A COMPOSITION AND METHOD TO ALTER LEAN BODY MASS AND BONE PROPERTIES IN A SUBJECT

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application, Serial Number 60/357,808 entitled "increase Body mass, decrease body fat proportion, increase bone density and improve bone healing rate," filed on 10/26/2001, the entire content of which is hereby incorporated by reference.

BACKGROUND

[0002] The present invention pertains to compositions and methods for plasmid-mediated gene supplementation. The present invention relates to a method of decreasing body fat proportions and increasing lean body mass ("LBM") in an animal subject. Overall, the embodiments of the invention can be accomplished by delivering a nucleic acid expression construct that encodes a GHRH or functional biological equivalent thereof into a tissue of a subject and allowing expression of the encoded gene in the subject. For example, when such a nucleic acid sequence is delivered into the specific cells of the subject tissue specific constitutive expression is achieved. Furthermore, external regulation of the GHRH or functional biological equivalent thereof gene can be accomplished by utilizing inducible promoters that are regulated by molecular switch molecules, which are given to the subject. The preferred method to deliver the constitutive or inducible nucleic acid encoding sequences of GHRH or the functional biological equivalents thereof is directly into the cells of the subject by the process of *in vivo* electroporation. In addition, this invention also relates to a method of increasing bone density and improvising the rate of bone healing in an animal subject. More specifically, this invention pertains to both an *in vivo* and an *ex vivo* method for delivering a heterologous nucleic acid sequence encoding growth hormone releasing hormone "GHRH" or functional biological equivalent thereof into the cells of the subject and allowing expression of the encoded gene to occur while the modified cells are within the subject. Another embodiment of the present invention relates to regulating the expression of the GHRH using a molecular switch (*e.g.* mifepistone).

[0003] <u>Growth Hormone ("GH") and Immune Function:</u> The central role of growth hormone ("GH") in controlling somatic growth in humans and other vertebrates, and the physiologically relevant pathways regulating GH secretion from the pituitary are well known. The GH production pathway is composed of a series of interdependent genes whose

products are required for normal growth. The GH pathway genes include: (1) ligands, such as GH and insulin-like growth factor-I ("IGF-I"); (2) transcription factors such as prophet of pit 1, or prop 1, and pit 1: (3) agonists and antagonists, such as growth hormone releasing hormone ("GHRH") and somatostatin ("SS"), respectively; and (4) receptors, such as GHRH receptor ("GHRH-R") and the GH receptor ("GH-R"). These genes are expressed in different organs and tissues, including the hypothalamus, pituitary, liver, and bone. Effective and regulated expression of the GH pathway is essential for optimal linear growth, as well as homeostasis of carbohydrate, protein, and fat metabolism GH synthesis and secretion from the anterior pituitary is stimulated by GHRH and inhibited by somatostatin, both hypothalamic hormones. GH increases production of IGF-I, primarily in the liver, and other target organs. IGF-I and GH, in turn, feedback on the hypothalamus and pituitary to inhibit GHRH and GH release. GH elicits both direct and indirect actions on peripheral tissues, the indirect effects being mediated mainly by IGF-I.

[0004] The principal feature of GH deficiencies in children is short stature. Similar phenotypes are produced by genetic defects at different points in the GH axis, as well as non-GH-deficient short stature. Non-GH-deficiencies have different etiology, such as: (1) genetic diseases, Turner syndrome, hypochondroplasia; and (2) chronic renal insufficiency. Cases where the GH axis is unaffected (*i.e.*, patients have normal hormones, genes and receptors) account for more than 50% of the total cases of growth retardation. In these cases GHRH and GH therapy has been shown to be effective (Gesundheit and Alexander, 1995).

[0005] Reduced GH secretion from the anterior pituitary causes skeletal muscle mass to be lost during aging from 25 years to senescence. The GHRH-GH-IGF-I axis undergoes dramatic changes through aging and in the elderly with decreased GH production rate and GH half-life, decreased IGF-I response to GH and GHRH stimuli leads to loss of skeletal muscle mass (sarcopenia), osteoporosis, and increase in fat and decrease in lean body mass. Previous studies have shown that in elderly the level of GH secretion is significant reduced by 70-80% of teenage level. It has been demonstrated that the development of sarcopenia can be offset by exogenous GH therapy. However, this remains a controversial therapy in the elderly because of its cost and frequent side effects.

[0006] The production of recombinant proteins allows a useful tool for the treatment of these conditions. Although GH replacement therapy is widely used in patients

with growth deficiencies and provides satisfactory growth, and may have positive psychological effects on the children being treated, this therapy has several disadvantages, including an impractical requirement for frequent administration of GH and undesirable secondary effects.

