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Combinatorial gene construct and non-viral delivery for anti-obesity in diet-induced obese mice

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ABSTRACT

The combinatorial peptidergic therapy of islet amyloid polypeptide (IAPP) and leptin (LEP) analogues was once an optimistic option in treating obese animals and patients. However, the need for frequent administrations and its negative side effects prevent it from being a viable choice. Here, we developed a combinatorial gene therapy of IAPP and LEP, where two genes are inserted into a single plasmid with self-cleaving furin and 2A sites to treat diet-induced obese (DIO) mice. The developed plasmid DNA (pDNA) individually produced both IAPP and LEP peptides in vitro and in vivo. The pDNA was delivered with a non-viral polymeric carrier, and its once-a-week administrations demonstrated a synergistic loss of body weight and significant reductions of fat mass, blood glucose, and lipid levels in DIO mice. The results suggest that the combinatorial gene therapy would have higher potential than the peptidergic approach for future translation due to its improved practicability.

1. Introduction

Excess weight and obesity have been complex and serious threats to global human health, associated with type 2 diabetes mellitus, cancer, and cardiovascular diseases [1,2]. Since the discovery of adipocyte-derived hormone Leptin (LEP) in 1994 [3], a variety of peptides and their corresponding receptors involved in energy homeostasis have been continuously discovered from the central and peripheral organs [4–6]. Even though obesity can be simply characterized by weight gain after the cumulative, excessive energy intake over energy expenditure for life and activity, it can also be caused by single gene mutations, and/or multiple environmental, psychological factors [5,7]. For the management of obesity, a host of single therapies have been investigated and considered so far. However, when a particular pathway is blocked by a single therapy, the body starts to activate alternative compensatory mechanisms to maintain the pre-existing energy balance state. This is relevant to developing resistance to monotherapy [8]. For example, LEP therapy was once thought to hold great promise in the battle against the obesity epidemic; however, LEP alone was highly effective in treating LEP-deficient subjects, but showed only limited efficacy in the major obese condition, diet-induced obesity, as a result of LEP resistance [9,10].

For a more effective and realistic approach to the treatment of obesity, combinatorial peptide therapy was strongly suggested to overcome resistance development. The LEP resistance was efficiently overcome by continuous or twice-a-day (b.i.d.) injections of LEP (metreleptin for humans) with islet amyloid polypeptide (IAPP; also known as amylin, pramlintide for humans). This approach received a great deal of attention as a solution to overcome LEP resistance through the interaction between the long-term adiposity signal by LEP and the short-term satiety signal by IAPP [8–12]. However, as noted, continuous or b.i.d. administrations of the dual therapeutic peptides limit patient compliance, cause irritation in the administrations sites, have a high cost burden, and even lead to antibody production against these peptides [10]. Moreover, when a combination of therapeutic peptides is identified to manage a particular obesity condition, its clinical tests and applications eventually become impractical due to the above reasons.

As a promising alternative to peptidergic therapy, the development of a combinatorial gene delivery system can be proposed as a safer and more workable approach for obesity treatment. Once the genes of required therapeutic peptides are identified, the gene combination can be utilized for treatment with less frequent injections and without the production of clinical-grade peptides in large quantity. The combinatorial

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gene delivery to supply multiple therapeutic peptides at one time can be established by inserting 2A and furin peptide linker genes between individual target genes in a single plasmid construction. The 2A linker-based technology enables a single transcript mRNA encoding multiple genes to express their corresponding peptides simultaneously in similar molar concentrations. It also renders stable, cell type-independent co-expression [13]. This application was exemplified in the field of induced pluripotent stem cell research [14], and generation of multi-transgenic animals [15,16]. Specifically, the self-cleaving porcine teschovirus-1 2A (P2A) presents the highest cleavage efficiency in mammalian cells among 2A family [17]. In addition, furin peptide (Arg-Ala-Lys-Arg amino acid sequence) has been known as a linker to prevent possible adverse effects derived from the residual 2A peptide in 2A-based-gene constructs [18]. Thus, the introduction of these two linker genes enables a simple gene-based combination therapy.

Since naked plasmid DNA (pDNA) is rapidly degraded following its systemic administration, an appropriate pDNA carrier is essential to prevent its degradation and prolong its circulation time. Two typical carriers, viral and non-viral delivery carriers, have been intensively used. Even though the former has ignited and propagated the area of gene therapy, it has still been confronted by many limitations, such as its tumorigenic potential from random insertion of genes and virus-induced immunogenicity. On the other hand, the non-viral delivery system has been suggested as an alternative to virus-based gene delivery by addressing safety issues [19–21]. Among the non-viral gene delivery carriers, linear polyethylenimine (lPEI) is deemed highly suitable for a proof-of the concept work, due to its wide-spread distribution of genes in body, negligible immunogenicity, low cytotoxicity, and efficient gene transport [19,20,22]. Furthermore, lPEI produced a nano-sized polyplex with pDNA, leading to improved gene induction [21,23].

Here, we have demonstrated that the combinatory bicistronic (two target genes-involved) plasmid encoding both IAPP and LEP genes accomplished a synergistic effect on body weight loss on LEP-resistant diet-induced obese (DIO) mice. The plasmid delivered with a non-viral polymeric gene vehicle, lPEI, provided anti-obesogenic effects through weekly injections. Therefore, this approach paves the way for an efficient, safe, and practical gene therapy to combat the worldwide obesity epidemic.

