcgc cctcgccggc agcaagaagc cacggaagtc    120
cgcccggagc agaaaatgcc cacgctactg cgggtttata tagacggtcc ccacgggatg    180
gggaaaacca ccaccacgca actgctggtg gccctgggtt cgcgcgacga tatcgtctac    240
gtacccgagc cgatgactta ctggcgggtg ctgggggctt ccgagacaat cgcgaacatc    300
tacaccacac aacaccgcct tgaccagggt gagatatcgg ccggggacgc ggcggtggta    360
atgacaagcg cccagataac aatgggcatg ccttatgccg tgaccgacgc cgttctggct    420
cctcatatcg ggggggaggc tgggagctca catgccccgc ccccggccct caccctcatc    480
ttcgaccgcc atcccatcgc cgccctcctg tgctacccgg ccgcgcgata ccttatgggc    540
agcatgaccc cccaggccgt gctggcgttc gtggccctca tcccgccgac cttgcccggc    600
acaaacatcg tgttgggggc ccttccggag gacagacaca tcgaccgcct ggccaaacgc    660
cagcgccccg gcgagcggct tgacctggct atgctggccg cgattcgccg cgtttacggg    720
ctgcttgcca atacggtgcg gtatctgcag ggcggcgggt cgtggcggga ggattgggga    780
cagctttcgg ggacggccgt gccgccccag ggtgccgagc cccagagcaa cgcgggccca    840
cgaccccata tcggggacac gttatttacc ctgtttcggg cccccgagtt gctggccccc    900
aacggcgacc tgtacaacgt gtttgcctgg gccttggacg tcttggccaa acgcctccgt    960
cccatgcacg tctttatcct ggattacgac caatcgcccg ccggctgccg ggacgccctg   1020
ctgcaactta cctccgggat gatccagacc cacgtcacca ccccaggctc cataccgacg   1080
atctgcgacc tggcgcgcac gtttgcccgg gagatggggg aggctaac                1128
```

<210> SEQ ID NO 53
<211> LENGTH: 474
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: yCD

<400> SEQUENCE: 53

```
atggtgaccg gcggcatggc ctccaagtgg gaccagaagg gcatggacat cgcctacgag     60
gaggccgccc tgggctacaa ggagggcggc gtgcccatcg gcggctgcct gatcaacaac    120
aaggacggct ccgtgctggg ccgcggccac aacatgcgct tccagaaggg ctccgccacc    180
ctgcacggcg agatctccac cctggagaac tgcggccgcc tggagggcaa ggtgtacaag    240
gacaccaccc tgtacaccac cctgtccccc tgcgacatgt gcaccggcgc catcatcatg    300
tacggcatcc cccgctgcgt ggtgggcgag aacgtgaact tcaagtccaa gggcgagaag    360
tacctgcaga cccgcggcca cgaggtggtg gtggtggacg acgagcgctg caagaagatc    420
atgaagcagt tcatcgacga gcgcccccag gactggttcg aggacatcgg cgag          474
```

<210> SEQ ID NO 54
<211> LENGTH: 1356
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: PAH

<400> SEQUENCE: 54

```
atgtccactg cggtcctgga aaacccaggc ttgggcagga aactctctga ctttggacag     60
```

-continued