[0007] GH is released in a distinctive pulsatile pattern that has profound importance for its biological activity (Argente et al., 1996). Secretion of GH is stimulated by the natural GH secretagogue, GHRH, and inhibited by somatostatin (SS), and both hypothalamic hormones (Thorner et al., 1990). GH pulses are a result of GHRH secretion that is associated with a diminution or withdrawal of somatostatin secretion. In addition, the pulse generator mechanism is timed by GH-negative feedback. The endogenous rhythm of GH secretion becomes entrained to the imposed rhythm of exogenous GH administration. Effective and regulated expression of the GH and insulin-like growth factor I ("IGF-I") pathway is essential for optimal linear growth, homeostasis of carbohydrate, protein, and fat metabolism, and for providing a positive nitrogen balance (Murray and Shalet, 2000). Numerous studies in humans, sheep or pigs showed that continuous infusion with recombinant GHRH protein restores the normal GH pattern without desensitizing GHRH receptors or depleting GH supplies as this system is capable of feed-back regulation, which is abolished in the GH therapies (Dubreuil et al., 1990a; Vance et al., 1985b; Vance, 1990; Vance et al., 1985a). Thus, GHRH recombinant protein treatment may be more physiologically relevant than GH therapy. However, due to the short half-life of GHRH *in vivo*, frequent (one to three times per day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administrations are necessary (Evans et al., 2001; Thorner et al., 1986). Thus, as a chronic therapy, recombinant GHRH protein administration is not practical.

[0008] Extracranially secreted GHRH, as mature peptide or truncated molecules (as seen with pancreatic islet cell tumors and variously located carcinoids) are often biologically active and can even produce acromegaly (Thorner et al., 1984). Administration of recombinant GHRH to GH-deficient children or adult humans augments IGF-I levels, increases GH secretion proportionally to the GHRH dose, yet still invokes a response to bolus doses of GHRH (Bercu et al., 1997). Thus, GHRH administration represents a more physiological alternative of increasing subnormal GH and IGF-I levels (Corpas et al., 1993).

[0009] Although GHRH protein therapy entrains and stimulates normal cyclical GH secretion with virtually no side effects, the short half-life of GHRH *in vivo* requires frequent (one to three times a day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administration. Thus, as a chronic treatment, GHRH administration is not practical. Extracranially secreted GHRH, as processed protein species GHRH(l-40) hydroxy or GHRH(l-44) amide or even as shorter truncated molecules, are biological active (Thorner et al., 1984). It has been reported that a low level of GHRH (100 pg/ml) in the blood supply stimulates GH secretion (Corpas et al., 1993). Direct plasmid DNA gene transfer is currently the basis of many emerging therapy strategies and thus does not require viral genes or lipid particles (Aihara and Miyazaki, 1998; Muramatsu et al., 1998). Skeletal muscle is target tissue, because muscle fiber has a long life span and can be transduced by circular DNA plasmids that express over months or years in an immunocompetent host (Davis et al., 1993; Tripathy et al., 1996). Previous reports demonstrated that human GHRH cDNA could be delivered to muscle by an injectable myogenic expression vector in mice where it transiently stimulated GH secretion to a modes extent over a period of two weeks (Draghia-Akli et al., 1997).

[0010] Wild type GHRH has a relatively short half-life in the circulatory system, both in humans (Frohman et al., 1984) and in farm animals. After 60 minutes of incubation in plasma 95% of the GHRH(1-44)NH2 is degraded, while incubation of the shorter (1-40)OH form of the hormone, under similar conditions, shows only a 77% degradation of the peptide after 60 minutes of incubation (Frohman et al., 1989). Incorporation of cDNA coding for a particular protease-resistant GHRH analog in a therapeutic nucleic acid vector results in a molecule with a longer half-life in serum, increased potency, and provides greater GH release in plasmid-injected animals (Draghia-Akli et al., 1999), herein incorporated by reference). Mutagenesis *via* amino acid replacement of protease sensitive amino acids prolongs the serum half-life of the GHRH molecule. Furthermore, the enhancement of biological activity of GHRH is achieved by using super-active analogs that may increase its binding affinity to specific receptors (Draghia-Akli et al., 1999).

