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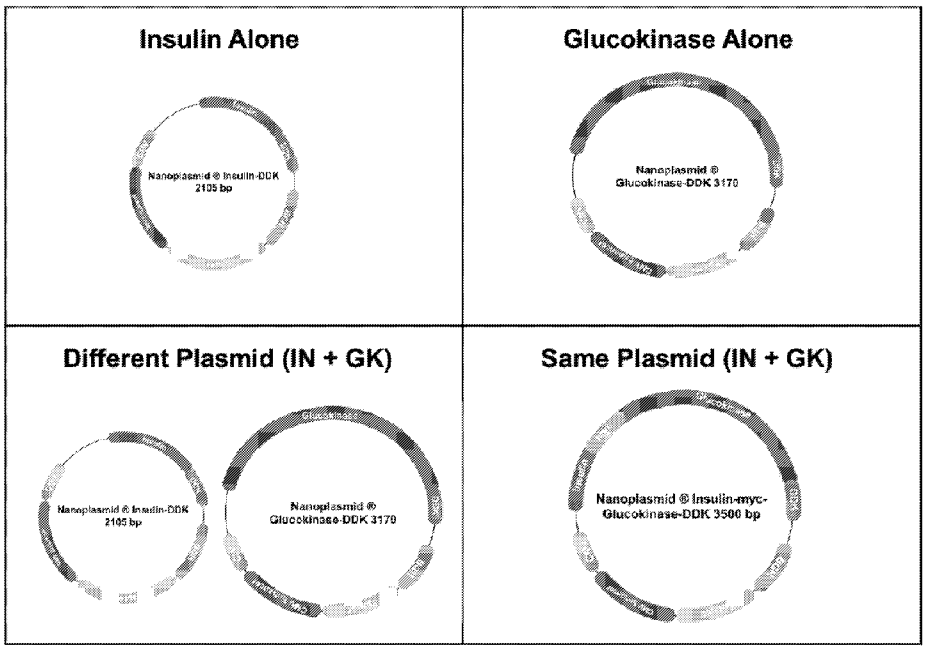

FIG. 1

(57) Abstract: Methods for type 1 diabetes (T1D) regulation include gene electrotransfer (GET) for the delivery of nucleic acid encoding at least one of insulin and glucokinase are provided herein. Avoiding the need for immunomodulation as required for standard gene therapy approaches, the application of electrical pulses enables plasmid uptake for the production of insulin and glucokinase in target cells. GET plasmid delivery provides long-term T1D treatment by regulating glucose levels.

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LOCALIZED IN VIVO ELECTRO-GENE THERAPY FOR TYPE 1 DIABETES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Application No. 63/506,631, filed June 7, 2023 and U.S. Provisional Application No. 63/559,928, filed March 1, 2024, the content of each which is incorporated by reference in its entirety.

BACKGROUND

[0002] Type 1 Diabetes (T1D) affects nearly 1.6 million Americans, with 64,000 newly diagnosed each year, at a $800B lifetime cost to the healthcare system. Patients with T1D require insulin injections to survive, but this treatment does not regulate glycemia in all cases. Chronic hyperglycemia can cause diabetes-associated microvascular, macrovascular, and neurologic complications. Thus, precise regulation of glucose homeostasis is a major challenge in diabetes management. Islet and stem cell transplantations have been explored in the clinic, but access to human islets and the necessary immunosuppressive therapy present limitations to this treatment type.

[0003] Genetic engineering represents an attractive approach for managing hyperglycemia via the introduction of insulin or glucokinase transcripts into cells. Traditional approaches for insulin and/or glucokinase gene therapy involve adeno-associated viral (AAV) vector delivery and transfection. However, a major drawback to AAV vector-mediated delivery is the need for immunosuppressive drugs to avoid triggering an unwanted immune response.

[0004] Thus, there is a need to develop therapies for the regulation of blood glucose that obviate expensive, burdensome, ineffective, or adverse aspects of existing T1D treatments.

SUMMARY

[0005] The present disclosure provides systems and methods that overcome the aforementioned drawbacks associated with T1D gene therapy by co-delivering genetic transcripts for insulin and glucokinase into cells using the non-viral, non-integrating, localized *in vivo* electro gene therapy (liveGT) approach.

[0006] In an aspect, a method for treating type 1 diabetes in a subject is provided, the method comprising: (a) administering to the subject at least one of: a therapeutically effective amount of a nucleic acid encoding insulin and a therapeutically effective amount of a nucleic acid encoding glucokinase at an administration site; and (b) applying an electrical pulse to the

administration site. The method may comprise administering both the nucleic acid encoding insulin and the nucleic acid encoding glucokinase.

[0007] The administration site may be in skeletal muscle.

[0008] The electrical pulse may be applied using a monopolar electrode.

[0009] The electrical pulse may be a monophasic pulse. The electrical pulse may be between about 1 V and about 1.5 kV. The electrical pulse may be about 90V. The electrical pulse may be applied for between about 50 μs and about 200 ms. The electrical pulse may be applied for about 150 ms.

The electrical pulse is applied between 1 and 100 times.

[0010] The nucleic acid encoding insulin and the nucleic acid encoding glucokinase may each be provided on a plasmid. In embodiments, the nucleic acid encoding insulin and the nucleic acid encoding glucokinase are provided on separate plasmids. In embodiments, the nucleic acid encoding insulin and the nucleic acid encoding glucokinase are both provided on the same plasmid.

[0011] Step (a) may be performed by intramuscular injection.

[0012] The method may further comprise (c) repeating steps (a) and (b) at least about every six months. Step (c) may be performed at between about every six months and about every twelve months.

[0013] These aspects are nonlimiting. Other aspects and features of the systems and methods described herein will be provided below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIG. 1 illustrates plasmids encoding insulin and glucokinase.

[0015] FIGS. 2A-2B show glucose consumption in B16F10 melanoma cells transfected with insulin and glucokinase over time (FIG. 2A), and on day 2 (FIG. 2B).

[0016] FIGS. 3A-3B show glucose consumption in C2C12 myoblasts transfected with insulin and glucokinase over time (FIG. 3A), and on day 2 (FIG. 3B).

[0017] FIGS. 4A-4F delivery of plasmid DNA encoding insulin (FIG. 4A), glucokinase (FIG. 4B), insulin and glucokinase on separate plasmids (FIG. 4C), insulin and glucokinase on the same plasmid (FIG. 4D), and no DNA control (FIG. 4E). FIG. 4F shows the transfection efficiency calculated from five fields of view.

[0018] FIG. 5 is a plot of a monophasic pulse parameter, according to aspects of the present disclosure.

[0019] FIGS. 6A-6B illustrate the proposed mechanism of gene electrotransfer (GET) of

pDNA encoding insulin and glucokinase. FIG. 6A is a schematic of the GET transfection process, according to aspects of the present disclosure. FIG. 6B is a schematic of insulin and glucokinase synthesis and resulting downstream processes, according to aspects of the present disclosure.

[0020] FIGS 7A-7F. GET significantly enhances plasmid DNA delivery. Immunofluorescence images show expression of human insulin (FIG. 7A), human glucokinase (FIG. 7B), insulin and glucokinase on the same plasmid (FIG. 7C), and insulin and glucokinase on separate plasmids (FIG. 7D). FIG. 7E shows transfection efficiency calculated from three fields of view. FIG. 7F shows co-localization of insulin and glucokinase for the two co-delivery conditions.

[0021] FIGS. 8A-8B. Monophasic GET delivery of insulin and glucokinase significantly increases cellular glucose consumption. Glucose consumption over six days in cells treated with GET compared to glucose metabolism in controls over time (FIG. 8A), and on day 3 (FIG. 8B).

[0022] FIGS 9A-9I. GET significantly enhances plasmid DNA delivery. The fluorescence images show protein expression in the same plasmid group in both high (FIG. 9A) and low (FIG. 9B) glucose conditions and different plasmid groups for in high glucose media (FIG. 9C) and low glucose media (FIG. 9D), when compared to high (FIG. 9E) and low (FIG. 9F) glucose controls. Protein expression was measured using immunofluorescence microscopy (FIG. 9G). Insulin expression was significantly enhanced in media as measured via ELISA (FIGS. 9H-9I).

[0023] FIGS. 10A-10C. Glucokinase acts as a glucose sensor preventing hypoglycemia. FIG. 10A shows glucose consumption is shown in high and low glucose conditions for cells exposed to GET with the same plasmid and different plasmid schemes. The change in glucose consumption in high and low glucose conditions for cells exposed to GET with the same plasmid scheme (FIG. 10B) and different plasmid scheme (FIG. 10C) is shown.

[0024] FIG. 11 is a plot of monophasic pulse parameters, according to aspects of the present disclosure.

[0025] FIGS. 12A-12F. Monophasic electrotransfer enhances gene delivery. Luciferase expression shown in applied electric field of 1300 V/cm (FIG. 12A) and 600 V/cm (FIG. 12B), lipofectamine (FIG. 12C), and control (FIG. 12D) treatment groups in C2C12 cells. FIG. 12E shows luciferase expression over five days. Transfection efficiency is shown in FIG. 12F.

[0026] FIGS. 13A-13H. Monophasic electrotransfer enhances delivery of plasmid DNA encoding insulin (FIG. 13A), glucokinase (FIG. 13B), insulin and glucokinase on separate plasmids (FIG. 13C), insulin and glucokinase on the same plasmid (FIG. 13D), and no DNA

control (FIG. 13E). Transfection efficiency was calculated from five fields of view (FIG. 13F). Media glucose depletion in C2C12 cells over three days is shown in FIG. 13G, and on day 3 in FIG. 13H.

[0027] FIG. 14. Gene expression (luciferase) after electrotransfer in skeletal muscle over 6 months.

[0028] FIGS. 15A-15B. Human insulin and glucokinase co-delivery via *liveGT* is safe. FIG. 15A is a Kaplan Meier Survival plot of animals subjected to *liveGT*. FIG. 15B is a plot of weight over time of animals subject to *liveGT*.

[0029] FIGS. 16A-16B. *LiveGT* mediated human insulin and glucokinase co-delivery significantly reduced serum glucose over 21 days. FIG. 16A shows exogenous human insulin levels in serum. FIG. 16B shows serum glucose levels.

DETAILED DESCRIPTION

[0030] This disclosure provides an innovative, non-viral, non-integrating, liveGT approach to deliver insulin- and glucokinase-encoding DNA as a treatment for T1D.

[0031] In an aspect, provided herein is a method for treating type 1 diabetes in a subject, the method comprising: (a) administering to the subject at least one of: a therapeutically effective amount of a nucleic acid encoding insulin and a therapeutically effective amount of a nucleic acid encoding glucokinase at an administration site; and (b) applying an electrical pulse to the administration site.

[0032] Type 1 diabetes is a chronic autoimmune disease in which the pancreas is unable to produce normal levels of insulin. As used herein, the terms “treating” and “to treat” mean to alleviate symptoms, eliminate the causation of resultant symptoms either on a temporary or permanent basis, and/or to prevent or slow the appearance or to reverse the progression or severity of resultant symptoms of the named disease or disorder. The terms “treating” and "to treat" further include the reduction in one or more symptom associated with type 1 diabetes, for example excessive hunger or thirst, frequent urination, unexplained weight loss, fatigue, blurred vision, slow healing of cuts and sores, vaginal yeast infections.

[0033] Insulin is a peptide hormone produced by beta cells of the pancreas that regulates the metabolism of carbohydrates, fats, and protein by promoting the absorption of glucose from the blood into cells of the liver, fat, and skeletal muscles. Glucose production and secretion by the liver are strongly inhibited by high concentrations of insulin in the blood. Decreased or absent insulin activity results in diabetes. The insulin nucleic acid administered in the methods described herein may encode human insulin or any other form of insulin having the desired

effect. The nucleic acid may encode long-lasting insulin.

[0034] Glucokinase is an enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate. It plays an important role in the regulation of carbohydrate metabolism by acting as a glucose sensor, triggering shifts in metabolism or cell function in response to rising or falling levels of glucose.

[0035] FIGS. 6A-6B illustrate the nucleic acid (pDNA) uptake and resulting glucose regulation in a cell exposed to the GET methods described herein. The electrical pulse induces cell membrane permeabilization, allowing the nucleic acid to enter the cell (FIG. 6A). Once in the cell, the nucleic acid is transported into the nucleus.

[0036] Referring to FIG. 6B, the nucleic acid is transcribed, then translated into the insulin and glucokinase proteins. The insulin is secreted by the cell and binds to extracellular insulin receptors on neighboring cells, initiating phosphoinositide 3-kinase (PI3K) signaling to activate and translocate glucose transporter type 4 (GLUT-4) to the cell membrane. GLUT-4 is an insulin-regulated glucose transporter that facilitates the diffusion of circulating glucose down its concentration gradient into cells. Once inside the cell, glucose binds to glucokinase to produce glucose-6-phosphate for participation in further metabolic pathways.

[0037] As used herein, the term "administering" an agent, such as a therapeutic entity like a nucleic acid encoding insulin or a nucleic acid encoding glucokinase, to a subject or cell, is intended to refer to dispensing, delivering or applying the substance to the intended target by any suitable route for delivery, including delivery by either the parenteral/oral route, intramuscular injection, subcutaneous/intradermal injection, intravenous injection, retro-orbital injection, intrathecal administration, buccal administration, transdermal delivery, topical administration, and administration by the intranasal or respiratory tract route. The nucleic acids may be administered to any tissue or cell type that expresses GLUT4, such as skeletal muscle, adipose connective tissue (fat cells), and liver tissue (hepatocytes). In exemplary embodiments, the nucleic acids are administered by intramuscular injection into the skeletal muscle.

[0038] The terms “nucleic acid”, “nucleic acid sequence”, “polynucleotide”, and “polynucleotide sequence” refer to a polymer of nucleotides, an oligonucleotide, a polynucleotide (which terms may be used interchangeably), or any fragment thereof. A polynucleotide may refer to a polydeoxyribonucleotide (containing 2-deoxy-D-ribose), a polyribonucleotide (containing D-ribose), and to any other type of polynucleotide that is an N glycoside of a purine or pyrimidine base. There is no intended distinction in length between the terms “nucleic acid”, “oligonucleotide” and “polynucleotide”, and these terms will be used

interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. For use in the present compositions and methods, an oligonucleotide also can comprise nucleotide analogs in which the base, sugar, or phosphate backbone is modified as well as non-purine or non-pyrimidine nucleotide analogs. These phrases also refer to DNA or RNA of genomic, natural, or synthetic origin (which may be single-stranded or double-stranded and may represent the sense or the antisense strand).

[0039] A "therapeutic polynucleotide" as used herein refers to DNA sequence encoding a polypeptide or an RNA that induces a positive therapeutic effect when expressed. A therapeutic polynucleotide may comprise several operably linked fragments, such as a promoter, a 5' leader sequence, a coding sequence and a 3' non-translated sequence, such as sequence encoding a polyadenylation site. "Expression" of a polynucleotide refers to the process wherein a gene is transcribed into an RNA and/or translated into a protein.

[0040] The nucleic acids described herein may be provided in a construct. The terms "construct" "nucleic acid construct" and "expression construct" are used herein to refer to a recombinant polynucleotide, i.e., a polynucleotide that was formed artificially by combining at least two polynucleotide components from different sources (natural or synthetic). For example, the constructs described herein comprise the coding region of a transgene of interest (a "therapeutic polynucleotide") operably linked to a promoter that (1) is associated with another gene found within the same genome, (2) is from the genome of a different species, or (3) is synthetic. Constructs can be generated using conventional recombinant DNA methods. A "transgene" refers to a gene that has been introduced into a host cell. The transgene may comprise sequences that are native to the cell, sequences that do not occur naturally in the cell, or combinations thereof. A transgene may contain sequences coding for one or more proteins that may be operably linked to appropriate regulatory sequences for expression of the coding sequences in the cell.