```
gaaacaagct atattgaaga caactgcaat caaaatggtg ccatatcact gatcttctca    120

ctcaaagaag aagttggtgc attggccaaa gtattgcgct tatttgagga gaatgatgta    180

aacctgaccc acattgaatc tagaccttct cgtttaaaga aagatgagta tgaatttttc    240

acccatttgg ataaacgtag cctgcctgct ctgacaaaca tcatcaagat cttgaggcat    300

gacattggtg ccactgtcca tgagctttca cgagataaga agaaagacac agtgccctgg    360

ttcccaagaa ccattcaaga gctggacaga tttgccaatc agattctcag ctatggagcg    420

gaactggatg ctgaccaccc tggttttaaa gatcctgtgt accgtgcaag acggaagcag    480

tttgctgaca ttgcctacaa ctaccgccat gggcagccca tccctcgagt ggaatacatg    540

gaggaagaaa agaaaacatg gggcacagtg ttcaagactc tgaagtcctt gtataaaacc    600

catgcttgct atgagtacaa tcacattttt ccacttcttg aaaagtactg tggcttccat    660

gaagataaca ttccccagct ggaagacgtt tctcaattcc tgcagacttg cactggtttc    720

cgcctccgac ctgtggctgg cctgctttcc tctcgggatt tcttgggtgg cctggccttc    780

cgagtcttcc actgcacaca gtacatcaga catggatcca agcccatgta tacccccgaa    840

cctgacatct gccatgagct gttgggacat gtgccccttgt tttcagatcg cagctttgcc    900

cagttttccc aggaaattgg ccttgcctct ctgggtgcac ctgatgaata cattgaaaag    960

ctcgccacaa tttactggtt tactgtggag tttgggctct gcaaacaagg agactccata   1020

aaggcatatg gtgctgggct cctgtcatcc tttggtgaat tacagtactg cttatcagag   1080

aagccaaagc ttctccccct ggagctggag aagacagcca tccaaaatta cactgtcacg   1140

gagttccagc ccctgtatta cgtggcagag agttttaatg atgccaagga gaaagtaagg   1200

aactttgctg ccacaatacc tcggcccttc tcagttcgct acgacccata cacccaaagg   1260

attgaggtct tggacaatac ccagcagctt aagattttgg ctgattccat taacagtgaa   1320

attggaatcc tttgcagtgc cctccagaaa ataaag                             1356
```

```
<210> SEQ ID NO 55
<211> LENGTH: 723
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: GTPCH

<400> SEQUENCE: 55

atggagaagc cgcggggagt caggtgcacc aatgggttct ccgagcgtga actacctcgt     60

cctggagcaa gcccacctgc agagaagtcc cgacctcctg aagcaaaggg cgcacagccg    120

gccgacgcct ggaaggcagg gcggcaccgc agcgaggagg aaaaccaggt gaacctcccc    180

aaactggcgg ccgcttactc gtccattctg ctctcgctgg gcgaggaccc ccagcggcag    240

gggctgctca agacgccctg gagggcggcc accgccatgc agtacttcac caagggatac    300

caggagacca tctcagatgt cctgaatgat gctatatttg atgaagatca tgacgagatg    360

gtgattgtga aggacataga tatgttctcc atgtgtgagc atcaccttgt tccatttgta    420

ggaagggtcc atattggcta tcttcctaac aagcaagtcc ttggtctcag taaacttgcc    480

aggattgtag aaatctacag tagacgacta caagttcaag agcgcctcac caaacagatt    540

gcggtggcca tcacagaagc cttgcagcct gctggcgttg gagtagtgat tgaagcgaca    600

cacatgtgca tggtaatgcg aggcgtgcag aaaatgaaca gcaagactgt cactagcacc    660
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atgctgggcg tgttccggga agaccccaag actcgggagg agttcctcac actaatcagg 720

agc 723

<210> SEQ ID NO 56
<211> LENGTH: 432
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: PTPS

<400> SEQUENCE: 56

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gcgagccacc ggctgcacag cccatctctg agcgatgaag agaacttaag agtgtttggg 120

aaatgcaaca atccgaatgg ccacgggcac aactataaag ttgtggtgac agtccatgga 180

gagattgatc ctgttacagg aatggttatg aatttgaccg acctcaaaga atacatggag 240

gaggccatca tgaagcctct tgatcacaag aacctggacc tggatgtgcc gtactttgcg 300

gatgctgtga gcacgacaga aaatgtagct gtctacatct gggaaagcct ccagaaactt 360

cttccagtgg gagctcttta taaagtaaaa gtgtttgaaa ccgacaacaa catcgtagtc 420

tataaaggag aa 432

<210> SEQ ID NO 57
<211> LENGTH: 783
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: SR (sepiapterin reductase)