[0011] Administering novel GHRH analog proteins (U.S. Pat Nos. 5,847,066; 5846,936; 5,792,747; 5,776,901; 5,696,089; 5,486,505; 5,137,872; 5,084,442, 5,036,045; 5,023,322; 4,839,344; 4,410,512, RE33,699) or synthetic or naturally occurring peptide fragments of GHRH (U.S. Pat. Nos. 4,833,166; 4,228,158; 4,228,156; 4,226,857; 4,224,316; 4,223,021; 4,223,020; 4,223, 019) for the purpose of increasing release of growth hormone

have been reported. A GHRH analog containing the following mutations has been reported (U.S. Patent No. 5,846,936): Tyr at position 1 to His; Ala at position 2 to Val, Leu, or others; Asn at position 8 to Gln, Ser, or Thr; Gly at position 15 to Ala or Leu; Met at position 27 to Nle or Leu; and Ser at position 28 to Asn. The GHRH analog is the subject of U.S. Patent Application Serial No. 09/624,268 ("the '268 patent application"), which teaches application of a GHRH analog containing mutations that improve the ability to elicit the release of growth hormone. In addition, the '268 patent application relates to the treatment of growth deficiencies; the improvement of growth performance; the stimulation of production of growth hormone in an animal at a greater level than that associated with normal growth; and the enhancement of growth utilizing the administration of growth hormone releasing hormone analog and is herein incorporated by reference. In the embodiments of the '268 patent application and specific embodiments herein, the mutated GHRH-encoding molecules lack the Gln, Ser or Thr mutations of the Asn at position 8.

[0012] U.S. Patent No. 5,061,690 is directed toward increasing both birth weight and milk production by supplying to pregnant female mammals an effective amount of human GHRH or one of it analogs for 10-20 days. Application of the analogs lasts only throughout the lactation period. However, multiple administrations are presented, and there is no disclosure regarding administration of the growth hormone releasing hormone (or factor) as a DNA molecule, such as with plasmid mediated supplementation techniques.

[0013] U.S. Patent Nos. 5,134,120 (" the '120 patent") and 5,292,721 (" the '721 patent") teach that by deliberately increasing growth hormone in swine during the last 2 weeks of pregnancy through a 3 week lactation resulted in the newborn piglets having marked enhancement of the ability to maintain plasma concentrations of glucose and free fatty acids when fasted after birth. In addition, the '120 and '721 patents teaches that treatment of the sow during lactation results in increased milk fat in the colostrum and an increased milk yield. These effects are important in enhancing survivability of newborn pigs and weight gain prior to weaning. However the '120 and '721 patents provide no teachings regarding administration of the growth hormone releasing hormone as a DNA form.

[0014] In contrast to protein therapy, nucleic acid transfer delivers polynucleotides to somatic tissue in a manner that, in some embodiments, can correct inborn or acquired deficiencies and imbalances. In other embodiments, vectors such as plasmids are

used to supplement basal levels of an expressed endogenous gene product. Gene-based drug delivery offers a number of advantages over the administration of recombinant proteins. These advantages include the conservation of native protein structure, improved biological activity, avoidance of systemic toxicities, and avoidance of infectious and toxic impurities. In addition, nucleic acid transfer, for therapeutic purposes, and plasmid-mediated supplementation of an endogenous gene product allow for prolonged exposure to the protein in the therapeutic range, because the newly secreted protein is present continuously in the blood circulation.

[0015] The primary limitation of using recombinant protein is the limited availability of protein after each administration. Plasmid-mediated gene supplementation using injectable DNA plasmid vectors overcomes this, because a single injection into the patient's skeletal muscle permits physiologic expression for extensive periods of time (WO 99/05300 and WO 01/06988). Injection of the vectors promotes the production of enzymes and hormones in animals in a manner that more closely mimics the natural process. Furthermore, among the non-viral techniques for gene transfer *in vivo*, the direct injection of plasmid DNA into muscle tissue is simple, inexpensive, and safe.

[0016] In a plasmid-mediated supplementation expression system, a non-viral nucleic acid vector, such as a plasmid, may comprise a synthetic nucleic acid delivery system in addition to a nucleic acid encoding the GHRH being supplemented. In this way, the risks associated with the use of most viral vectors can be avoided. The non-viral expression vector products generally have low toxicity due to the use of "species-specific" components for nucleic acid delivery, which minimizes the risks of immunogenicity generally associated with viral vectors. Additionally, no integration of plasmid sequences into host chromosomes has been reported *in vivo* to date, so that this type of plasmid-mediated supplementation of GHRH, should neither activate oncogenes nor inactivate tumor suppressor genes. As episomal systems residing outside the chromosomes, plasmids have defined pharmacokinetics and elimination profiles, leading to a finite duration of gene expression in target tissues. Furthermore, the plasmid DNA could be engineered so it would be delivered to the cells in a linear rather than circular form (which would further prevent any genomic integration event); the plasmid could be deleted of the antibiotic resistance gene and bacterial origin of replication, making it completely safe for *in vivo* therapy.