[0041] A "promoter" or "transcription regulatory sequence" refers to a nucleic acid fragment that functions to control the transcription of one or more coding sequences, such as a therapeutic polynucleotide sequence, and is typically located upstream with respect to the direction of transcription of the coding sequence. A promoter is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from

the promoter, including e.g. attenuators or enhancers, but also silencers. A "constitutive" promoter is a promoter that is active under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer.

[0042] The construct may be part of a vector. A “vector” is a nucleic acid molecule capable of transporting another nucleic acid to which it is linked. The four major types of vectors are plasmids, viral vectors, cosmids, and artificial chromosomes. Certain vectors are capable of autonomous replication in a host cell into which they are introduced. Other vectors can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome (e.g., lentiviral vectors). Moreover, certain vectors are capable of directing the expression of exogenous genes to which they are operatively linked. Suitable vectors are known in the art and contain the necessary elements in order for the gene encoded within the vector to be expressed as a protein in the host cell. The term "plasmid", and also “minicircle DNA” as well as “nanoplasmid” refers to a circular double stranded DNA loop into which additional DNA segments may be ligated, specifically exogenous DNA segments encoding the mutant α-gal protein. The term “viral vector” is used to describe a virus particle that is used to deliver genetic material (e.g., the constructs of the present invention) into cells, wherein additional DNA segments may be ligated into the viral genome. Viral vectors include replication defective retroviruses (including lentiviruses), adenoviruses, and adeno-associated viruses (AAV)), which serve equivalent functions. In exemplary embodiments, the construct is provided on a plasmid. In embodiments, the nucleic acid encoding insulin and the nucleic acid encoding glucokinase are each provided on a plasmid. Insulin and glucokinase may be encoded on separate plasmids. Both insulin and glucokinase be encoded on the same plasmid.

[0043] The nucleic acids encoding insulin and glucokinase may be formulated in one or two pharmaceutical compositions. As used herein, the term “pharmaceutical composition” refers to a chemical or biological composition suitable for administration to a mammal. Such compositions typically include the active agent and a pharmaceutically acceptable carrier. As used herein the term “pharmaceutically acceptable carrier” includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions. Examples of compositions appropriate for such therapeutic applications include preparations for intramuscular administration, such as sterile suspensions and emulsions. In some cases, pharmaceutical

compositions appropriate for therapeutic applications may be in admixture with one or more pharmaceutically acceptable excipients, diluents, or carriers such as sterile water, physiological saline, glucose or the like.

[0044] As used herein, the terms "protein" or "polypeptide" or "peptide" may be used interchangeable to refer to a polymer of amino acids. Typically, a "polypeptide" or "protein" is defined as a longer polymer of amino acids, of a length typically of greater than 50, 60, 70, 80, 90, or 100 amino acids. A "peptide" is defined as a short polymer of amino acids, of a length typically of 50, 40, 30, 20 or less amino acids. A protein typically comprises a polymer of naturally or non-naturally occurring amino acids (*e.g.*, alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine).

[0045] A "subject" or "subject in need thereof" refers to a subject in need of treatment for a disease or disorder. The disease or disorder may be type 1 diabetes or a disorder associated with type 1 diabetes. The term "subject" may be used interchangeably with the terms "individual" and "patient" and includes human and non-human mammalian subjects. In preferred embodiments, the subject is a human.

[0046] In preferred embodiments, the nucleic acid encoding insulin and the nucleic acid encoding glucokinase are administered to an administration site in skeletal muscle, after which the electrical pulse is applied to or near the administration site such that the electrical field produced by the electrode covers the entire volume of the administration site. An intramuscular injection refers to an injection of the nucleic acids into the muscle. However, the electrical pulse may be applied externally (e.g. to the skin) at or near the administration site.

[0047] As used herein the terms "therapeutically effective amount" and "effective amount" refer to the amount or dose of the nucleic acids encoding insulin and glucokinase that provides the desired effect. In some embodiments, the effective amount is the amount or dose of the agent, upon single or multiple dose administration to the subject, which provides the desired effect in the subject under diagnosis or treatment.

[0048] The step of applying an electrical pulse to the administration site refers to the application of an electric field resulting in induction of a transmembrane voltage across the plasma membrane of cells at the site of application. The transmembrane voltage must be sufficiently strong to render the cell membrane transiently permeable, allowing the entry of the injected nucleic acids encoding insulin and glucokinase into the cells.

[0049] The electrical pulse may be applied using one or more electrodes. The electrode may be a monopolar electrode. The electrode may be a platinum electrode. In exemplary

embodiments, the electrode is a 10 mm^2 platinum monopolar electrode. Other electrodes may be used, e.g. needle electrodes, bipolar electrodes, calipers, or multi-electrode arrays.

[0050] In an embodiment, a monophasic electrical pulse of between about 1 V and about 1.5 kV is applied. The monophasic electrical pulse may be about 90V. The monophasic electrical pulse may be applied for between about 50 μs and about 200 ms. For example, the monophasic electrical pulse may be applied for about 150 ms. The monophasic electrical pulse may be applied between 1 and 100 times.

[0051] The method may be performed more than one time. For example, the method may be performed on a regimen of once every about six months or about every twelve months, or at any interval in between. Each time the method is performed, the administration site may be different. For example, the second and subsequent administrations may be done at a site that is not covered by the electrical field applied at the first administration.

[0052] As used in this specification and the claims, the singular forms "a," "an," and "the" include plural forms unless the context clearly dictates otherwise.

[0053] As used herein, "about", "approximately," "substantially," and "significantly" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, "about" and "approximately" will mean up to plus or minus 10% of the particular term and "substantially" and "significantly" will mean more than plus or minus 10% of the particular term.

[0054] As used herein, the terms "include" and "including" have the same meaning as the terms "comprise" and "comprising." The terms "comprise" and "comprising" should be interpreted as being "open" transitional terms that permit the inclusion of additional components further to those components recited in the claims. The terms "consist" and "consisting of" should be interpreted as being "closed" transitional terms that do not permit the inclusion of additional components other than the components recited in the claims. The term "consisting essentially of" should be interpreted to be partially closed and allowing the inclusion only of additional components that do not fundamentally alter the nature of the claimed subject matter.

[0055] The phrase "such as" should be interpreted as "for example, including." Moreover, the use of any and all exemplary language, including but not limited to "such as", is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed.

[0056] Furthermore, in those instances where a convention analogous to "at least one of A, B and C, etc." is used, in general such a construction is intended in the sense of one having

ordinary skill in the art would understand the convention (*e.g.*, "a system having at least one of A, B and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description or figures, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

[0057] All language such as "up to," "at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can subsequently be broken down into ranges and subranges. A range includes each individual member. Thus, for example, a group having 1-3 members refers to groups having 1, 2, or 3 members. Similarly, a group having 6 members refers to groups having 1, 2, 3, 4, or 6 members, and so forth.

[0058] The modal verb "may" refers to the preferred use or selection of one or more options or choices among the several described embodiments or features contained within the same. Where no options or choices are disclosed regarding a particular embodiment or feature contained in the same, the modal verb "may" refers to an affirmative act regarding how to make or use an aspect of a described embodiment or feature contained in the same, or a definitive decision to use a specific skill regarding a described embodiment or feature contained in the same. In this latter context, the modal verb "may" has the same meaning and connotation as the auxiliary verb "can."

[0059] The following examples illustrate uses of the method described herein, and are not intended to be limiting.

[0060] EXAMPLES

[0061] Example 1. Exogenous Insulin and Glucokinase Expression Enhance Glucose Depletion.

[0062] Lipofectamine transfection of plasmid DNA encoding for insulin, glucokinase was performed to observe their effects on glucose depletion.

[0063] Methods

[0064] Cells were plated at a density of 2000 cells/well and settled for 24 hours with mitomycin C (causes DNA crosslinking, not allowing DNA replication). Cells were lipofectamine transfected with Nanoplasmid® DNA encoding for a) insulin, b) glucokinase, c) insulin and glucokinase on different plasmids, or d) insulin and glucokinase on the same plasmid. The plasmids are shown at FIG. 1. Glucose was measured using GlucCell™ (mg/dL). Media was

replaced after measurement and taken every day for three days. Immunofluorescence was performed to observe transfection efficiency (One-way ANOVA and Tukey multiple comparison test).

[0065] Results

[0066] As shown in FIGS. 2A-2B, co-expression of insulin and glucokinase led to significantly higher media glucose depletion over 3 days in B16F10 melanoma cells (FIG. 2A), with peak expression on day 2 (FIG. 2B). Similar results were observed in C2C12 myoblast cells (FIGS. 3A-3B). FIGS. 4A-4F show the efficiency of insulin and glucokinase lipofectamine transfection.

[0067] Discussion

[0068] Exogenous co-expression of insulin and glucokinase led to significantly enhanced

[0069] glucose depletion compared to controls. Glucokinase or insulin delivery alone, also enhanced glucose depletion, however to a lower degree. The most significant decrease between all test groups appeared on day two as both insulin and glucokinase delivery alone and co-expression was significantly different than media glucose levels of untreated cells. Cells expressing both insulin and glucokinase delivered on the same plasmid had the lowest transfection rate, likely due to the increased size of the plasmid and minimized endocytosis. These observations are consistent with our hypothesis that glucose transporter 4 expressing cells can be reprogramed to modulate glucose in an insulin dependent manner, leading to an immunomodulatory drug-free, potential treatment for T1D.

[0070] Example 2. Gene Electrotransfer Increases Mammalian Cell Glucose Consumption in an Insulin-Dependent Manner.

[0071] Methods

[0072] Experimental groups. *In vitro* experiments utilized four experimental groups of C2C12 myoblasts treated with different Nanoplasmid® plasmids (5 μg). The groups include (1) Insulin only (IN), (2) Glucokinase only (GK), (3) IN and GK in the same plasmid, (4) IN and GK different plasmids as shown in FIG. 1.

[0073] Gene Electrotransfer. Prior to Gene Electrotransfer (GET) C2C12 myoblast replication was inactivated utilizing Mitomycin C (10 μg/mL). GET parameters were based on previously optimized experiments utilizing reporter gene expression, and shown in FIG. 5. Specifically, 5 μg of the plasmid DNA was added to the cells, and the cells were subject to six monophasic pulses lasting 100 μs at 130 V with 250 ms intervals between each pulse.

[0074] The cells were then incubated with High Glucose Media (450 mg/dL) or Low Glucose Media (100 mg/dL). Media glucose levels were measured using a GlucCell™ system.

[0075] Immunofluorescence (IF) Microscopy Imaging and Analysis. Immunofluorescence microscopy was performed on the cells following day 7. Human Insulin ELISA was performed to quantify insulin expression. Transfection efficiency and percent colocalization were calculated. Groups were compared with an ordinary two-way ANOVA, and Tukey's multiple comparisons test, with $p < 0.05$ considered significant. The proposed mechanism of plasmid DNA delivery is illustrated in FIGS. 6A and 6B.

[0076] Results

[0077] As shown in FIGS. 7A-7F, GET significantly enhanced plasmid DNA delivery in each treatment group as shown by glucokinase and insulin immunofluorescence. The transfection efficiency calculated from three fields of view was significantly higher over a control group in which plasmid DNA (pDNA) encoding insulin and glucokinase were provided with no pulses (FIG. 7E). High co-localization of insulin and glucokinase was observed for both co-delivery conditions (FIG. 7F).

[0078] Monophasic GET delivery of insulin and glucokinase significantly increased cellular glucose consumption as seen in FIGS. 8A-8B. FIG. 8A shows the glucose consumption over six days compared to glucose metabolism in controls. The highest levels of media glucose depletion are seen on day three (FIG. 8B).

[0079] FIGS. 9A-9I further show that GET significantly enhanced plasmid DNA delivery. The fluorescence images show increased protein expression in the treatment group in which the insulin and glucokinase were provided on the same plasmid in high glucose media (FIG. 9A) and low glucose media (FIG. 9B), and in the treatment group in which the insulin and glucokinase were provided on different plasmids in high glucose media (FIG. 9C) and low glucose media (FIG. 9D), when compared to a control group (C2C12 cells not exposed to plasmid or electrotransfer) in high glucose media (FIG. 9E) and low glucose media (FIG. 9F). Insulin and glucokinase expression were increased in all treatment groups in both high and low glucose media (FIG. 9G). Media insulin levels for cells maintained in high glucose and low glucose are shown in FIGS. 9H and 9I, respectively.

[0080] As shown in FIGS. 10A-10C, transfer of glucokinase prevented hypoglycemia glucose levels in cells.

[0081] Discussion

[0082] Exogenous co-expression of insulin and glucokinase led to significantly enhanced glucose depletion compared to controls. Glucokinase or insulin delivery alone also enhanced glucose depletion, however to a lower degree. The most significant decrease between all test groups appeared on day three and four as both same plasmid and different plasmid co-

expression of insulin and glucokinase was significantly different than media glucose levels of untreated cells. There were no measured differences between protein expression in all groups. When stimulating a fasting state, glucokinase modulated glucose consumption ensuring the media glucose levels stayed above hypoglycemic conditions. These observations are consistent with our hypothesis that glucose transporter expressing cells can be reprogramed to modulate glucose in an insulin dependent manner, leading to an immunomodulatory drug-free, potential treatment for T1D.

[0083] Example 3. Gene Electrotransfer Mediated Insulin and Glucokinase Delivery Modulates Glucose

[0084] Methods

[0085] For monophasic electrotransfer delivery of plasmid DNA, cells were plated at 12,000 cells/well with 10 μg plasmid DNA (with luciferin reporter). The electrotransfer parameters tested were 1300 V/cm and 600 V/cm. MIRUS Lipofectamine transfection was used as a positive control. Cells with DNA and no applied voltage were used as a negative control. Luciferin luminescence was measured over five days when treated with luciferase. Immunofluorescence was measured (One-way ANOVA and Tukey multiple comparison test).

[0086] For assessing glucose regulation, cells were plated at a density of 12,000 cells/well with 10 μg plasmid DNA. 1300 V/cm was applied. The plasmid groups tested were: insulin, glucokinase, insulin and glucokinase (different plasmids), insulin and glucokinase (same plasmid) (FIG. 1). Cells with DNA and no applied voltage were used as a negative control. Media glucose levels (GlucCell) were measured each day for three consecutive days. Immunofluorescence was measured (One-way ANOVA and Tukey multiple comparison test).

[0087] For electrotransfer delivery of plasmid DNA, cells were plated at 12,000 cells/well with 10 μg plasmid DNA (with luciferin reporter). A monophasic wave (1300 V/cm) was used. Cells with DNA and no applied voltage were used as a negative control. Luciferin luminescence was measured over five days when treated with luciferase. Immunofluorescence was measured (One-way ANOVA and Tukey multiple comparison test). The applied pulse protocols are shown at FIG. 11.

[0088] Results

[0089] As shown in FIG. 12, traditional monophasic pulse protocols enhanced gene delivery. Applied electric fields of 1300 V/cm and 600 V/cm yielded comparable delivery and expression as lipofectamine in C2C12 cells. (FIGS. 12A-12D). Both GET and lipofectamine enhanced luciferase expression over five days (FIG. 12E) and transfection efficiency (FIG. 12F).

[0090] As shown in FIGS. 13A-13D, insulin and glucokinase co-expression modulated extracellular glucose. Co-expression of insulin and glucokinase led to significantly higher media glucose depletion over three days in C2C12 cells (FIG. 13G), with peak expression on day 3 (FIG. 13H).