<400> SEQUENCE: 57

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cggacgctgg ccccgctcct ggcctcgctg ctgtcgcccg gctccgtgct tgtccttagc 120

gcccgcaacg acgaggcact gcgccagctg gaggccgagc tgggcgccga gcggtctggc 180

ctgcgcgtgg tgcgggtgcc cgccgacctg ggcgccgagg ccggcttgca gcagctgctc 240

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aacaactact gggcactgaa cttgacctcc atgctctgcc tgacttccag cgtcctgaag 420

gccttcccgg acagtcctgg cctcaacaga accgtggtta acatctcgtc cctctgtgcc 480

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ttccaggtcc tggcgctgga ggaacctaat gtgagggtgc tgaactatgc cccaggtcct 600

ctggacacag acatgcagca gttggcccgg gagacctccg tggacccaga catgcgaaaa 660

gggctgcagg agctgaaggc aaaggggaag ctggtggatt gcaaggtgtc agcccagaaa 720

ctgctgagct tactggaaaa ggacgagttc aagtctggag cccacgtgga cttctatgac 780

aaa 783

<210> SEQ ID NO 58
<211> LENGTH: 4413
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Factor VIII minus B

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<400> SEQUENCE: 58

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accagaagat actacctggg tgcagtggaa ctgtcatggg actatatgca aagtgatctc 120
ggtgagctgc ctgtggacgc aagatttcct cctagagtgc caaaatcttt tccattcaac 180
acctcagtcg tgtacaaaaa gactctgttt gtagaattca cggatcacct tttcaacatc 240
gctaagccaa ggccaccctg gatgggtctg ctaggtccta ccatccaggc tgaggtttat 300
gatacagtgg tcattacact taagaacatg gcttcccatc ctgtcagtct tcatgctgtt 360
ggtgtatcct actggaaagc ttctgaggga gctgaatatg atgatcagac cagtcaaagg 420
gagaaagaag atgataaagt cttccctggt ggaagccata catatgtctg gcaggtcctg 480
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atggtctatg aagacacact caccctattc ccattctcag gagaaactgt cttcatgtcg 2100
atggaaaacc caggtctatg gattctgggg tgccacaact cagactttcg gaacagaggc 2160
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gcagtggaga ggctctggga ttatgggatg agtagctccc cacatgttct aagaaacagg   2580
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acctggtctc cttcaaaagc tcgacttcac ctccaaggga ggagtaatgc ctggagacct   4080
caggtgaata atccaaaaga gtggctgcaa gtggacttcc agaagacaat gaaagtcaca   4140
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atctccagca gtcaagatgg ccatcagtgg actctctttt ttcagaatgg caaagtaaag   4260
gtttttcagg gaaatcaaga ctccttcaca cctgtggtga actctctaga cccaccgtta   4320
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<210> SEQ ID NO 59
<211> LENGTH: 7053
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Factor VIII

<400> SEQUENCE: 59

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ggtgagctgc ctgtggacgc aagatttcct cctagagtgc caaaatcttt tccattcaac     180

acctcagtcg tgtacaaaaa gactctgttt gtagaattca cggatcacct tttcaacatc     240

gctaagccaa ggccaccctg gatgggtctg ctaggtccta ccatccaggc tgaggtttat     300

gatacagtgg tcattacact taagaacatg gcttcccatc ctgtcagtct tcatgctgtt     360

ggtgtatcct actggaaagc ttctgaggga gctgaatatg atgatcagac cagtcaaagg     420

gagaaagaag atgataaagt cttccctggt ggaagccata catatgtctg gcaggtcctg     480

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tttgatgaag ggaaaagttg gcactcagaa acaaagaact ccttgatgca ggatagggat     720

gctgcatctg ctcgggcctg gcctaaaatg cacacagtca atggttatgt aaacaggtct     780

ctgccaggtc tgattggatg ccacaggaaa tcagtctatt ggcatgtgat tggaatgggc     840

accactcctg aagtgcactc aatattcctc gaaggtcaca catttcttgt gaggaaccat     900

cgccaggcgt ccttggaaat ctcgccaata actttcctta ctgctcaaac actcttgatg     960

gaccttggac agtttctact gttttgtcat atctcttccc accaacatga tggcatggaa    1020

gcttatgtca aagtagacag ctgtccagag gaaccccaac tacgaatgaa aaataatgaa    1080

gaagcggaag actatgatga tgatcttact gattctgaaa tggatgtggt caggtttgat    1140

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tgggtacatt acattgctgc tgaagaggag gactgggact atgctccctt agtcctcgcc    1260

cccgatgaca gaagttataa aagtcaatat ttgaacaatg gccctcagcg gattggtagg    1320

aagtacaaaa aagtccgatt tatggcatac acagatgaaa cctttaagac tcgtgaagct    1380

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ttgattatat ttaagaatca agcaagcaga ccatataaca tctaccctca cggaatcact    1500