[0017] Efforts have been made to enhance the delivery of plasmid DNA to cells by physical means including electroporation, sonoporation, and pressure. Injection by electroporation involves the application of a pulsed electric field to create transient pores in the cellular membrane without causing permanent damage to the cell. It thereby allows for the introduction of exogenous molecules (Smith and Nordstrom, 2000). By adjusting the electrical pulse generated by an electroporetic system, nucleic acid molecules can travel through passageways or pores in the cell that are created during the procedure. U.S. Patent No. 5,704,908 describes an electroporation apparatus for delivering molecules to cells at a selected location within a cavity in the body of a patient. These pulse voltage injection devices are also described in U.S. Patent Nos. 5,439,440 and 5,702,304, and PCT WO 96/12520, 96/12006, 95/19805, and 97/07826.

[0018] Recently, significant progress has been obtained using electroporation to enhance plasmid delivery *in vivo*. Electroporation has been used very successfully to transfect tumor cells after injection of plasmid (Lucas et al., 2002; Matsubara et al., 2001) or to deliver the anti-tumor drug bleomycin to cutaneous and subcutaneous tumors in humans (Gehl et al., 1998; Heller et al., 1996). Electroporation also has been extensively used in mice (Lesbordes et al., 2002; Lucas et al., 2001; Vilquin et al., 2001), rats (Terada et al., 2001; Yasui et al., 2001), and dogs (Fewell et al., 2001) to deliver therapeutic genes that encode for a variety of hormones, cytokines or enzymes. Our previous studies using growth hormone releasing hormone ("GHRH") showed that plasmid therapy with electroporation is scalable and represents a promising approach to induce production and regulated secretion of proteins in large animals and humans (Draghia-Akli et al., 1999; Draghia-Akli et al., 2002).

[0019] The ability of electroporation to enhance plasmid uptake into the skeletal muscle has been well documented, as described above. In addition, plasmid formulated with poly-L-glutamate ("PLG") or polyvinylpyrolidone (PVP) has been observed to increase plasmid transfection and consequently expression of the desired transgene. The anionic polymer sodium PLG could enhance plasmid uptake at low plasmid concentrations, while reducing any possible tissue damage caused by the procedure. The ability of electroporation to enhance plasmid uptake into the skeletal muscle has been well documented, as previously described. PLG is a stable compound and resistant to relatively high temperatures (Dolnik et al., 1993). PLG has been previously used to increase stability in vaccine preparations (Matsuo et al., 1994) without increasing their immunogenicity. It also has been used as an anti-toxin

post-antigen inhalation or exposure to ozone (Fryer and Jacoby, 1993). In addition, plasmid formulated with PLG or polyvinylpyrrolidone (PVP) has been observed to increase gene transfection and consequently gene expression to up to 10 fold in the skeletal muscle of mice, rats and dogs (Fewell et al., 2001; Mumper et al., 1998). PLG has been used to increase stability of anti-cancer drugs (Li et al., 2000) and as "glue" to close wounds or to prevent bleeding from tissues during wound and tissue repair (Otani et al., 1996; Otani et al., 1998).

[0020] Although not wanting to be bound by theory, PLG will increase the transfection of the plasmid during the electroporation process, not only by stabilizing the plasmid DNA, and facilitating the intracellular transport through the membrane pores, but also through an active mechanism. For example, positively charged surface proteins on the cells could complex the negatively charged PLG linked to plasmid DNA through protein-protein interactions. When an electric field is applied, the surface proteins reverse direction and actively internalize the DNA molecules, process that substantially increases the transfection efficiency.

[0021] The use of directly injectable DNA plasmid vectors has been limited in the past. The inefficient DNA uptake into muscle fibers after simple direct injection has led to relatively low expression levels (Prentice et al., 1994; Wells et al., 1997) In addition, the duration of the transgene expression has been short (Wolff et al., 1990). The most successful previous clinical applications have been confined to vaccines (Danko and Wolff, 1994; Tsurumi et al., 1996).

[0022] Although there are references in the art directed to electroporation of eukaryotic cells with linear DNA (McNally et al., 1988; Neumann et al., 1982) (Toneguzzo et al., 1988) (Aratani et al., 1992; Nairn et al., 1993; Xie and Tsong, 1993; Yorifuji and Mikawa, 1990), these examples illustrate transfection into cell suspensions, cell cultures, and the like, and the transfected cells are not present in a somatic tissue.

[0023] U.S. Patent No. 4,956,288 is directed to methods for preparing recombinant host cells containing high copy number of a foreign DNA by electroporating a population of cells in the presence of the foreign DNA, culturing the cells, and killing the cells having a low copy number of the foreign DNA.