[0091] Discussion

[0092] The results show that traditional monophasic pulse parameters significantly enhance luciferase, insulin, and glucokinase gene delivery in C2C12 cells. Extracellular glucose levels were significantly reduced with exogenous co-delivery of insulin and glucose encoding plasmid DNA with traditional monophasic pulse protocol.

[0093] Traditional monophasic pulsing parameters utilized were effective for effector gene delivery experiments, as both 1300 V/cm and 600 V/cm significantly increased expression. Exogenous co-expression of both insulin and glucokinase significantly reduced media glucose levels compared to respective controls, indicating that GET delivery of insulin and glucokinase is a viable therapeutic pathway. C2C12 cells that express glucose transporter-4 modulate glucose in an insulin dependent manner. These observations are consistent with our hypothesis that skeletal muscle cells can be reprogrammed to modulate blood glucose levels, resulting in a potential treatment for type 1 diabetes without the use of immunomodulatory drugs.

[0094] Example 4. Skeletal Muscle *liveGT* Enhances Therapeutic Expression Levels over Several Months

[0095] Methods

[0096] *In vivo* gene electrotransfer procedure. Non-diabetic Sprague Dawley rats were anesthetized with isoflurane inhalation. Both flanks were carefully shaved to remove as much hair as possible to allow for direct electrode contact with the skin. The animals were placed on their side, and the return plate electrode was placed under the opposite flank with ultrasound gel applied between the skin and grounding plate to ensure contact. A 50 µl intradermal injection of 2 mg/ml plasmid DNA encoding firefly luciferase, or pDNA encoding both human insulin and glucokinase (FIG. 1) was administered. Immediately after injection, a 10 mm^2 monopolar electrode was used to apply monophasic pulses.

[0097] Bioluminescence imaging. Bioluminescence imaging was performed on days 1, 2, 7, 14, 21, 28, 91, and 182 after injection. After isoflurane inhalation anesthesia induction, animals received 150 mg/kg subcutaneous injections of D-luciferin (Gold Biotechnology, Inc., St. Louis, MO) at treatment sites. An In Vivo Imaging System (PerkinElmer, Akron OH) was used to capture and quantitate bioluminescence signal. Peak flux was recorded for each pulsing condition (n=4). Groups were compared with an ordinary two-way ANOVA, and Tukey's

multiple comparisons test, with $p < 0.05$ considered significant.

[0098] Blood glucose survival. Fed blood glucose was measured via glucometer on days 1, 2, 7, 14, 21, 28 and 91 after injection. Serum human insulin levels were measured via ELISA (n=4). Groups were compared with an ordinary two-way ANOVA, and Tukey's multiple comparisons test, with $p < 0.05$ considered significant. Kaplan Meier Survival Analysis was performed, and weights were recorded.

[0099] Results

[0100] As shown in FIG. 14, gene electrotransfer significantly enhanced luciferase encoding gene delivery and expression in skeletal muscle over 6 months. Monopolar, monophasic pulses resulted in the highest expression (>1000 fold increase over injection only group).

[0101] FIGS. 15A-15B show that human insulin and glucokinase co-delivery via (localized in vivo electro gene therapy) *liveGT* is safe. The Kaplan Meier Survival analysis of FIG. 15A indicates no significant difference in survival of *liveGT* and control animals ($p=0.3173$). Individual rats continued to grow normally throughout the duration of this experiment as illustrated by FIG. 15B.

[0102] Referring to FIGS. 16A-16B, *LiveGT* mediated human insulin and glucokinase co-delivery significantly reduced serum glucose over 21 days. Exogenous (human) insulin levels in serum were significantly elevated over 21 days (FIG. 16A). Serum glucose levels were significantly lower with insulin and glucokinase co-deliver via *liveGT* (FIG. 16B). Exogenous insulin and glucokinase gene delivery mediated with *liveGT* significantly reduced blood glucose levels without hypoglycemia.

[0103] Discussion

The presented approach allows for insulin independence, and potentially substantially minimizes the treatment burden for patients eliminating the need for daily insulin protein injection and eliminating the need for immunosuppressive drugs. The results indicate that co-expression and glucose control can be maintained for several months. Since it is well established that plasmid DNA delivery is non-integrating and non-immunogenic, repeat treatments are feasible. Additionally, *liveGT* can be used as a platform technology as an alternative to other protein replacement therapies.

CLAIMS

We claim:

1. A method for treating type 1 diabetes in a subject, the method comprising:
(a) administering to the subject at least one of: a therapeutically effective amount of a nucleic acid encoding insulin and a therapeutically effective amount of a nucleic acid encoding glucokinase at an administration site; and
(b) applying an electrical pulse to the administration site.

2. The method of claim 1, wherein step (a) comprises administering both the nucleic acid encoding insulin and the nucleic acid encoding glucokinase.

3. The method of claim 1 or 2, wherein the administration site is in skeletal muscle.

4. The method of any one of claims 1-3, wherein the electrical pulse is applied using a monopolar electrode.

5. The method of any one of claims 1-4, wherein the electrical pulse is a monophasic pulse.

6. The method of claim 5, wherein the electrical pulse is between about 1 V and about 1.5 kV.

7. The method of claim 6, wherein the electrical pulse is about 90V.

8. The method of any one of claims 5-7, wherein the electrical pulse is applied for between about 50 μs and about 200 ms.

9. The method of claim 8, wherein the electrical pulse is applied for about 150 ms.

10. The method of any one of claims 5-9, wherein the electrical pulse is applied between 1 and 100 times.

11. The method of any one of claims 1-10, wherein the nucleic acid encoding insulin and

the nucleic acid encoding glucokinase are each provided on a plasmid.

12. The method of claim 11, wherein the nucleic acid encoding insulin and the nucleic acid encoding glucokinase are provided on separate plasmids.

13. The method of claim 11, wherein the nucleic acid encoding insulin and the nucleic acid encoding glucokinase are both provided on the same plasmid.

14. The method of any one of claims 1-13, wherein step (a) is performed by intramuscular injection.

15. The method of any one of claims 1-14, further comprising: (c) repeating steps (a) and (b) at least about every six months.

16. The method of claim 15, wherein step (c) is performed at between about every six months and about every twelve months.

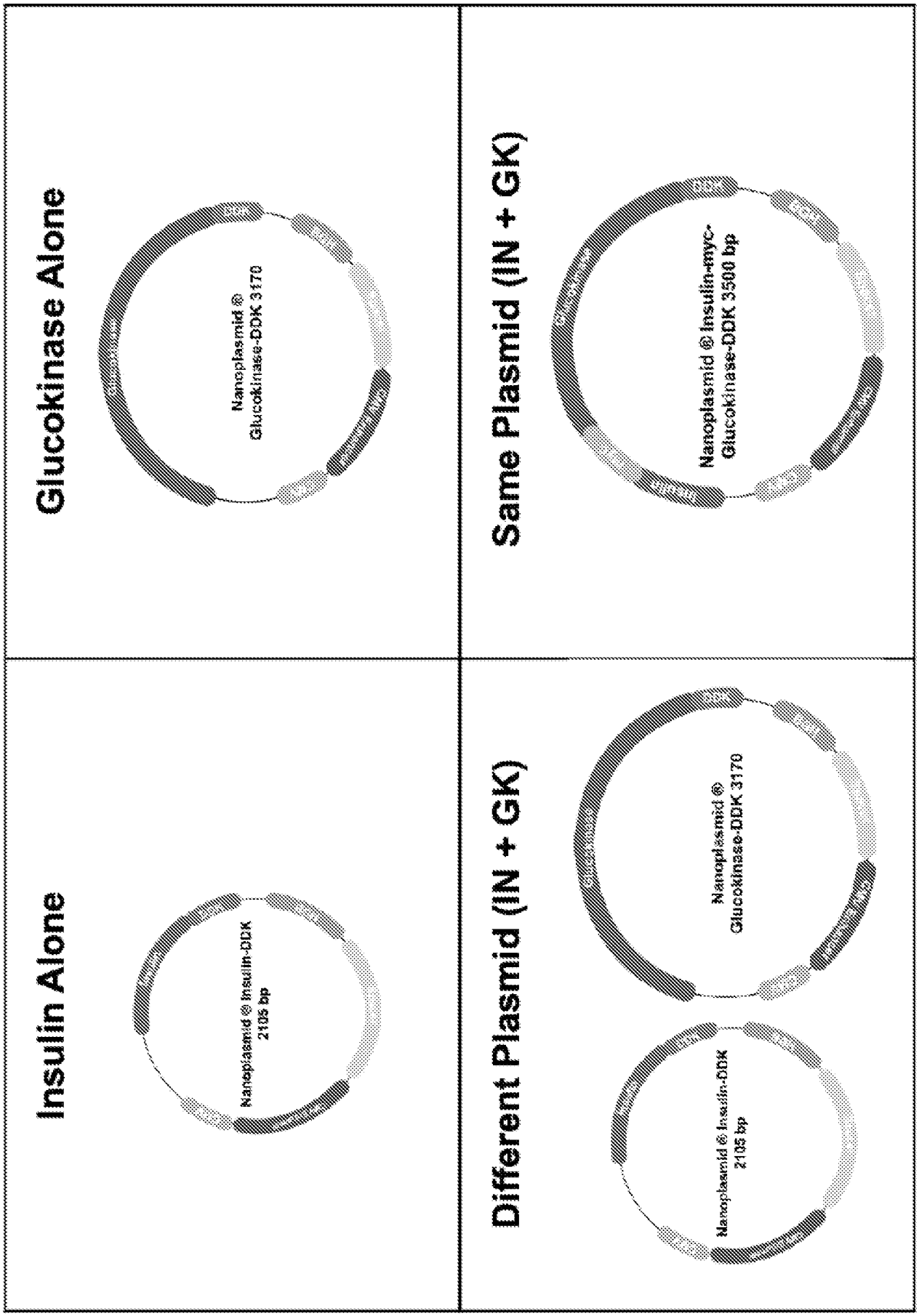
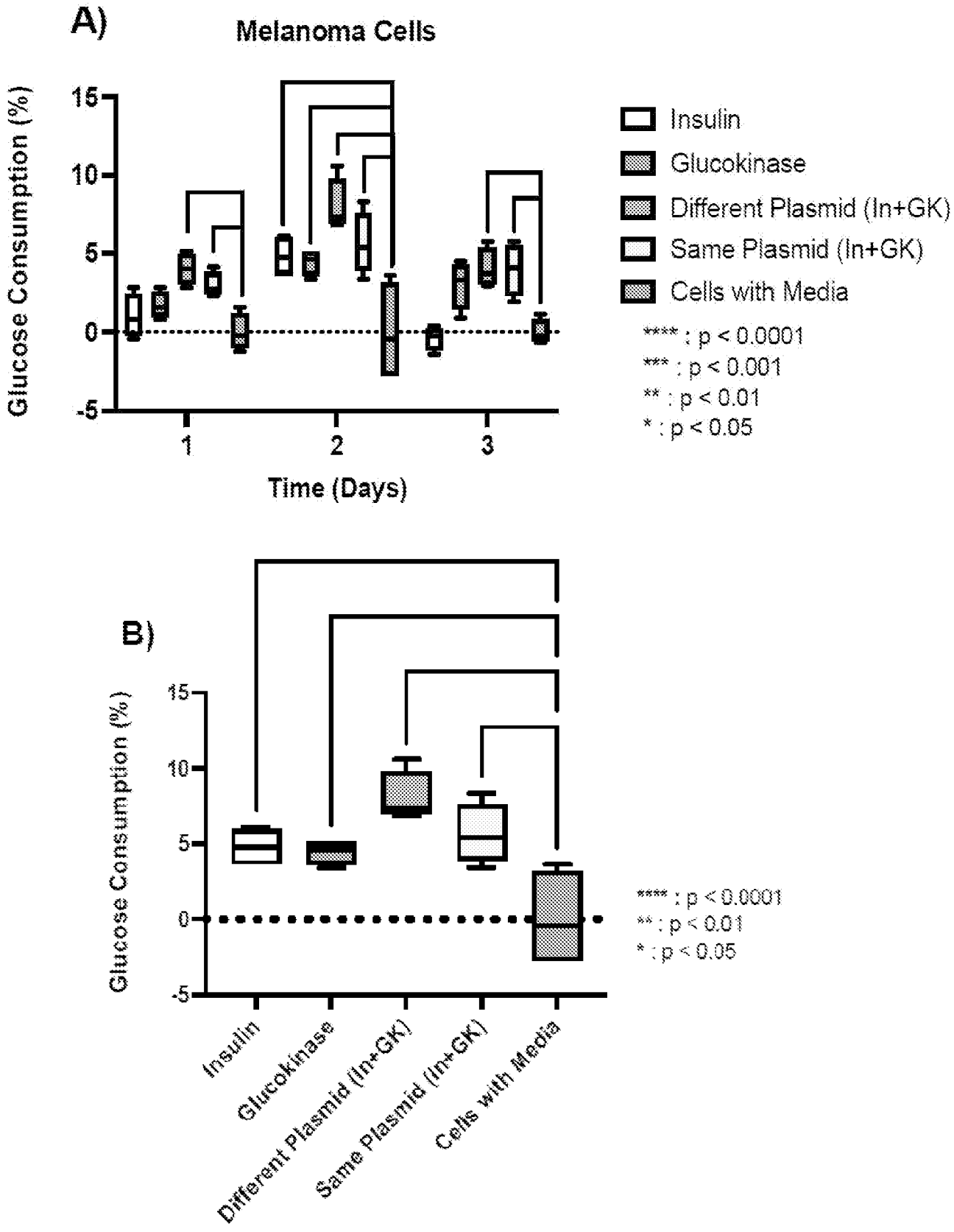
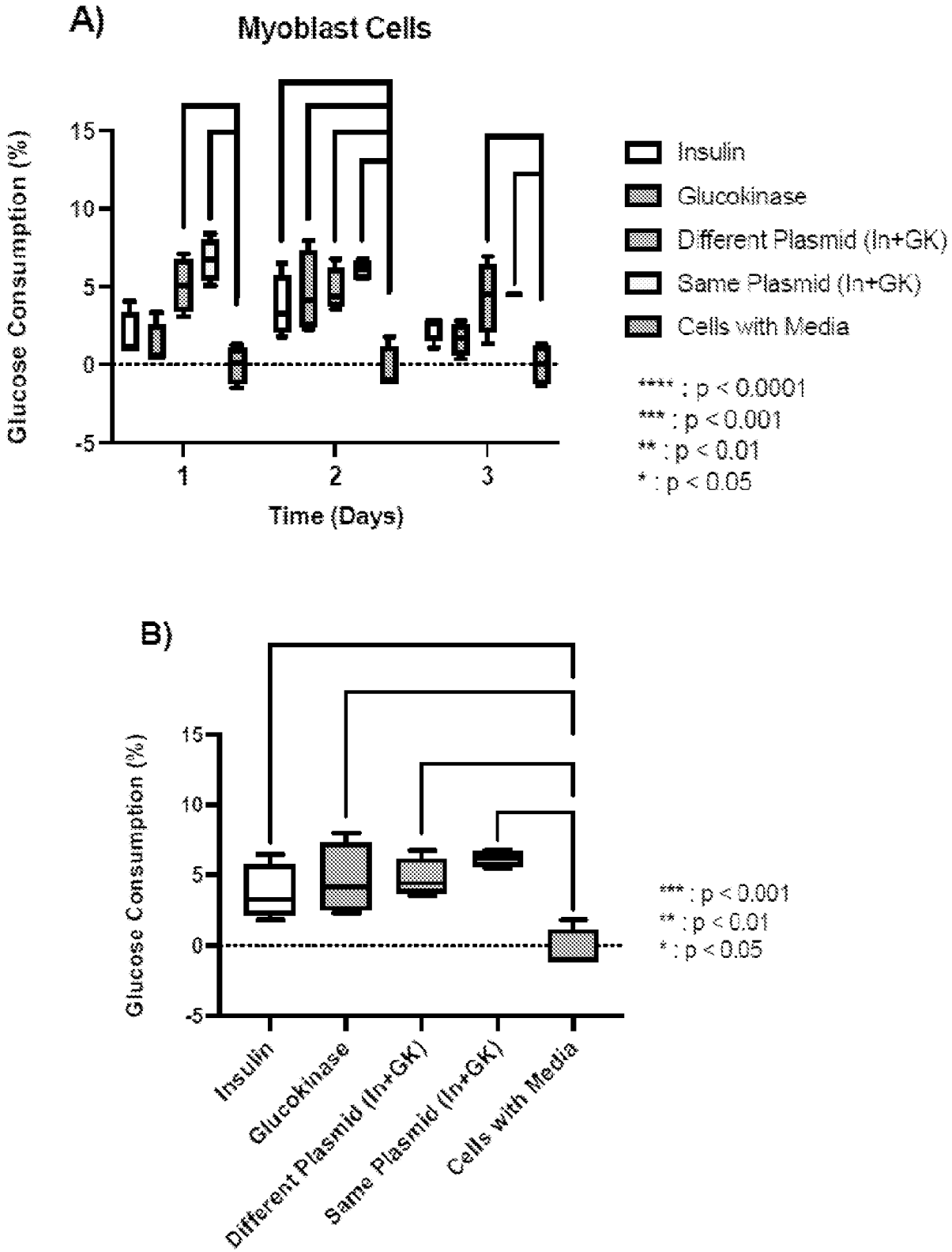