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agaggaaacc agataatgtc agacaagagg aatgtcatcc tgttttctgt atttgatgag    1800

aaccgaagct ggtacctcac agagaatata caacgctttc tccccaatcc agctggagtg    1860

cagcttgagg atccagagtt ccaagcctcc aacatcatgc acagcatcaa tggctatgtt    1920

tttgatagtt tgcagttgtc agtttgtttg catgaggtgg catactggta cattctaagc    1980

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caggtgaata atccaaaaga gtggctgcaa gtggacttcc agaagacaat gaaagtcaca 6780

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atctccagca gtcaagatgg ccatcagtgg actctctttt ttcagaatgg caaagtaaag 6900

gtttttcagg gaaatcaaga ctccttcaca cctgtggtga actctctaga cccaccgtta 6960

ctgactcgct accttcgaat tcacccccag agttgggtgc accagattgc cctgaggatg 7020

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gaagatcatg taaaattagt gaatgaagta actgaatttg caaaaacatg tgttgctgat 240

gagtcagctg aaaattgtga caaatcactt catacccttt ttggagacaa attatgcaca 300

gttgcaactc ttcgtgaaac ctatggtgaa atggctgact gctgtgcaaa acaagaacct 360

gagagaaatg aatgcttctt gcaacacaaa gatgacaacc caaacctccc ccgattggtg 420

agaccagagg ttgatgtgat gtgcactgct tttcatgaca atgaagagac atttttgaaa 480

aaatacttat atgaaattgc cagaagacat ccttactttt atgccccgga actccttttc 540

tttgctaaaa ggtataaagc tgcttttaca gaatgttgcc aagctgctga taaagctgcc 600

tgcctgttgc caaagctcga tgaacttcgg gatgaaggga aggcttcgtc tgccaaacag 660

agactcaagt gtgccagtct ccaaaaattt ggagaaagag ctttcaaagc atgggcagta 720

gctcgcctga gccagagatt tcccaaagct gagtttgcag aagtttccaa gttagtgaca 780

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tacgttccca aagagtttaa tgctgaaaca ttcaccttcc atgcagatat atgcacactt   1620

tctgagaagg agagacaaat caagaaacaa actgcacttg ttgagctcgt gaaacacaag   1680

cccaaggcaa caaaagagca actgaaagct gttatggatg atttcgcagc ttttgtagag   1740

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```

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aaaattctga atcggccaaa gaggtataat tcaggtaaat tggaagagtt tgttcaaggg    180