[0024] U.S. Patent No. 5,874,534 ("the '534 patent") and U.S. Patent No. 5,935,934 ("the '934 patent") describe mutated steroid receptors, methods for their use and a molecular switch for nucleic acid transfer, the entire content of each is hereby incorporated by reference. A molecular switch for regulating expression in nucleic acid transfer and methods of employing the molecular switch in humans, animals, transgenic animals and plants (e.g. GeneSwitch®) are described in the '534 patent and the '934 patent. The molecular switch is described as a method for regulating expression of a heterologous nucleic acid cassette for nucleic acid transfer and is comprised of a modified steroid receptor that includes a natural steroid receptor DNA binding domain attached to a modified ligand binding domain. The modified binding domain usually binds only non-natural ligands, anti-hormones or non-native ligands. One skilled in the art readily recognizes natural ligands do not readily bind the modified ligand-binding domain and consequently have very little, if any, influence on the regulation and/or expression of the gene contained in the nucleic acid cassette.

[0025] Thus, the present invention is directed to a novel method of increasing lean body mass, decreasing body fat proportions, increasing bone density, and/or increasing the rate of bone healing in an animal by plasmid-mediated supplementation of GHRH.

SUMMARY

[0026] One embodiment of the present invention pertains to a method for decreasing the body fat proportion, increasing lean body mass ("LBM"), increasing bone density, and increasing the rate of bone healing of a subject by utilizing a nucleic acid sequence containing both a constitutive promoter and an encoding sequence for growth hormone releasing hormone ("GHRH") or analog thereof. When this nucleic acid sequence is delivered into the specific cells of the subject (*e.g.* somatic cells, stem cells, or germ cells), tissue specific constitutive expression of GHRH is achieved. The preferred method to deliver the nucleic acid sequence with the constitutive promoter and the encoding sequence of GHRH or the analog thereof is directly into the cells of the subject by the process of *in vivo* electroporation. Electroporation may involve externally supplied electrodes, or in the case of needles, internally supplied electrodes to aid in the inclusion of desired nucleotide sequences into the cells of a subject while the cells are within a tissue of the subject.

[0027] Another embodiment of the present invention pertains to a method for decreasing the body fat proportion, increasing LBM, increasing bone density, and increasing bone healing rate of a subject by utilizing the ability to regulate the expression of GHRH or analog thereof. Regulation is achieved by delivering into the cells of the subject a first nucleic acid sequence, and a second nucleic acid sequence, followed by a molecular switch; where the first nucleic acid sequence contains an inducible-promoter with a coding region for a growth-hormone-releasing-hormone ("inducible-GHRH") or an analog thereof and the second nucleic acid sequence has a constitutive promoter with a coding region for an inactive regulator protein. By delivering a molecular switch molecule (*e.g.* mifepistone) into the subject, the inactive regulator protein becomes active and initiates transcription of the inducible-GHRH in the subject. The expression and ensuing release of GHRH or analog thereof by the modified-cells within the subject will decrease the body fat proportion and increase the LBM of the subject in a manner that can be regulated by external molecular switch molecules (*e.g.* mifepistone). The delivery of the nucleic acid sequences that allow external regulation of GHRH or the analog thereof directly into the cells of the subject can be accomplished by the process of *in vivo* electroporation.

[0028] A further embodiment of the present invention pertains to a method for increasing lean body mass, decreasing body fat proportion, increasing bone density, increasing

the rate of bone healing, or a combination thereof, of a subject by utilizing therapy that introduces specific recombinant GHRH-analog protein into the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0030] FIGURE 1 shows the amino acid sequence of GHRH or functional biological equivalent thereof. All mutant sequences were obtained by site directed mutagenesis of the porcine wild type sequence.

[0031] FIGURE 2 shows the body weight of SCID mice that were injected with 7.5 micrograms of pSP-GHRH mutants.

[0032] FIGURE 3 shows the body composition of SCID mice that were injected with 7.5 micrograms of plasmid expressing either one of the GHRH mutants or a pSP-beta-galactosidase as control.

[0033] FIGURE 4 shows the bone area of SCID mice that were injected with 7.5 micrograms of plasmid expressing either one of the GHRH mutants or a pSP-beta-galactosidase as control.

[0034] FIGURE 5 shows the IGF-I levels of SCID mice that were injected with 7.5 micrograms of plasmid expressing either one of the GHRH mutants or a pSP-beta-galactosidase as control.

[0035] FIGURE 6 shows a schematic of the mifepristone-dependent GHRH/GeneSwitch® system in primary myoblasts *in vitro*. Plasmid structures and schematic for how the GeneSwitch® system works are illustrated. Plasmid p1633 encodes for the GeneSwitch® regulator protein, which is a chimera of yeast GAL4 DNA binding domain ("GAL4"), truncated human progesterone receptor ligand-binding domain ("hPR LBD"), and activation domain from the p65 subunit of human NF-κB ("p65"). The protein is synthesized as an inactive monomer. Binding of mifepristone triggers a conformational change that leads to activation and dimerization. Activated homodimers bind to GAL4 sites in the inducible promoter and stimulate transcription of the GHRH gene.