Glucokinase Alone
Nanoplasmid ®
Glucokinase-DDK 3170
Insulin Alone
Nanoplasmid ® Insulin-DDK
2105 bp
Same Plasmid (IN + GK)
Nanoplasmid ® Insulin-myc-
Glucokinase-DDK 3500 bp
Different Plasmid (IN + GK)
Nanoplasmid ®
Glucokinase-DDK 3170
Nanoplasmid ® Insulin-DDK
2105 bp

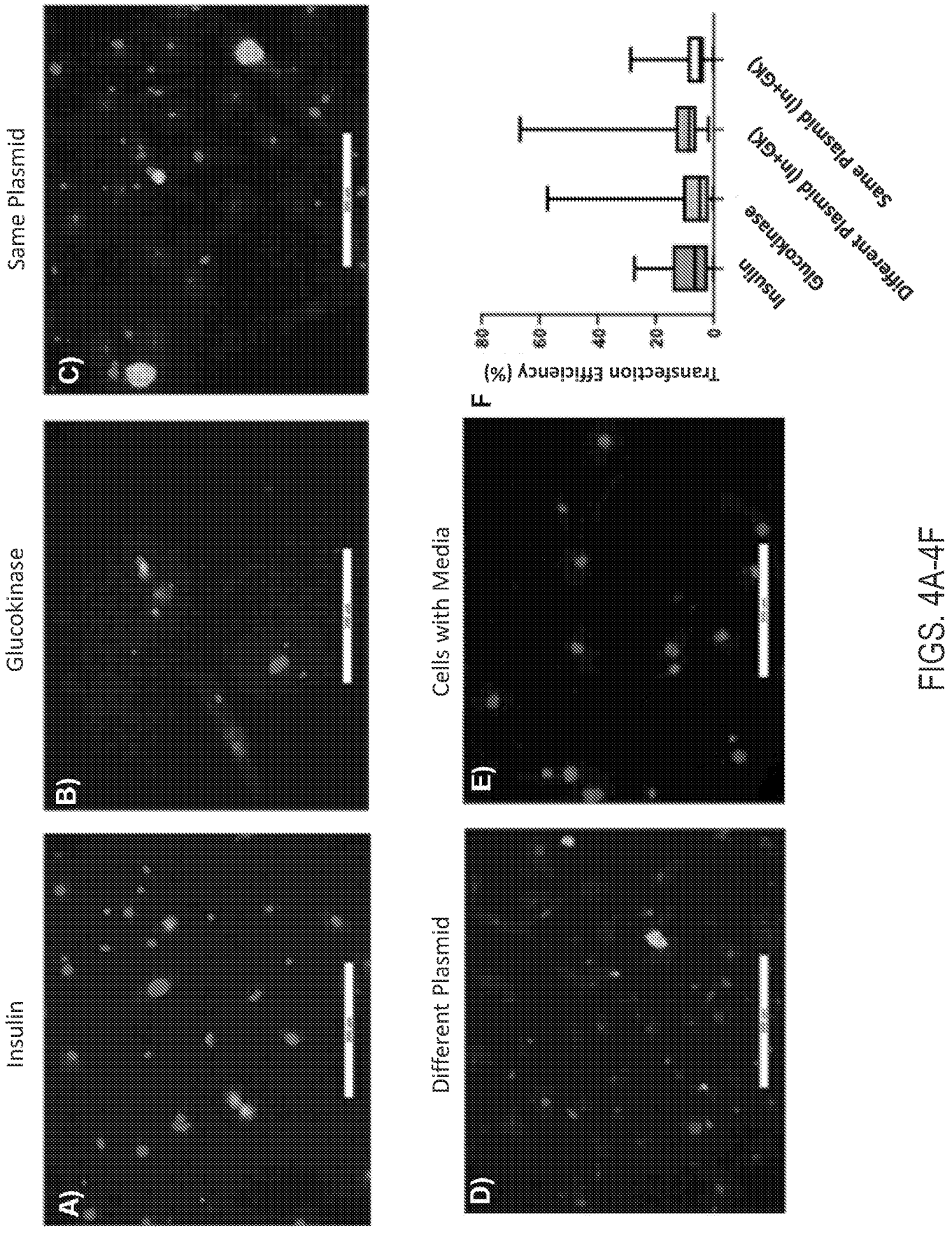
FIG. 1


A)
Melanoma Cells
Glucose Consumption (%)
15
10
5
0
-5
1
2
3
Time (Days)
Insulin
Glucokinase
Different Plasmid (In+GK)
Same Plasmid (In+GK)
Cells with Media
**** : p < 0.0001
*** : p < 0.001
** : p < 0.01
* : p < 0.05
B)
Glucose Consumption (%)
15
10
5
0
-5
Insulin
Glucokinase
Different Plasmid (In+GK)
Same Plasmid (In+GK)
Cells with Media
**** : p < 0.0001
** : p < 0.01
* : p < 0.05

FIGS. 2A-2B


A)
Myoblast Cells
Glucose Consumption (%)
15
10
5
0
-5
1
2
3
Time (Days)
Insulin
Glucokinase
Different Plasmid (In+GK)
Same Plasmid (In+GK)
Cells with Media
**** : p < 0.0001
*** : p < 0.001
** : p < 0.01
* : p < 0.05
B)
Glucose Consumption (%)
15
10
5
0
-5
Insulin
Glucokinase
Different Plasmid (In+GK)
Same Plasmid (In+GK)
Cells with Media
*** : p < 0.001
** : p < 0.01
* : p < 0.05

FIGS. 3A-3B


Insulin
Glucokinase
Same Plasmid
A)
B)
C)
Different Plasmid
Cells with Media
D)
E)
F
Transfection Efficiency (%)
0
20
40
60
80
Insulin
Glucokinase
Different plasmid (In+Gk)
Same plasmid (In+Gk)

FIGS. 4A-4F

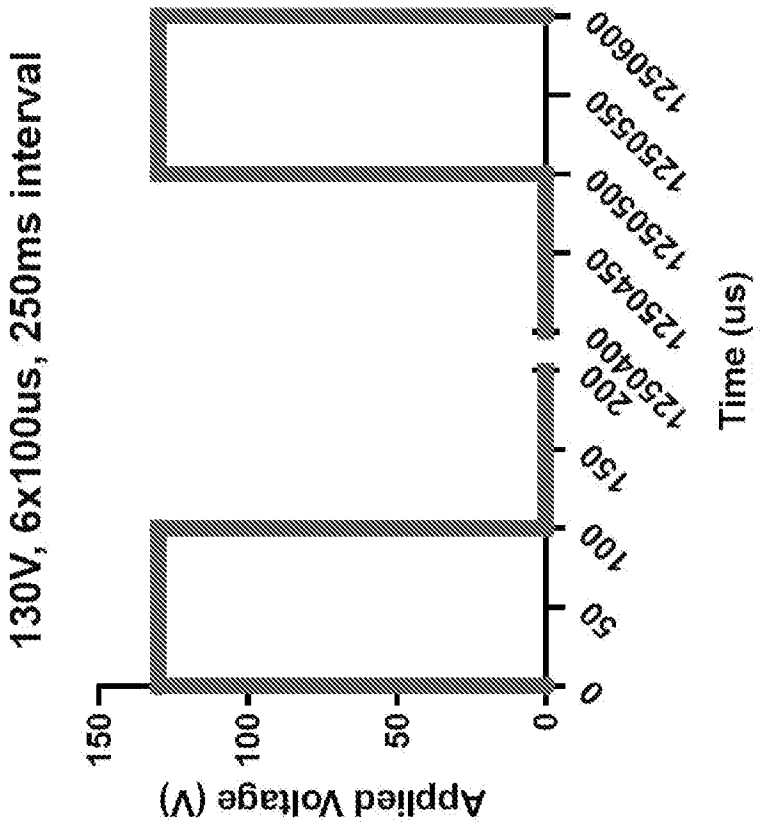

130V, 6x100us, 250ms interval
Applied Voltage (V)
150
100
50
0
0
50
100
150
200
1250400
1250450
1250500
1250550
1250600
Time (us)