aaccttgaga gagaatgtat ggaagaaaag tgtagttttg aagaagcacg agaagttttt    240

gaaaacactg aaagaacaac tgaattttgg aagcagtatg ttgatggaga tcagtgtgag    300

tccaatccat gtttaaatgg cggcagttgc aaggatgaca ttaattccta tgaatgttgg    360

tgtccctttg gatttgaagg aaagaactgt gaattagatg taacatgtaa cattaagaat    420

ggcagatgcg agcagttttg taaaaatagt gctgataaca aggtggtttg ctcctgtact    480

gagggatatc gacttgcaga aaaccagaag tcctgtgaac cagcagtgcc atttccatgt    540

ggaagagttt ctgtttcaca aacttctaag ctcacccgtg ctgagactgt ttttcctgat    600

gtggactatg taaattctac tgaagctgaa accattttgg ataacatcac tcaaagcacc    660

caatcattta atgacttcac tcgggttgtt ggtggagaag atgccaaacc aggtcaattc    720

ccttggcagg ttgttttgaa tggtaaagtt gatgcattct gtggaggctc tatcgttaat    780

gaaaaatgga ttgtaactgc tgcccactgt gttgaaactg gtgttaaaat tacagttgtc    840

gcaggtgaac ataatattga ggagacagaa catacagagc aaaagcgaaa tgtgattcga    900

attattcctc accacaacta caatgcagct attaataagt acaaccatga cattgccctt    960

ctggaactgg acgaaccctt agtgctaaac agctacgtta cacctatttg cattgctgac   1020

aaggaataca cgaacatctt cctcaaattt ggatctggct atgtaagtgg ctggggaaga   1080

gtcttccaca aagggagatc agctttagtt cttcagtacc ttagagttcc acttgttgac   1140

cgagccacat gtcttcgatc tacaaagttc accatctata acaacatgtt ctgtgctggc   1200

ttccatgaag gaggtagaga ttcatgtcaa ggagatagtg gggaccccca tgttactgaa   1260

gtggaaggga ccagtttctt aactggaatt attagctggg gtgaagagtg tgcaatgaaa   1320

ggcaaatatg gaatatatac caaggtatcc cggtatgtca actggattaa ggaaaaaaca   1380

aagctcact                                                           1389
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<210> SEQ ID NO 62
<211> LENGTH: 432
<212> TYPE: DNA
<213> ORGANISM: Unknown
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<220> FEATURE:
<223> OTHER INFORMATION: H-FABP

<400> SEQUENCE: 62

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atgaagtcac tcgctcatat actcataacc ttcccctac cctcaggtgt gggttttgct 120

accaggcagg tggccagcat gaccaagcct accacaatca tcgaaaagaa tggggacatt 180

ctcaccctaa aaacacacag caccttcaag aacacagaga tcagctttaa gttgggggtg 240

gagttcgatg agacaacagc agatgacagg aaggtcaagt ccattgtgac actggatgga 300

gggaaacttg ttcacctgca gaaatgggac gggcaagaga ccacacttgt gcgggagcta 360

attgatggaa aactcatcct gacactcacc cacggcactg cagtttgcac tcgcacttat 420

gagaaagagg ca 432

<210> SEQ ID NO 63
<211> LENGTH: 462
<212> TYPE: DNA
<213> ORGANISM: Unknown
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<223> OTHER INFORMATION: Myocardial myoglobin

<400> SEQUENCE: 63

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gacatcccag gccatgggca ggaagtcctc atcaggctct ttaagggtca cccagagact 120

ctggagaagt ttgacaagtt caagcacctg aagtcagagg acgagatgaa ggcgtctgag 180

gacttaaaga agcatggtgc caccgtgctc accgccctgg gtggcatcct taagaagaag 240

gggcatcatg aggcagagat taagcccctg gcacagtcgc atgccaccaa gcacaagatc 300

cccgtgaagt acctggagtt catctcggaa tgcatcatcc aggttctgca gagcaagcat 360

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aaggacatgg cctccaacta caaggagctg ggcttccagg gc 462

<210> SEQ ID NO 64
<211> LENGTH: 1293
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: GFAP

<400> SEQUENCE: 64

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ggcttcaagg agacccgggc cagtgagcgg gcagagatga tggagctcaa tgaccgcttt 240

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ctgaaccagc tgcgggccaa ggagcccacc aagctggcag acgtctacca ggctgagctg 360

cgagagctgc ggctgcggct cgatcaactc accgccaaca gcgcccggct ggaggttgag 420

agggacaatc tggcacagga cctggccact gtgaggcaga agctccagga tgaaaccaac 480

ctgaggctgg aagccgagaa caacctggct gcctatagac aggaagcaga tgaagccacc 540

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cagtatgagg caatggcgtc cagcaacatg catgaagccg aagagtggta ccgctccaag     780

tttgcagacc tgacagacgc tgctgcccgc aacgcggagc tgctccgcca ggccaagcac     840

gaagccaacg actaccggcg ccagttgcag tccttgacct gcgacctgga gtctctgcgc     900

ggcacgaacg agtccctgga gaggcagatg cgcgagcagg aggagcggca cgtgcgggag     960

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cacaaggtca caagatatct caaaagcctc acaatacgag ttataccaat acaggctcac    1260

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<212> TYPE: DNA
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<223> OTHER INFORMATION: S100B

<400> SEQUENCE: 65

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ctttcccatt tcttagagga aatcaaagag caggaggttg tggacaaagt catggaaaca     180

ctggacaatg atggagacgg cgaatgtgac ttccaggaat tcatggcctt tgttgccatg     240

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```

<210> SEQ ID NO 66
<211> LENGTH: 1069
<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: PYY (Homo sapiens)

<400> SEQUENCE: 66

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cttcagactt tctaagaatg actcaggtca ttggtggagt gaagtcaaga tttccaactc     240