[0036] FIGURE 7 shows the function of a mifepristone-dependent GHRH/GeneSwitch® system in primary myoblasts *in vitro*. Northern blot analysis of inducible GHRH constructs. Primary chicken myoblast cultures were obtained and transfected as described previously (Bergsma et al., 1986; Draghia-Akli et al., 1997), with 4 micrograms of a 10:1 mixture of inducible GHRH ("pGR1774") and GeneSwitch® plasmids ("pGS1633"). A Muscle specific synthetic promoter (Li et al., 1999) driven construct coding for *E. coli* beta-galactosidase, □ βgal, is used as a negative control. As a positive control, cells were transfected with a constitutively active pSP-GHRH construct (Draghia-Akli et al., 1999). In the figure, Nt = non-transfected cells; β-gal = cells transfected with pSP-β-gal construct; SP-GHRH = cells transfected with a constitutively active GHRH construct; +MFP = mifepristone was added to the culture media; and - MFP = mifepristone was not added to the culture media. Ethidium bromide gels are included as loading controls.

[0037] FIGURE 8 shows that the mifepristone dosing induces serum IGF-I levels in SCID mice that received a single administration of GHRH/GeneSwitch® plasmids. Values are presented as fold activation over control levels. The area under the dark line represents normal variability of IGF-I levels in adult animals. The table contains the p values for the induced peaks. The p values C v. A indicate comparison between animals injected with the β-gal construct versus animals injected with the IS + MFP; C v. B. indicates comparison between animals injected with the IS with and without the MFP.

[0038] FIGURE 9 shows the enhanced weight gain during a chronic 149 day MFP induction. Average weight increased in injected mice upon chronic activation of the GHRH/GeneSwitch® system (* $p < 0.027$).

[0039] FIGURE 10 shows the increase in pituitary weight with a chronic 149 day MFP induction. Pituitary weight / total body weight in +MFP injected animals (* $p < 0.035$).

[0040] FIGURE 11 shows the improved body composition in chronically induced GHRH/GeneSwitch® mice. Body composition measurements were performed either under anesthesia, at day 149 post-injection ("PIXImus") or post-mortem (organ, carcass, body fat, direct dissection of the body). Lean non-bone mass is significantly increased (* $p < 0.022$).

[0041] FIGURE 12 shows the improved fat body mass/ total weight in chronically induced GHRH/GeneSwitch® mice. Fat body mass/ total weight measurements were performed either under anesthesia, at day 149 post-injection ("PIXImus") or post-mortem (organ, carcass, body fat, direct dissection of the body). Fat body mass/ total weight is significantly decreased in induced animals (* $p < 0.05$).

[0042] FIGURE 13 shows the increased bone area in chronically induced GHRH/GeneSwitch® mice. Bone area measurements were performed either under anesthesia, at day 149 post-injection ("PIXImus") or post-mortem (organ, carcass, body fat, direct dissection of the body). Bone area is increased by PIXImus (*$p < 0.0006$).

[0043] FIGURE 14 shows the increased mineral content in chronically induced GHRH/GeneSwitch® mice. Bone mineral content measurements were performed either under anesthesia, at day 149 post-injection ("PIXImus") or post-mortem (organ, carcass, body fat, direct dissection of the body). Bone mineral content is increased in induced animals (* $p < 0.002$).

[0044] FIGURE 15 shows the secreted embryonic alkaline phosphatase ("SEAP") plasma concentration in pigs at 0 to 7 days post-injection. Different needle-type electrodes were compared with calipers electrodes following the plasmid injection into the muscle. In the figure, N6 = Six-needle array electrode: 21 gauge needles, 2 cm length mounted on a 1 cm - diameter array; N3 = three-needle array device: two solid needles, 1 median hypodermic needle, 21 gauge, 2 cm length; and C = caliper electrode: 2 solid square plate electrodes, 1.5 cm. Voltage and number of pulses are also indicated. At 7 days post-injection $p < 0.006$ for N3/ 200V/ 6 pulses and N6/100V/ 6 pulses groups, and $p < 0.0035$ for N6/200V/ 6 pulses group.

[0045] FIGURE 16 shows the body weights of pigs injected at 10 days of age with 3, 1 and 0.1 mg of pSP-HV-GHRH or vehicle. The greatest weight gain was achieved by pigs injected with the lowest dose, with statistically significant differences from the controls at all time points tested ($p < 0.02$). Values are means ± s.e.m.

[0046] FIGURE 17 shows the body weights of pigs injected with 2 milligrams of pSP-HV-GHRH at 0, 7, 14 and 21 days of age. Animal injected at 14 days of age showed

the greatest weight gain, statistically different from the controls at all time points tested ($p < 0.02$). Values are means ± s.e.m.