2. Materials and methods

2.1. Materials

In-Fusion™ kits were purchased from Clontech (Mountain View, CA, USA). Mouse-originated IAPP and LEP cDNAs were bought from OriGene (Rockville, MD, USA). BamHI, EcoRI, and NotI restriction enzymes were from New England Biolabs (Ipswich, MA, USA). lPEI (weight average molecular weight 25 kDa) was purchased from Polysciences (Warrington, PA, USA).

2.2. Preparation of plasmids

We constructed pcDNA3-IAPP, pcDNA3-LEP, and pcDNA3-FP2A-IAPP-LEP with mouse-originated IAPP and LEP genes. For bicistronic plasmid, pcDNA3-FP2A-IAPP-LEP, the IAPP and LEP genes as main target genes and furin and P2A genes (FP2A) as a linker were assembled by In-Fusion™ reaction [24]. Briefly, forward and reverse PCR primers were designed, including more than 15 base pair overlap with the neighboring segment of pcDNA3 plasmid (Invitrogen, Carlsbad, CA, USA) and more than 20 base pairs of target gene-specific sequence. Additionally, short pieces of P2A, furin, restriction enzyme sites, and translation initiation site (Kozak sequence) were added in the primer sequences. The PCR amplification was performed with the primers, mouse-originated IAPP and LEP cDNA using CloneAmp™ HiFi Premix (Clontech). After pcDNA3 was digested by BamHI and EcoRI, the PCR amplicons were ligased into the linearized pcDNA3 by In-Fusion HD Enzyme Premix (Clontech). After that, the reaction mixture was transformed into Stellar™ Competent Cells (Clontech), followed by ampicillin-contained LB agar plate colony identification. For pcDNA3-LEP construction, the procedure was similar to pcDNA3-FP2A-IAPP-LEP preparation using In-Fusion™ reaction. Following pcDNA3 digestion by BamHI and NotI, the PCR product containing LEP ORF was sub-cloned into the pcDNA3. pcDNA3-IAPP was produced by simple double enzyme digestion of IAPP gene with EcoRI and NotI from IAPP ORF-associated cDNA plasmid, followed by the insertion of the gene into linearized pcDNA3. The gene sequence of pcDNA3-FP2A-IAPP-LEP was confirmed by Sanger DNA sequencing for authenticity. The primer sequences for pcDNA3-FP2A-IAPP-LEP and pcDNA3-LEP plasmids are provided (Table s1).

2.3. Characterization of plasmids by restriction enzyme digestion and of mRNA transcription using gel electrophoresis

pcDNA3, pcDNA3-IAPP, pcDNA3-LEP, and pcDNA3-FP2A-IAPP-LEP were prepared using maxiprep kits (Macherey-Nagel, Bethlehem, PA, USA). Following the incubation with complementary restriction enzymes (New England Biolabs) and each plasmid, the size of gene inserts was verified with a 1% (w/v) agarose gel. For a single mRNA transcript characterization of each plasmid, every plasmid was initially transduced by Xfect™ Transfection Reagent (Clontech) into HEK293T (ATCC, Manassas, VA, USA) according to manufacturer's protocol. Briefly, HEK293T was incubated in the six-well plate at initial density of 5×10^5 per well for 1 d. The complex of 1.5 μL of Xfect™ and 5 μg of pDNA was added to the cells. After 2 d additional incubation, cells were harvested and centrifuged to obtain cell pellet. Total RNA was extracted from the pellet using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The first strand cDNA was synthesized from the RNA sample using M-MuLV Enzyme Mix and M-MuLV Reaction Mix (New England Biolabs). Subsequently, reverse transcriptase-polymerase chain reaction (RT-PCR) was completed with OneTaq® RT-PCR Kit (New England Biolabs) and pcDNA3-complementary T7 and SP6 primers (obtained from the University of Utah Core Facility). Each RT-PCR product was analyzed through agarose gel electrophoresis.

2.4. Formation and characterization of lPEI-pDNA polyplexes

lPEI (100 mg) was added to de-ionized water (90 ml) and the mixture was adjusted to pH 7.0 with HCl, stirred for an hour with mild heating (30–35 °C) until a clear solution was obtained. The lPEI solution was further adjusted with de-ionized water to make a final concentration of 1 mg/ml. lPEI and each developed pDNA in HEPES buffer (20 mM) were mixed by rapidly vortexing with a N:P ratio of 5 (N:P ratio indicates the mole ratio of cationic amines to anionic nucleic acid phosphates). Following incubation for 25–30 min at RT, the prepared polyplexes were used in further studies. The particle size and surface charge of polyplexes were measured using a Nano ZS (Malvern Instruments) with a He–Ne ion laser at wavelength of 633 nm.

2.5. IAPP and LEP expression assay in vitro

HEK293T cells were seeded on 6-well plates (5×10^5 cells/well) one day prior to transfection with complexes of Xfect™-pDNA. After transfection, the cells were incubated for an additional 2 d. With the culture media, IAPP and LEP expression was confirmed using IAPP EIA kit and LEP ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA), respectively. To examine lPEI as the gene delivery carrier, lPEI-pDNA polyplex nanoparticles were formed in HEPES buffer (20 mM) at N:P ratio of 5. The next procedure with respect to the lPEI-pDNA polyplex addition for IAPP and LEP identification was the same as described in Xfect™-pDNA complex transfection. For both experiments, we used media containing 10% fetal bovine serum (FBS) for the entire experimental