FIG. 5

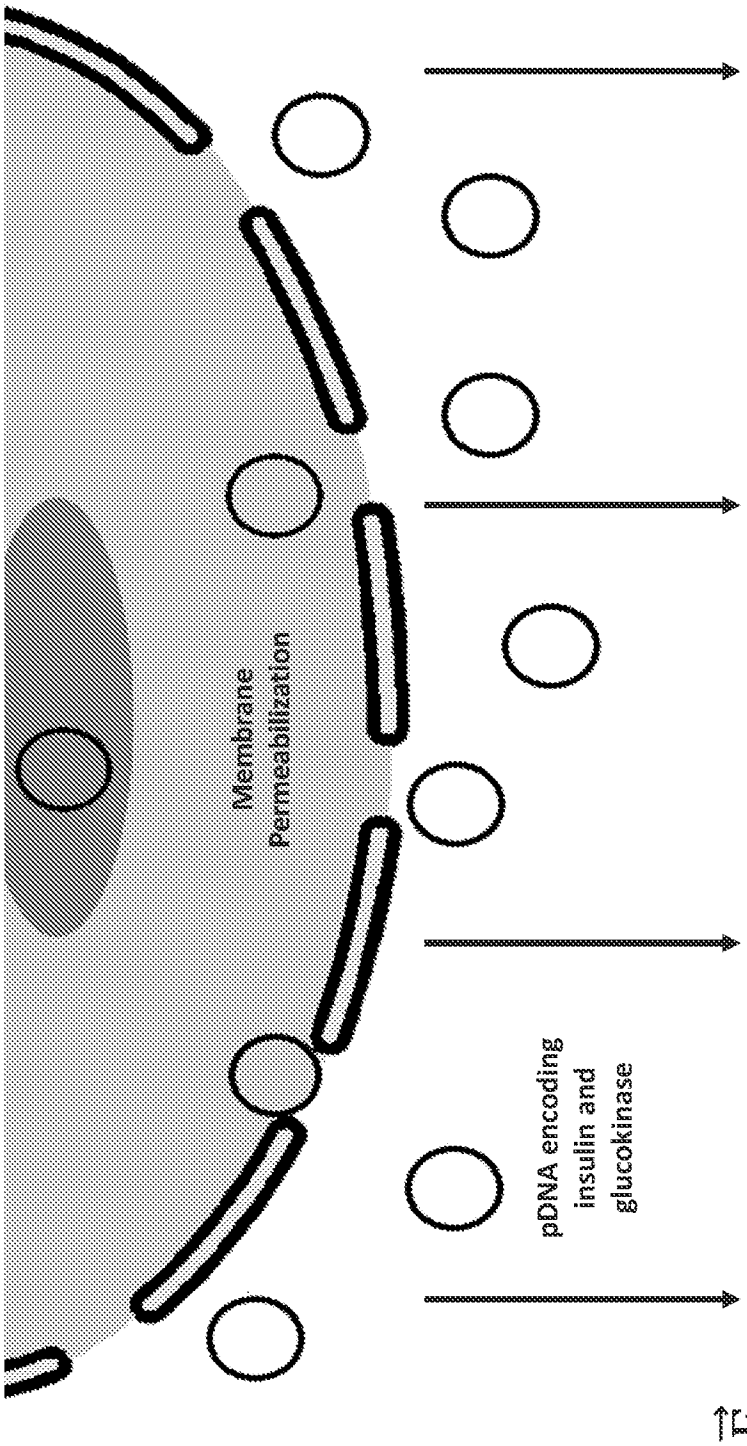

Membrane
Permeabilization
pDNA encoding
insulin and
glucokinase

FIG. 6A

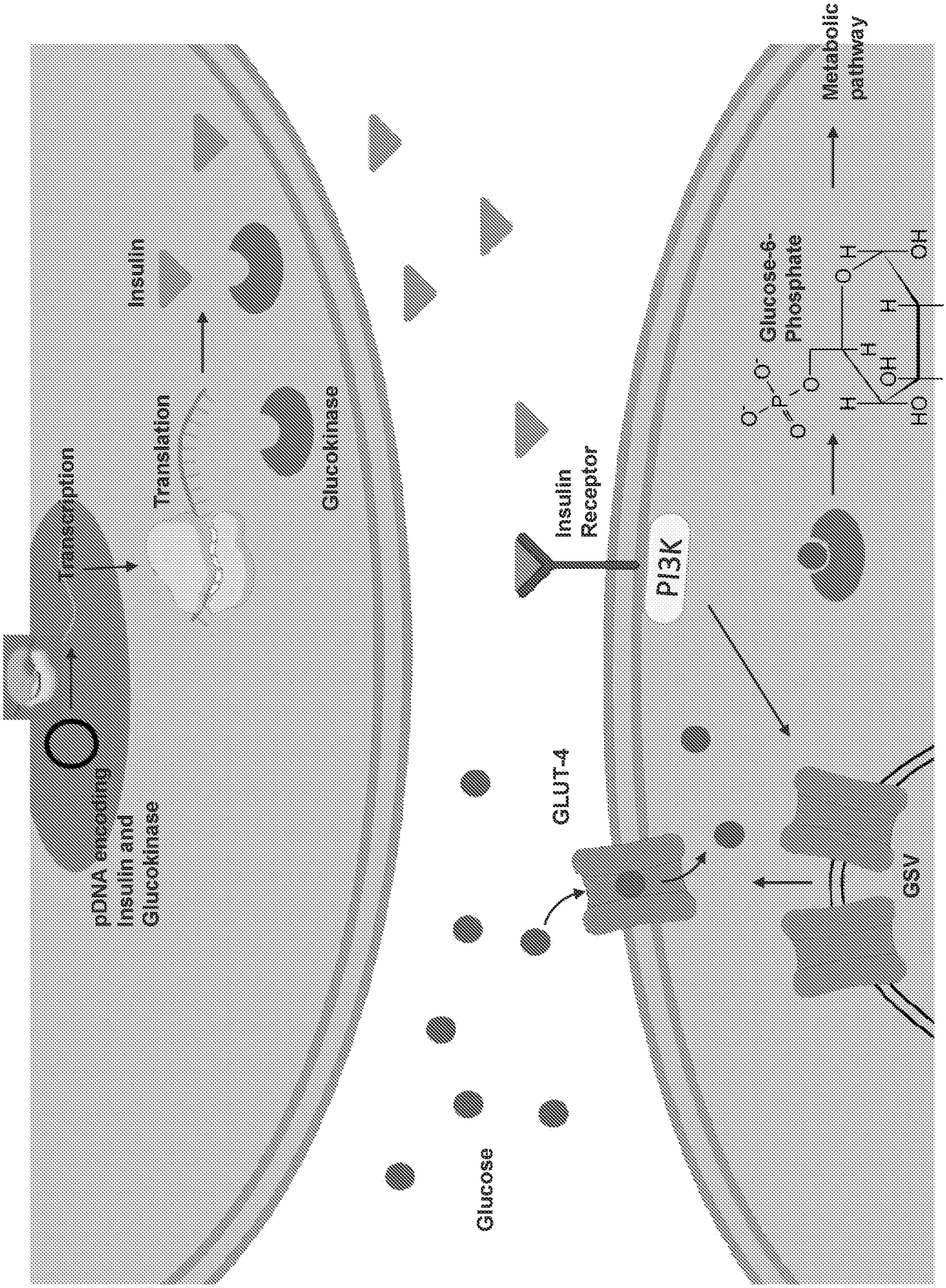
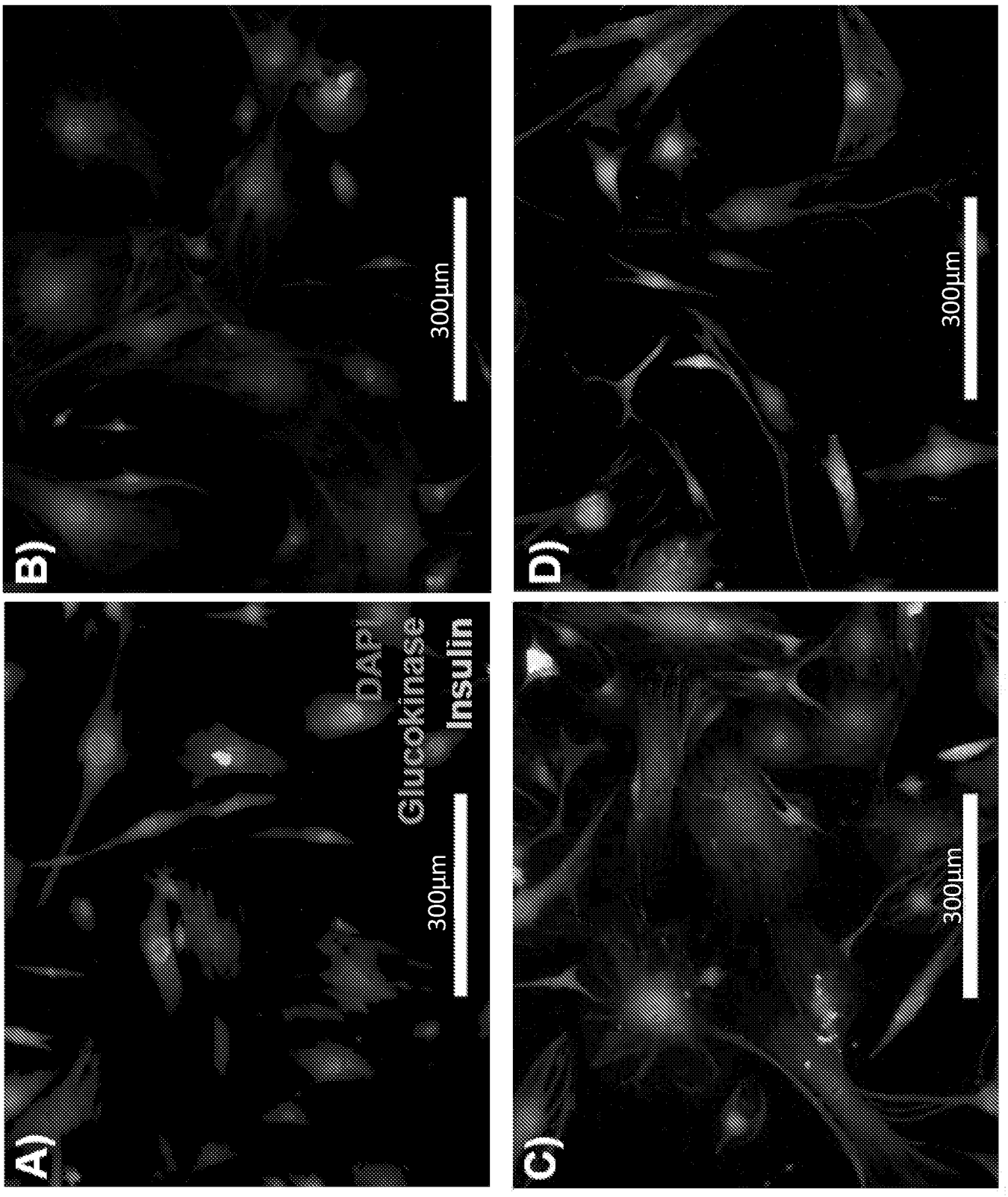
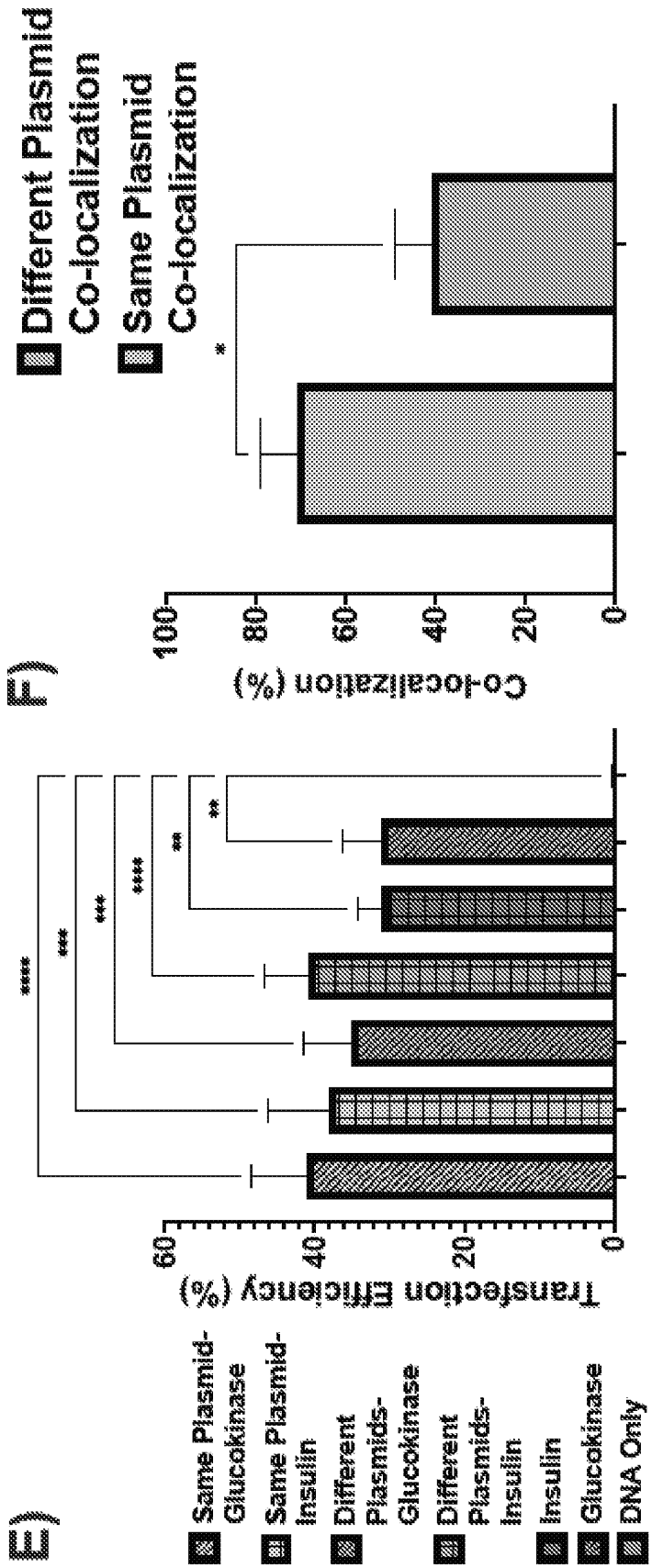

pDNA encoding Insulin and Glucokinase
Transcription
Translation
Insulin
Glucokinase
Insulin Receptor
PI3K
GLUT-4
Glucose
GSV
Glucose-6-Phosphate
Metabolic pathway

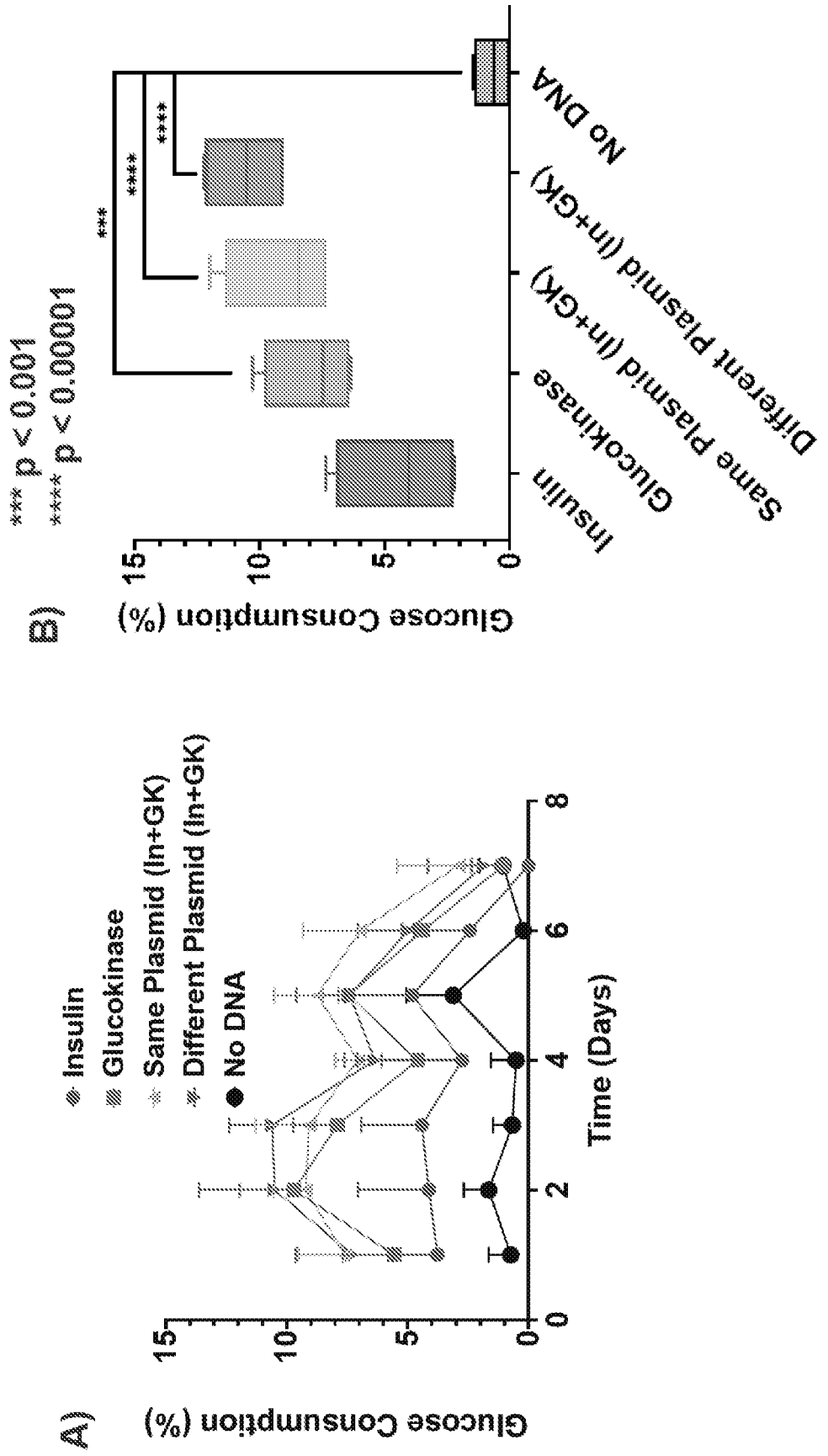
FIG. 6B



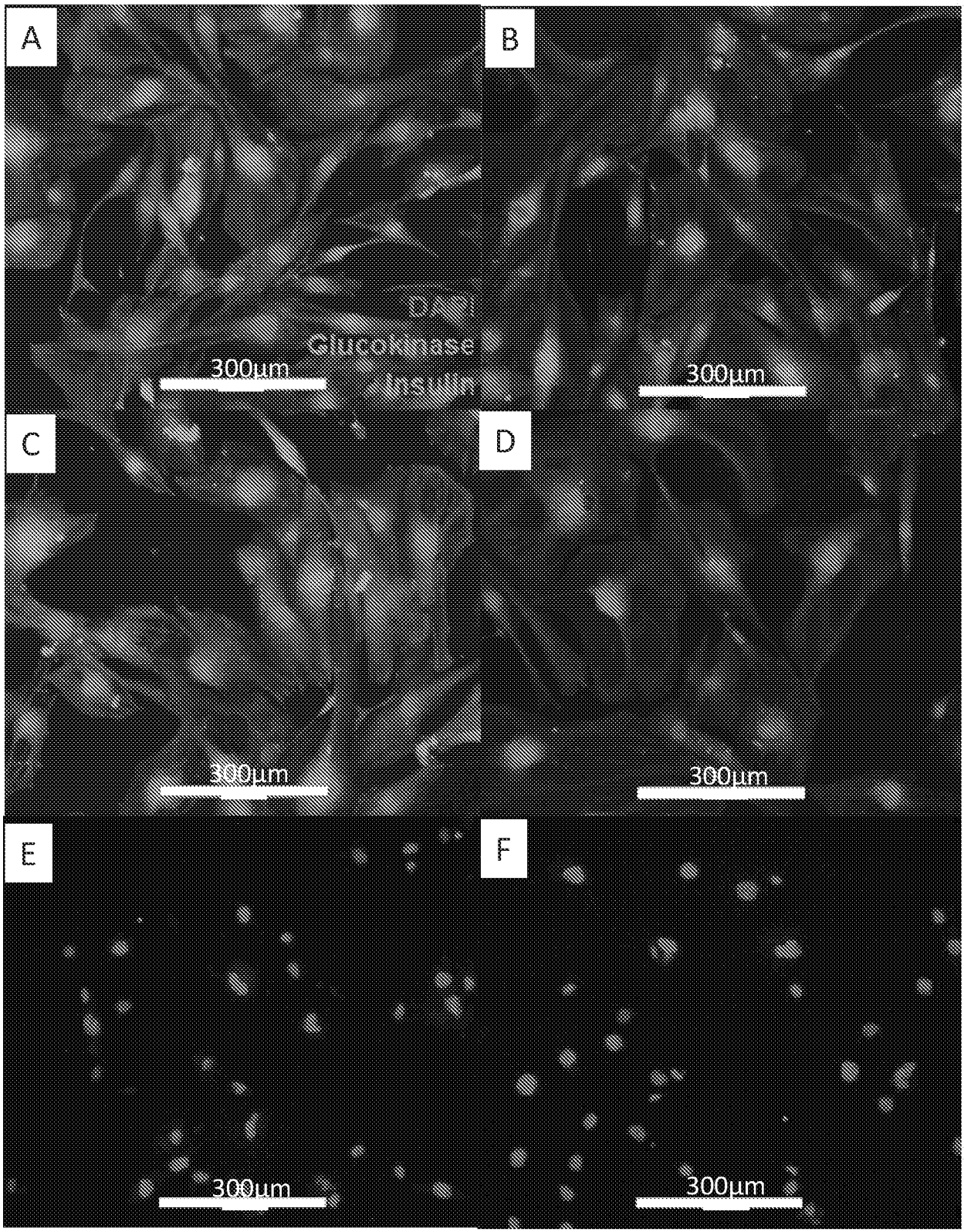
FIGS. 7A-7D


E)
Transfection Efficiency (%)
0
20
40
60
****
***
***
****
**
**
Same Plasmid-Glucokinase
Same Plasmid-Insulin
Different Plasmids-Glucokinase
Different Plasmids-Insulin
Insulin
Glucokinase
DNA Only
F)
Co-localization (%)
0
20
40
60
80
100
*
Same Plasmid Co-localization
Different Plasmid Co-localization