[0047] FIGURE 18 shows the plasma IGF-I concentration after direct intramuscular injection of the different quantities of pSP-HV-GHRH construct. Values are means ± s.e.m.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0048] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DEFINITIONS

[0049] The term "a" or "an" as used herein in the specification may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

[0050] The term "any range derivable therein" as used herein means a range selected from the numbers described in the specification, and "any integer derivable therein" means any integer between such a range.

[0051] The term "analog" as used herein includes any mutant of GHRH, or synthetic or naturally occurring peptide fragments of GHRH, such as HV-GHRH (SeqID No: 1), TI-GHRH (SeqID No: 2), TV-GHRH (SeqID No: 3), 15/27/28-GHRH (SeqID No: 4), (1-44)NH_2 (SeqID No: 5) or (1-40)OH (SeqID No: 6) forms, or any shorter form to no less than (1-29) amino acids.

[0052] The term "bone density" as used herein is defined as the density of minerals in the bone as measured by a standard means in the art, such as x-ray, MRI, dual-energy x-ray absorbitometry (DEXA), or any advanced imaging system in the art.

[0053] The term "cassette" as used herein is defined as one or more transgene expression vectors.

[0054] The term "cell-transfecting pulse" as used herein is defined as a transmission of a force which results in transfection of a vector, such as a linear DNA fragment, into a cell. In some embodiments, the force is from electricity, as in electroporation, or the force is from vascular pressure.

[0055] The term "coding region" as used herein refers to any portion of the DNA sequence that is transcribed into messenger RNA (mRNA) and then translated into a sequence of amino acids characteristic of a specific polypeptide.

[0056] The term "delivery" or "delivering" as used herein is defined as a means of introducing a material into a tissue, a subject, a cell or any recipient, by means of chemical or biological process, injection, mixing, electroporation, sonoporation, or combination thereof, either under or without pressure.

[0057] The term "donor-cells" as used herein refers to any cells that have been removed and maintained in a viable state for any period of time outside the donor-subject.

[0058] The term "donor-subject" as used herein refers to any species of the animal kingdom wherein cells have been removed and maintained in a viable state for any period of time outside the subject.

[0059] The term "DNA fragment" or "nucleic acid expression construct" as used herein refers to a substantially double stranded DNA molecule. Although the fragment may be generated by any standard molecular biology means known in the art, in some embodiments the DNA fragment or expression construct is generated by restriction digestion of a parent DNA molecule. The terms "expression vector," "expression cassette," or "expression plasmid" can also be used interchangeably. Although the parent molecule may be any standard molecular biology DNA reagent, in some embodiments the parent DNA molecule is a plasmid.

[0060] The term "electroporation" as used herein refers to a method that utilizes electrical pulses to deliver a nucleic acid sequence into cells.

[0061] The terms "electrical pulse" and "electroporation" as used herein refer to the administration of an electrical current to a tissue or cell for the purpose of taking up a nucleic acid molecule into a cell. A skilled artisan recognizes that these terms are associated with the terms "pulsed electric field" "pulsed current device" and "pulse voltage device." A skilled artisan recognizes that the amount and duration of the electrical pulse is dependent on the tissue, size, and overall health of the recipient subject, and furthermore knows how to determine such parameters empirically.

[0062] The term "encoded GHRH" as used herein is a biologically active polypeptide of GHRH.

[0063] The term "functional biological equivalent" of GHRH as used herein is a polypeptide that has a distinct amino acid sequence from a wild type GHRH polypeptide while simultaneously having similar or improved biological activity when compared to the GHRH polypeptide. The functional biological equivalent may be naturally occurring or it may be modified by an individual. A skilled artisan recognizes that the similar or improved biological activity as used herein refers to facilitating and/or releasing growth hormone or other pituitary hormones. A skilled artisan recognizes that in some embodiments the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biological activity when compared to the GHRH polypeptide. Methods known in the art to engineer such a sequence include site-directed mutagenesis.

[0064] The term "GeneSwitch®" (which is a registered trademark of Valentis, Inc. (Burlingame, CA)) as used herein refers to the technology of mifepristone-inducible heterologous nucleic acid sequences encoding regulator proteins, GHRH, functional biological equivalent or combination thereof. Such a technology is schematically diagramed in FIGURE 1A. A skilled artisan recognizes that antiprogesterone agent alternatives to mifepristone are available, including onapristone, ZK112993, ZK98734, and 5α pregnane-3,2-dione.

[0065] The term "growth hormone" ("GH") as used herein is defined as a hormone that relates to growth and acts as a chemical messenger to exert its action on a target cell. In a specific embodiment, the growth hormone is released by the action of growth hormone releasing hormone.