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cttgggcgcg cagcacacca caagtatctg ttactgtgtt ttgcagaagc ttcaggcggg     420

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gcagcccttg ggacttccct cgccttccac ctcctgctcg tctgcttcac aagctatcgc     540

tatggtgttc gtgcgcaggc cgtggcccgc cttgaccaca gtgcttctgg ccctgctcgt     600

ctgcctaggg gcgctggtcg acgcctaccc catcaaaccc gaggctcccg gcgaagacgc     660
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ctccgcccgc gtctcggtgc cccgcccccc tgggctggag ggctgtgtgt ggtccttccc    1020

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<211> LENGTH: 3670
<212> TYPE: DNA
<213> ORGANISM: Unknown
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<400> SEQUENCE: 67

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acatttatgg caaccctatc aagaggatcc agtatgagat caagcagata aagatgttca     540

aagggcctga gaaggatata gagtttatct acacggcccc ctcctcggca gtgtgtgggg     600

tctcgctgga cgttggagga aagaaggaat atctcattgc aggaaaggcc gaggggggacg    660

gcaagatgca catcaccctc tgtgacttca tcgtgccctg ggacaccctg agcaccaccc     720

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cctgtgcgtg gtaccgcggc gcggcgcccc ccaagcagga gtttctcgac atcgaggacc     960

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aaaaaaaaaa                                                          3670
```

1. A physiologically-tailored tissue organoid, comprising:

a plurality of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent,

wherein when the therapeutic agent is administered to an individual in need thereof, the therapeutic agent improves the individual's health.

2. The physiologically-tailored tissue organoid of claim **1**, wherein the tissue organoid comprises a stratified skin graft grown from cells taken from an individual.

3. The physiologically-tailored tissue organoid of claim **1**, wherein the tissue organoid comprises a cultured skin graft grown from embryonic stem cells, human induced pluripotent stem cells, epidermal stem cells, or keratinocytes.

4-9. (canceled)

10. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent comprises an enzyme, a protein, a clotting factor, a vitamin, a peptide, a lipid, a toxin, or a combination thereof.

11. The physiologically-tailored tissue organoid of claim **10**, wherein expression of the therapeutic agent is inducible by an inducer.

12-28. (canceled)

29. A method of treating an individual in need thereof for a disease, disorder, or addiction, comprising:

contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agents.

30. The method of claim **29**, wherein the tissue organoid is biointegrated into the individual by grafting or surgical implantation.

31. The method of claim **29**, wherein the disease or disorder is PKU or hemophilia, and

wherein the addiction is one or more of cocaine addiction, alcoholism, nicotine addiction, or amphetamine addiction.

32-49. (canceled)

50. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent comprises phenylalanine ammonia lyase (PAL).

51. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent improves the individual's health by treating phenylketonuria (PKU).

52. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent comprises Factor VIII or Factor IX.

53. The physiologically-tailored tissue organoid of claim **52**, wherein the therapeutic agent is conjugated with albumin.

54. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent improves the individual's health by treating hemophilia.

55. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent comprises hBChE.

56. The physiologically-tailored tissue organoid of claim **55**, wherein the therapeutic agent improves the individual's health by treating cocaine addiction.

57. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent comprises one or more of DkTx, VaTx, glucagon-like peptide 1 (GLP-1), a GLP-1 analog, a modified GLP-1 (mGLP1), and a mGLP1 analog.

58. The physiologically-tailored tissue organoid of claim **57**, wherein the therapeutic agent is inducible by alcohol consumption by the individual.

59. The physiologically-tailored tissue organoid of claim **58**, wherein the therapeutic agent improves the individual's health by treating alcoholism.

60. The physiologically-tailored tissue organoid of claim **1**, wherein the therapeutic agent improves the individual's health by treating nicotine or amphetamine addiction.

61. A method of treating an individual in need thereof for a disease, disorder, or addiction, comprising:

contacting a tissue organoid to the individual, the tissue organoid comprising a population of genetically engineered cells comprising at least one recombinant gene encoding a therapeutic agent, wherein expression of the therapeutic agent is inducible by an inducer; and administering an inducer to the individual to induce expression of the therapeutic agent.

* * * * *