FIGS. 7E-7F


A)
Glucose Consumption (%)
15
10
5
0
0
2
4
6
8
Time (Days)
Insulin
Glucokinase
Same Plasmid (In+GK)
Different Plasmid (In+GK)
No DNA
B)
Glucose Consumption (%)
15
10
5
0
*** p < 0.001
**** p < 0.00001
***
****
****
Insulin
Glucokinase
Same plasmid (In+GK)
Different plasmid (In+GK)
No DNA

FIGS. 8A-8B


A
B
C
D
E
F
DAPI
Glucokinase
Insulin
300µm
300µm
300µm
300µm
300µm
300µm

FIGS. 9A-9F

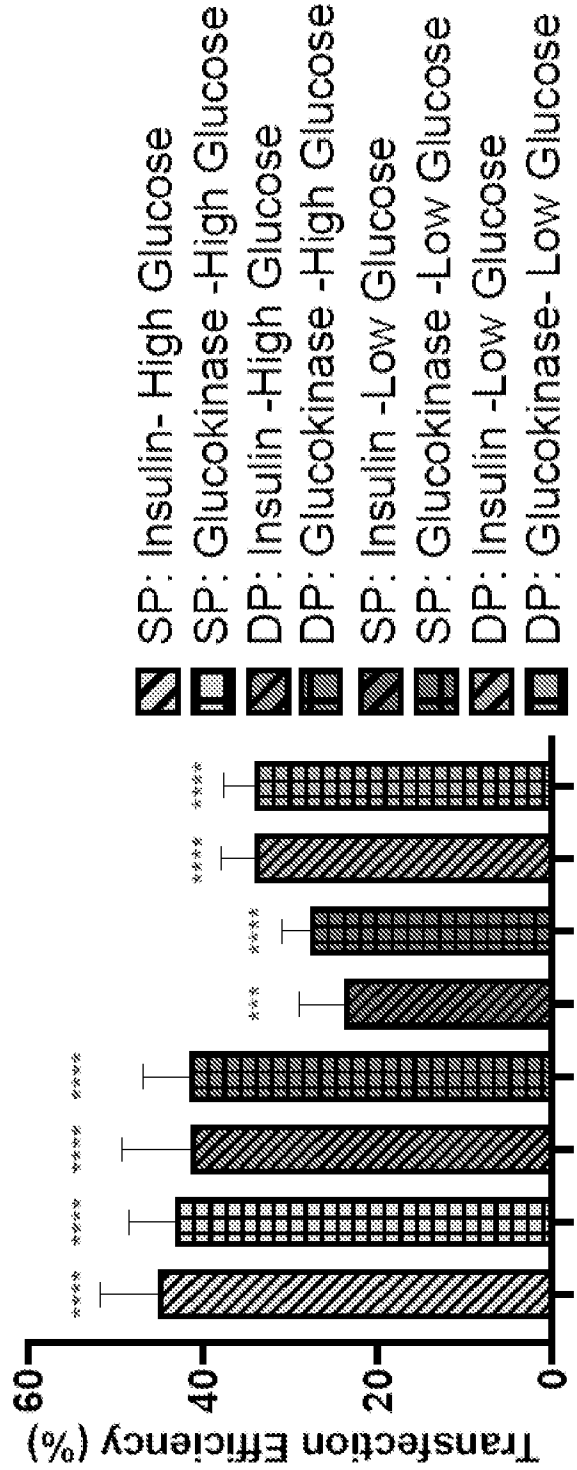
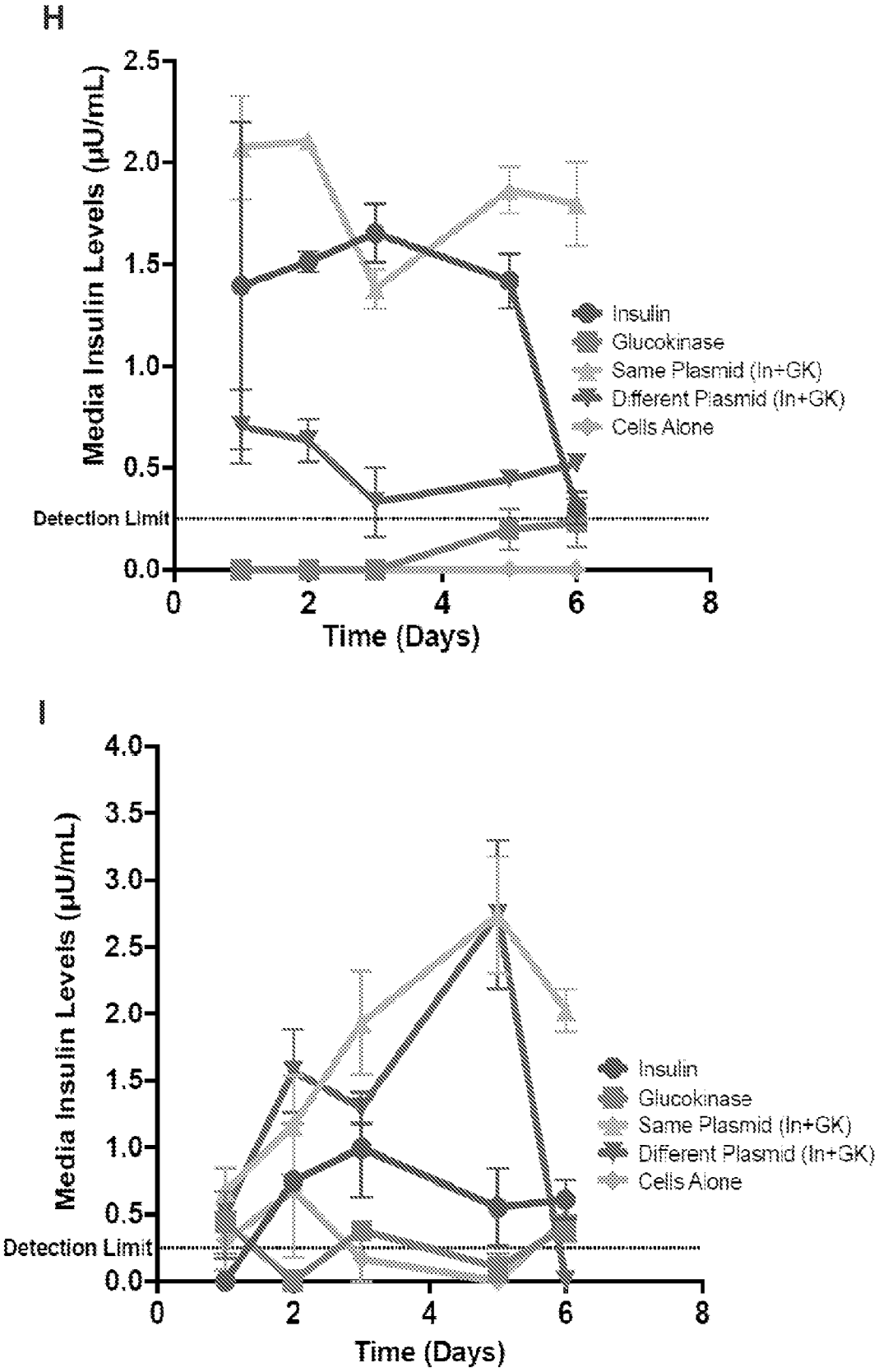

Transfection Efficiency (%)
0
20
40
60
SP: Insulin- High Glucose
SP: Glucokinase -High Glucose
DP: Insulin -High Glucose
DP: Glucokinase -High Glucose
SP: Insulin -Low Glucose
SP: Glucokinase -Low Glucose
DP: Insulin -Low Glucose
DP: Glucokinase- Low Glucose

FIG. 9G


H
Media Insulin Levels (µU/mL)
2.5
2.0
1.5
1.0
0.5
0.0
Detection Limit
0
2
4
6
8
Time (Days)
Insulin
Glucokinase
Same Plasmid (In+GK)
Different Plasmid (In+GK)
Cells Alone
I
Media Insulin Levels (µU/mL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Detection Limit
0
2
4
6
8
Time (Days)
Insulin
Glucokinase
Same Plasmid (In+GK)
Different Plasmid (In+GK)
Cells Alone

FIGS. 9H-9I

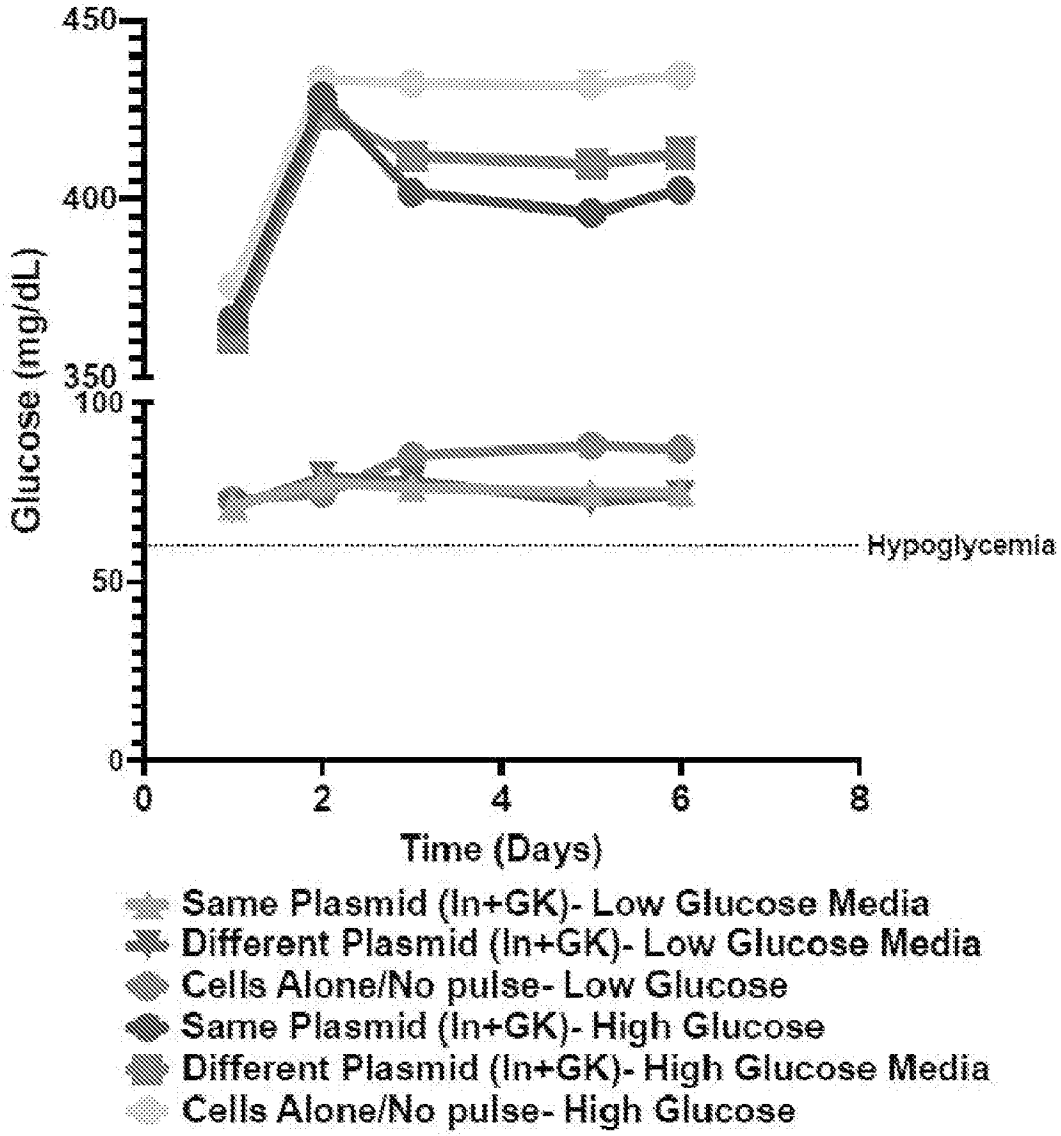
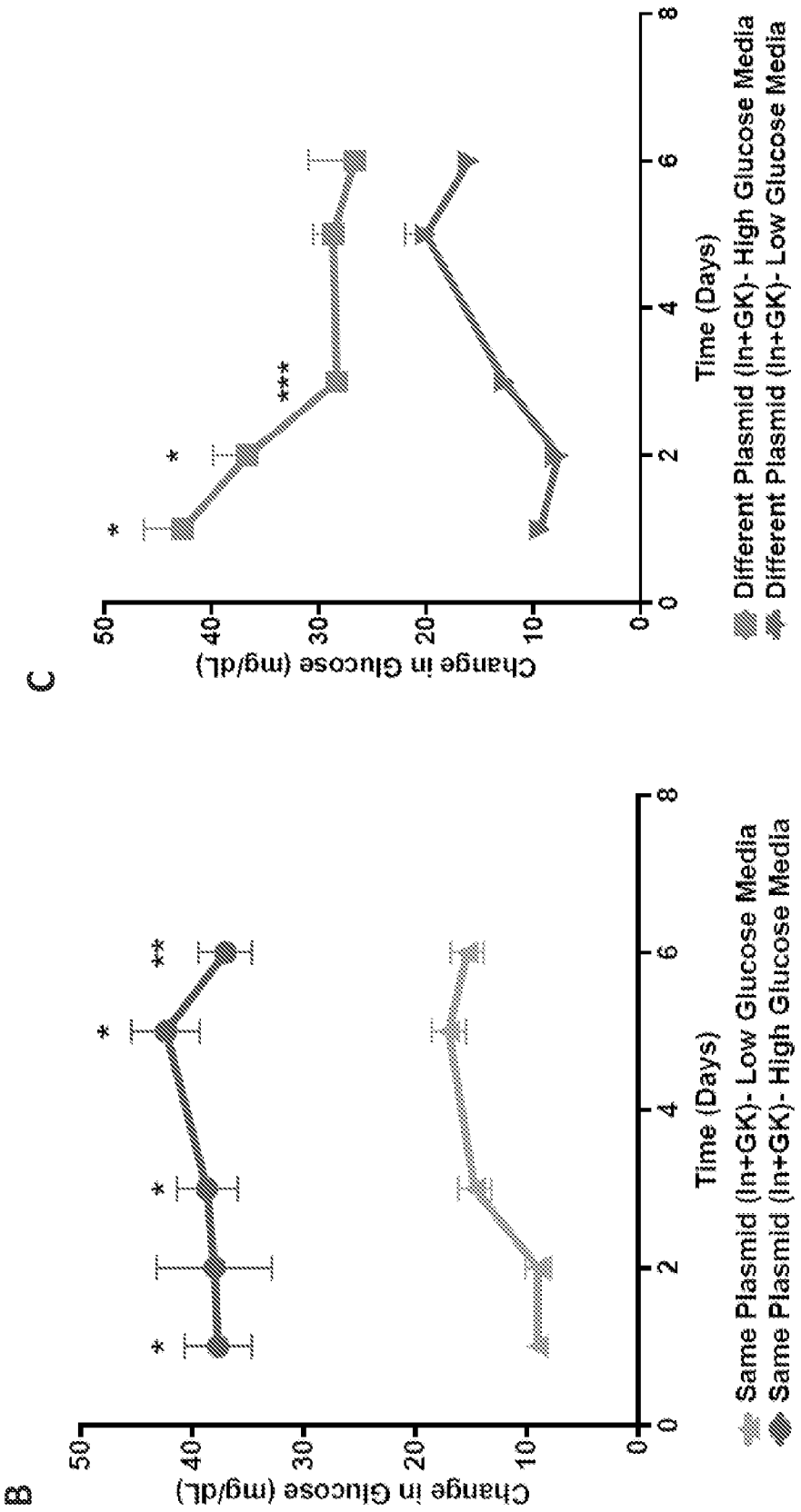

450
400
350
100
50
0
Glucose (mg/dL)
Hypoglycemia
0
2
4
6
8
Time (Days)
Same Plasmid (In+GK)- Low Glucose Media
Different Plasmid (In+GK)- Low Glucose Media
Cells Alone/No pulse- Low Glucose
Same Plasmid (In+GK)- High Glucose
Different Plasmid (In+GK)- High Glucose Media
Cells Alone/No pulse- High Glucose

FIG. 10A


B
Change in Glucose (mg/dL)
Time (Days)
Same Plasmid (In+GK)- Low Glucose Media
Same Plasmid (In+GK)- High Glucose Media
C
Change in Glucose (mg/dL)
Time (Days)
Different Plasmid (In+GK)- High Glucose Media
Different Plasmid (In+GK)- Low Glucose Media

FIGS. 10B-10C

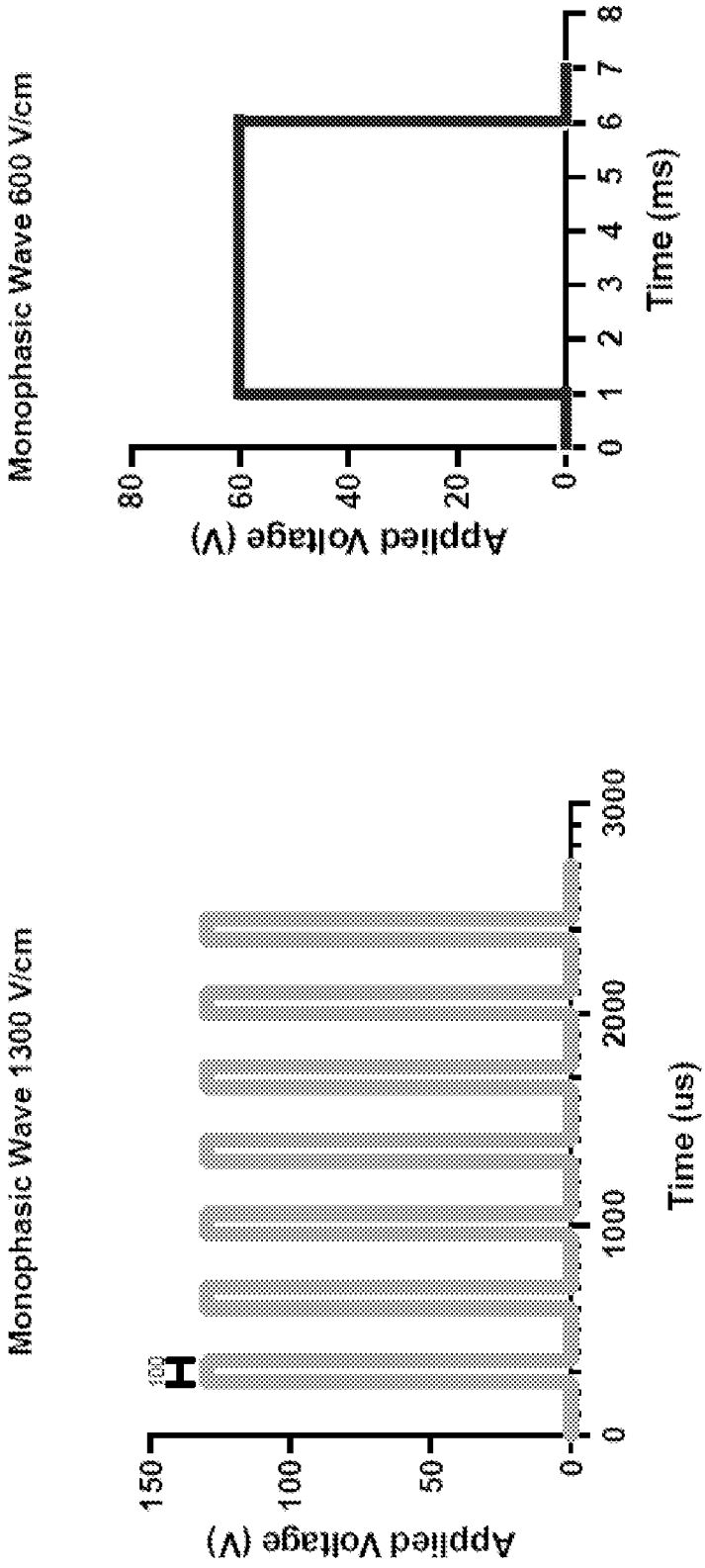
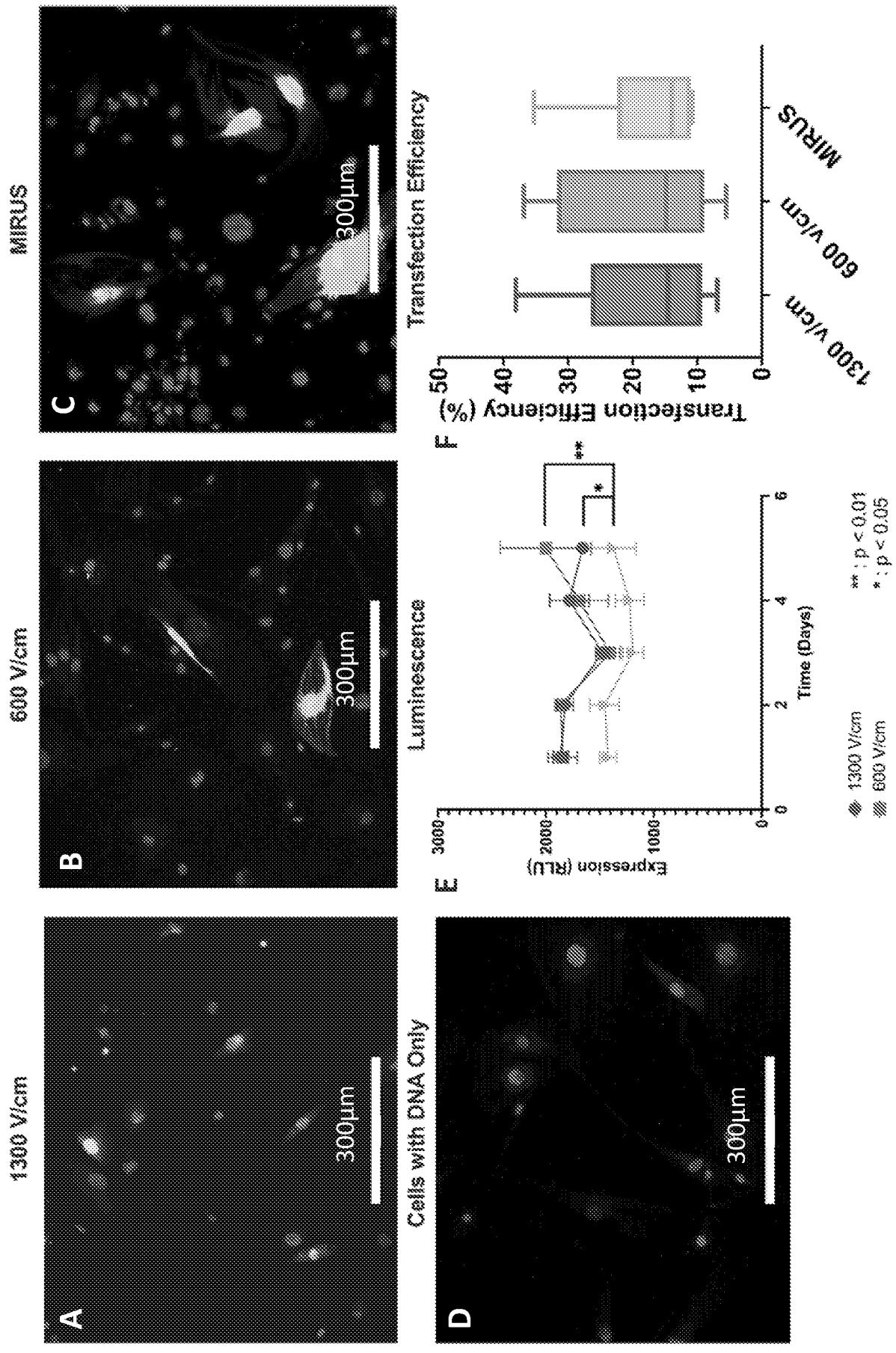
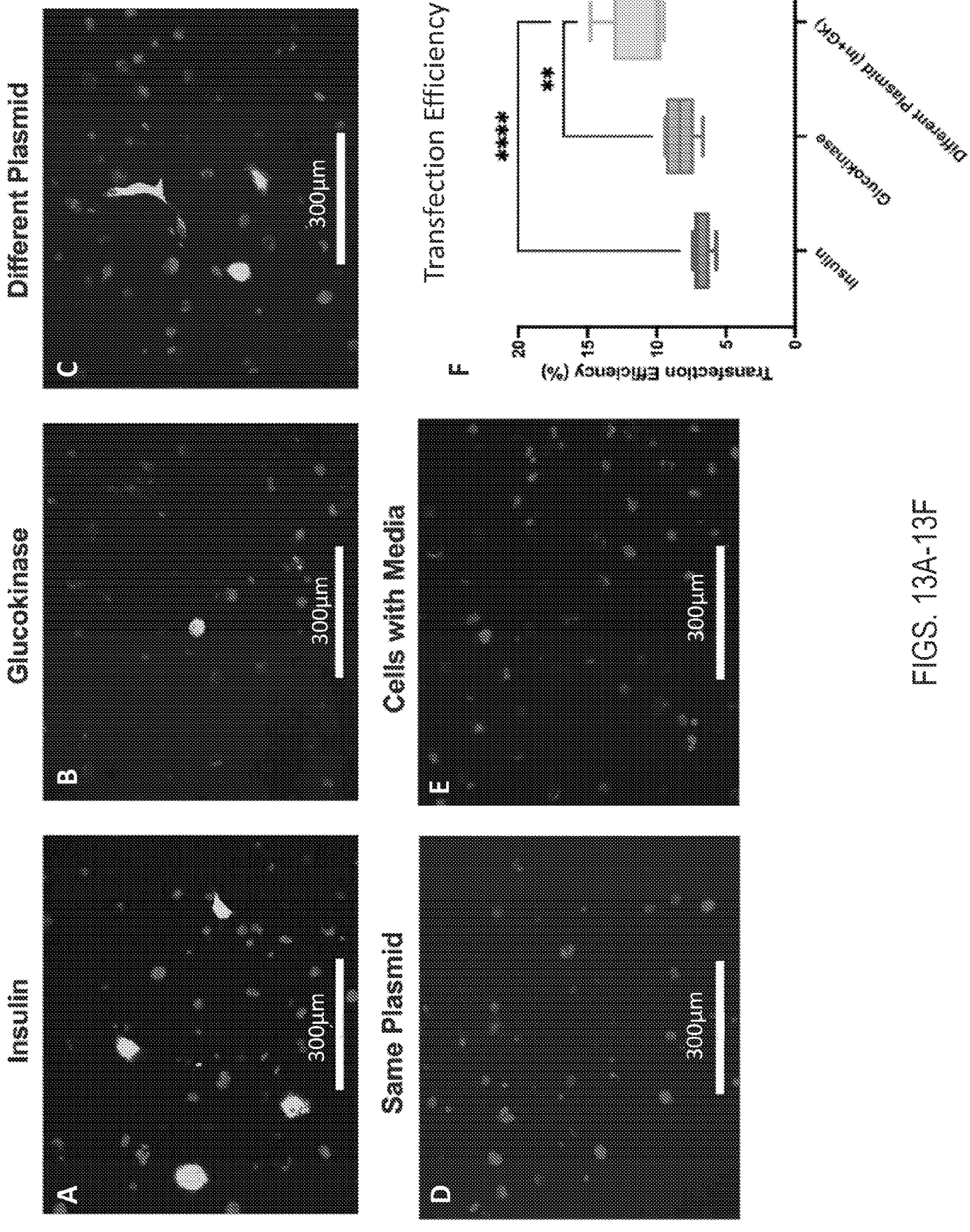

Monophasic Wave 600 V/cm
Applied Voltage (V)
0
20
40
60
80
0 1 2 3 4 5 6 7 8
Time (ms)
Monophasic Wave 1300 V/cm
Applied Voltage (V)
0
50
100
150
H
0 1000 2000 3000
Time (µs)

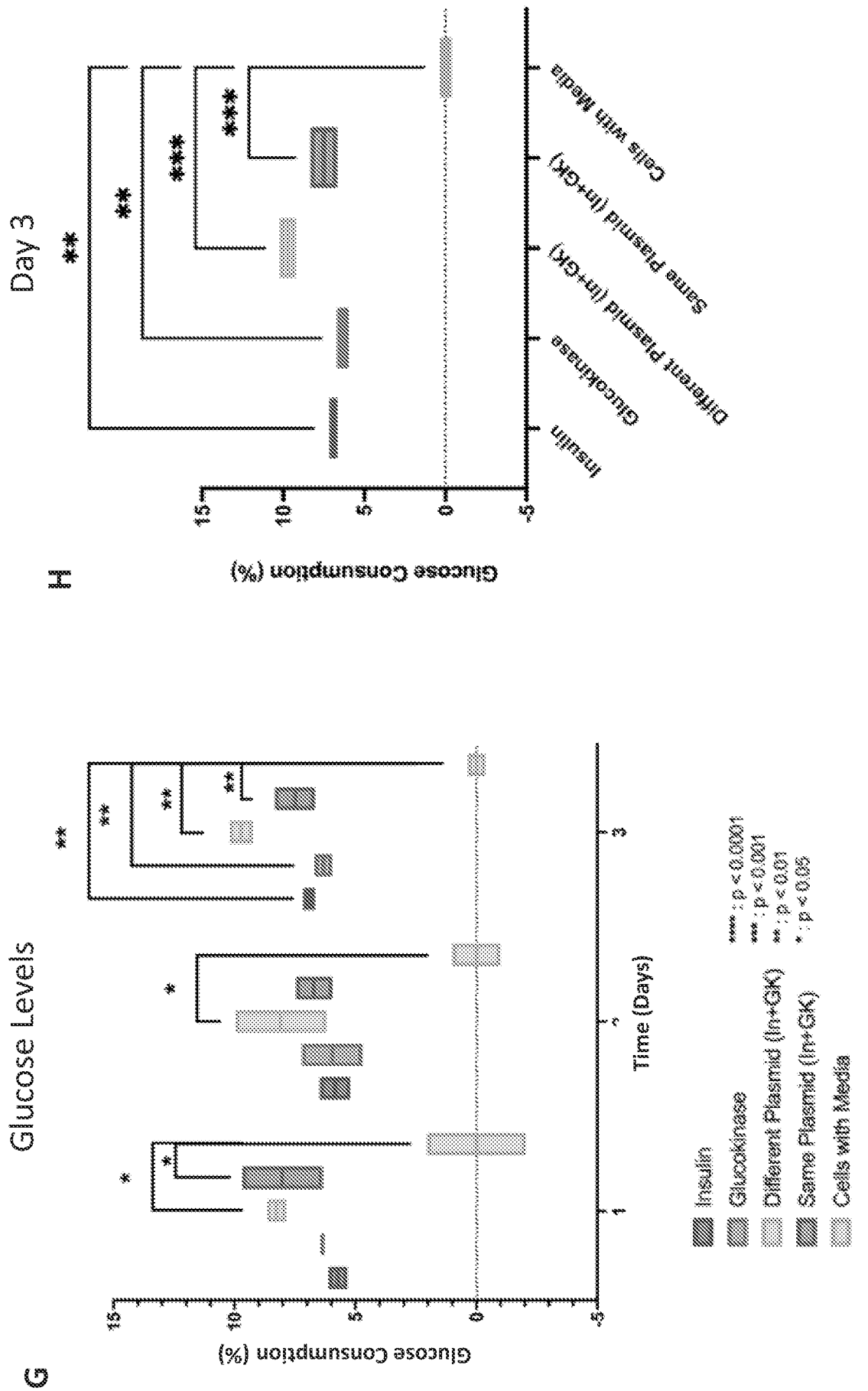
FIG. 11


1300 V/cm
600 V/cm
MIRUS
A
B
C
300µm
300µm
300µm
Cells with DNA Only
D
E
Luminescence
Expression (RLU)
3000
2000
1000
0
2
4
6
Time (Days)
**
*
1300 V/cm
600 V/cm
Cells with DNA Only
** : p < 0.01
* : p < 0.05
F
Transfection Efficiency
Transfection Efficiency (%)
50
40
30
20
10
0
1300 V/cm
600 V/cm
MIRUS

FIGS. 12A-12F


Insulin
Glucokinase
Different Plasmid
Same Plasmid
Cells with Media
A
B
C
D
E
F
300µm
Transfection Efficiency
Transfection Efficiency (%)
20
15
10
5
0
****
**
Insulin
Glucokinase
Different plasmid (In+Gk)

FIGS. 13A-13F


G
Glucose Levels
Glucose Consumption (%)
15
10
5
0
-5
1
2
3
Time (Days)
Insulin
Glucokinase
Different Plasmid (In+GK)
Same Plasmid (In+GK)
Cells with Media
**** : p < 0.0001
*** : p < 0.001
** : p < 0.01
* : p < 0.05
H
Day 3
Glucose Consumption (%)
15
10
5
0
-5
Insulin
Glucokinase
Different Plasmid (In+GK)
Same Plasmid (In+GK)
Cells with Media

FIGS. 13G-13H

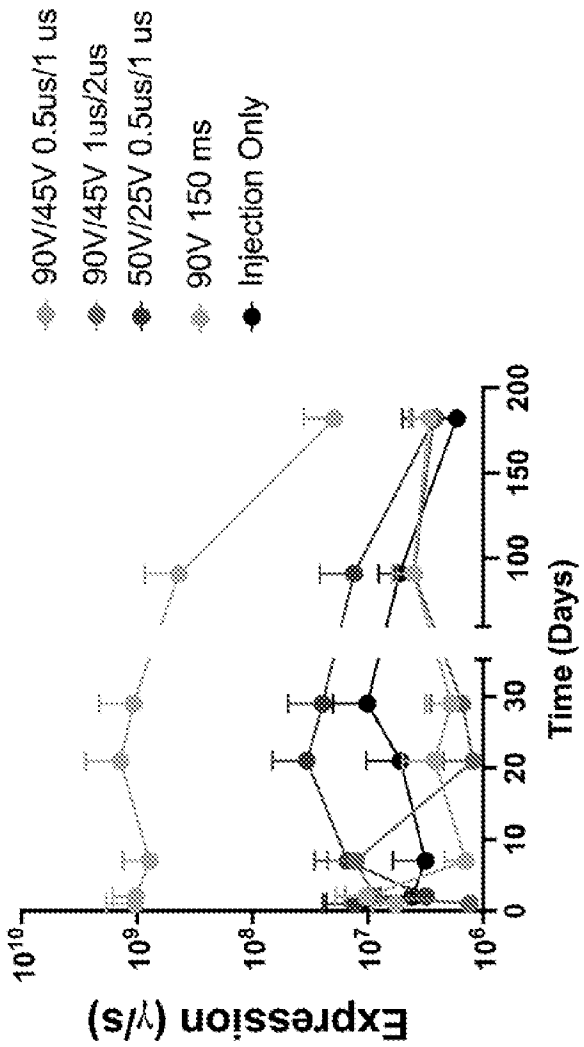
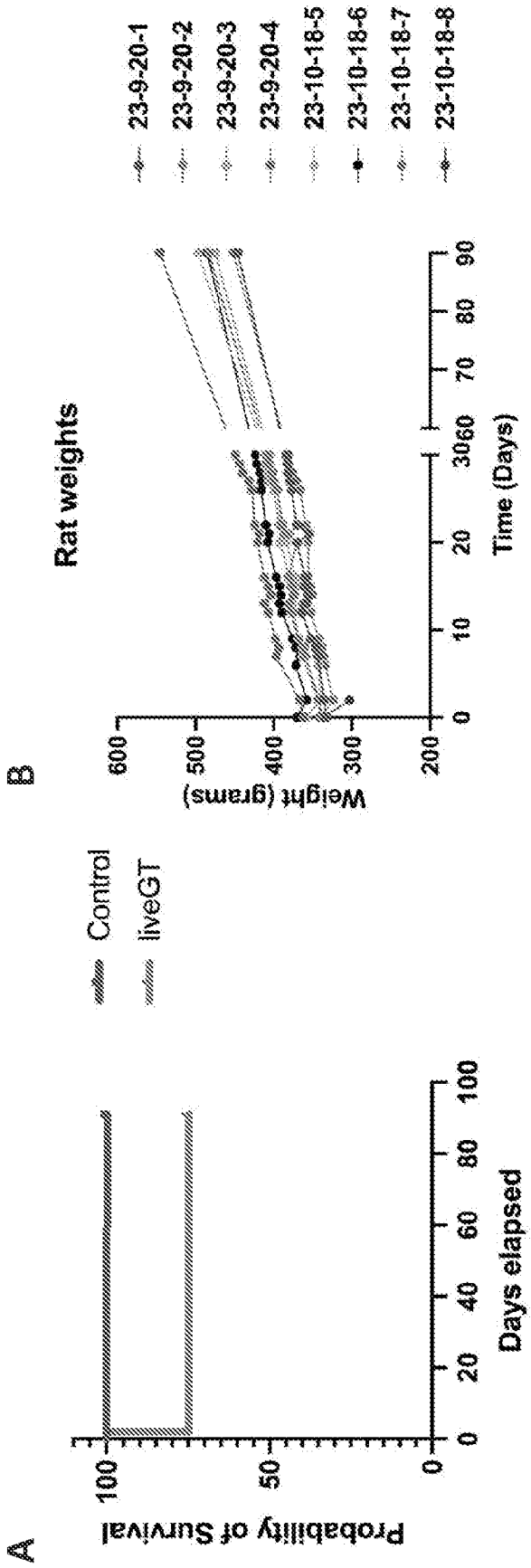
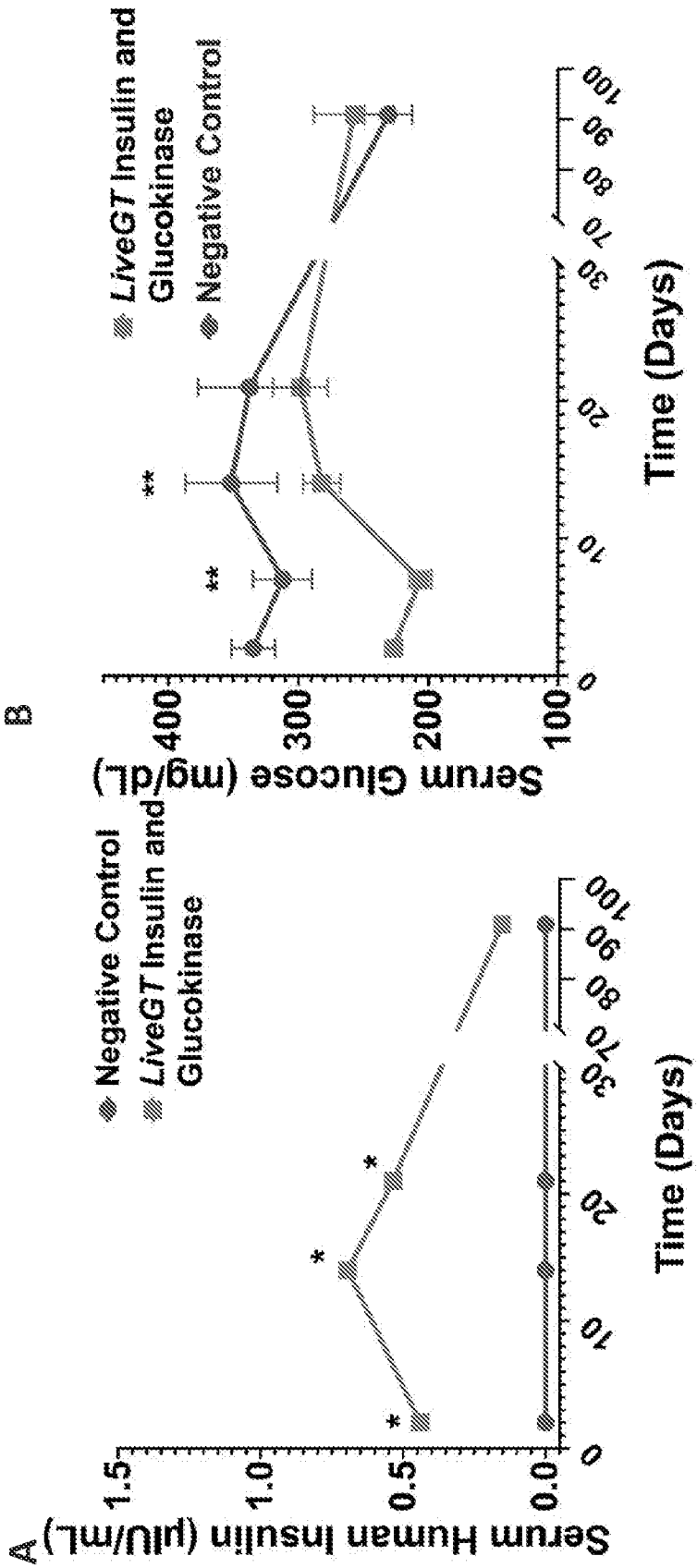

90V/45V 0.5us/1 us
90V/45V 1us/2us
50V/25V 0.5us/1 us
90V 150 ms
Injection Only
Expression (γ/s)
10^10
10^9
10^8
10^7
10^6
0
10
20
30
100
150
200
Time (Days)

FIG. 14


A
Probability of Survival
100
50
0
0
20
40
60
80
100
Days elapsed
Control
liveGT
B
Rat weights
Weight (grams)
600
500
400
300
200
0
10
20
30
60
70
80
90
Time (Days)
23-9-20-1
23-9-20-2
23-9-20-3
23-9-20-4
23-10-18-5
23-10-18-6
23-10-18-7
23-10-18-8

FIGS. 15A-15B


A
Serum Human Insulin (μIU/mL)
1.5
1.0
0.5
0.0
0
10
20
30
70
80
90
100
Time (Days)
Negative Control
LiveGT Insulin and Glucokinase
*
B
Serum Glucose (mg/dL)
400
300
200
100
0
10
20
30
70
80
90
100
Time (Days)
LiveGT Insulin and Glucokinase
Negative Control
**

FIGS. 16A-16B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/033138

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/28 A61K48/00 A61N1/32
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Bosch I Tubert Fàtima: "GENE THERAPY APPROACHES FOR DIABETES MELLITUS CENTRED ON GENETIC MANIPULATION OF THE PANCREAS AND EXTRAPANCREATIC TISSUES", V Symposium, 1 January 2004 (2004-01-01), XP093191527, Retrieved from the Internet: URL:https://statics.ccma.cat/recursos/marato/simposiums/marato1998/eng/p_27.html#	1-3, 11-16
Y	p. 1, paragraphs 5-6 (section 2), p. 2, paragraph 5, p. 3, paragraph 8 (section 3)	4-10

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[x] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 August 2024	10/09/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Empl, Laura

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BULYSHEVA ANNA ET AL: "Monopolar gene electrotransfer enhances plasmid DNA delivery to skin", BIOELECTROCHEMISTRY, ELESEVIER, AMSTERDAM, NL, vol. 140, 17 April 2021 (2021-04-17), XP086602727, ISSN: 1567-5394, DOI: 10.1016/J.BIOELECHEM.2021.107814 [retrieved on 2021-04-17] abstract, p.3, left column, paragraph 2, figure 3B-C,p. 4, left column paragraph1 and right column paragraph 2,p.6, left column,figure 5, p.5	4-10
Y	PASQUET LISE ET AL: "Safe and efficient novel approach for non-invasive gene electrotransfer to skin", SCIENTIFIC REPORTS, [Online] vol. 8, no. 1, 15 November 2018 (2018-11-15), XP093191243, US ISSN: 2045-2322, DOI: 10.1038/s41598-018-34968-6 Retrieved from the Internet: URL:https://www.nature.com/articles/s41598-018-34968-6> abstract, p. 2, paragraph 1-3, fig. 2 and5, p.8, paragraph 2	4-10
Y	MARTINENGHI S ET AL: "Human insulin production and amelioration of diabetes in mice by electrotransfer-enhanced plasmid DNA gene transfer to the skeletal muscle", GENE THERAPY, NATURE PUBLISHING GROUP, LONDON, GB, vol. 9, no. 21, 14 October 2002 (2002-10-14), pages 1429-1437, XP037770575, ISSN: 0969-7128, DOI: 10.1038/SJ.GT.3301804 [retrieved on 2002-10-14] abstract, p.1430-1433, p. 1435-1436	5-10
Y	Young Jennifer L. ET AL: "Electroporation-Mediated Gene Delivery" In: "Advances in Genetics", 1 January 2015 (2015-01-01), Academic Press, US, XP093191583, ISSN: 0065-2660 vol. 89, pages 49-88, DOI: 10.1016/bs.adgen.2014.10.003, p. 2 paragraph 1,p.6, last paragraph - p. 7, paragraph 2,p. 10-11	5-10

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/033138

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BATISTA NAPOTNIK TINA ET AL: "Cell death due to electroporation - A review", BIOELECTROCHEMISTRY, ELESEVIER, AMSTERDAM, NL, vol. 141, 6 June 2021 (2021-06-06), XP086713914, ISSN: 1567-5394, DOI: 10.1016/J.BIOELECHEM.2021.107871 [retrieved on 2021-06-06] table 1 -2, p.11 -----	1-16
A	HOJMAN PERNILLE: "Basic Principles and Clinical Advancements of Muscle Electrotransfer", CURRENT GENE THERAPY, BENTHAM SCIENCE PUBLISHERS LTD, NL, vol. 10, no. 2, 1 April 2010 (2010-04-01), pages 128-138, XP009185948, ISSN: 1566-5232, DOI: 10.2174/156652310791110994 p.129-130 -----	1-16

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