[0066] The term "growth hormone releasing hormone" ("GHRH") as used herein is defined as a hormone that facilitates or stimulates release of growth hormone, and in a lesser extent other pituitary hormones, such as prolactin. It is understood that the GHRH, the recombinant GHRH, or a functional biological equivalent are biologically active.

[0067] The term "heterologous nucleic acid sequence" as used herein is defined as a DNA sequence comprising differing regulatory and expression elements.

[0068] The term "lean body mass" ("LBM") as used herein is defined as the mass of the body of an animal attributed to non-fat tissue such as muscle.

[0069] The term "modified cells" as used herein is defined as the cells from a subject that have an additional nucleic acid sequence introduced into the cell.

[0070] The term "modified-donor-cells" as used herein refers to any donor-cells that harbor a GHRH encoding nucleic acid sequence.

[0071] The term "molecular switch" as used herein refers to a molecule that is delivered into a subject that can regulate transcription of a gene. A skilled artisan recognizes that there are many such switches known in the art, such as a tetracycline switch, a zinc finger switch, a glucocorticoid switch, and so forth.

[0072] The term "nucleic acid expression construct" as used herein refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. The term "expression vector" can also be used interchangeably herein. In specific embodiments, the nucleic acid expression construct comprises: a promoter; a nucleotide sequence of interest; and a 3′ untranslated region; wherein the promoter, the nucleotide sequence of interest, and the 3' untranslated region are operatively linked; and *in vivo* expression of the nucleotide sequence of interest is regulated by the promoter.

[0073] The term "operatively linked" as used herein refers to elements or structures in a nucleic acid sequence that are linked by operative ability and not physical location. The elements or structures are capable of, or characterized by accomplishing a desired operation. It is recognized by one of ordinary skill in the art that it is not necessary for elements or structures in a nucleic acid sequence to be in a tandem or adjacent order to be operatively linked.

[0074] The term "poly-L-glutamate ("PLG")" as used herein refers to a biodegradable polymer of L-glutamic acid that is suitable for use as a vector or adjuvant for DNA transfer into cells with or without electroporation.

[0075] The term "post-injection" as used herein refers to a time period following the introduction of a nucleic acid cassette (that contains heterologous nucleic acid sequence encoding GHRH or functional biological equivalent thereof) into the cells of a subject and

the allowing of the expression of the encoded gene to occur while the modified cells are within the living organism.

[0076] The term "plasmid" as used herein refers generally to a construction comprised of extra-chromosomal genetic material, usually of a circular duplex of DNA that can replicate independently of chromosomal DNA. Plasmids, or fragments thereof, may be used as vectors. Plasmids are double-stranded DNA molecule that occur or are derived from bacteria and (rarely) other microorganisms. However, mitochondrial and chloroplast DNA, yeast killer and other cases are commonly excluded.

[0077] The term "plasmid mediated gene supplementation" as used herein refers a method to allow a subject to have prolonged exposure to a therapeutic range of a therapeutic protein by utilizing a nucleic acid expression construct *in vivo*.

[0078] The term "pulse voltage device," or "pulse voltage injection device" as used herein relates to an apparatus that is capable of causing or causes uptake of nucleic acid molecules into the cells of an organism by emitting a localized pulse of electricity to the cells. The cell membrane then destabilizes, forming passageways or pores. Conventional devices of this type are calibrated to allow one to select or adjust the desired voltage amplitude and the duration of the pulsed voltage. The primary importance of a pulse voltage device is the capability of the device to facilitate delivery of compositions of the invention, particularly linear DNA fragments, into the cells of the organism.

[0079] The term "plasmid backbone" as used herein refers to a sequence of DNA that typically contains a bacterial origin of replication, and a bacterial antibiotic selection gene, which are necessary for the specific growth of only the bacteria that are transformed with the proper plasmid. However, there are plasmids, called mini-circles, that lack both the antibiotic resistance gene and the origin of replication (Darquet et al., 1997; Darquet et al., 1999; Soubrier et al., 1999). The use of *in vitro* amplified expression plasmid DNA (i.e. non-viral expression systems) avoids the risks associated with viral vectors. The non-viral expression systems products generally have low toxicity due to the use of "species-specific" components for gene delivery, which minimizes the risks of immunogenicity generally associated with viral vectors. One aspect of the current invention is that the plasmid backbone does not contain viral nucleotide sequences.

[0080] The term "promoter" as used herein refers to a sequence of DNA that directs the transcription of a gene. A promoter may direct the transcription of a prokaryotic or eukaryotic gene. A promoter may be "inducible", initiating transcription in response to an inducing agent or, in contrast, a promoter may be "constitutive", whereby an inducing agent does not regulate the rate of transcription. A promoter may be regulated in a tissue-specific or tissue-preferred manner, such that it is only active in transcribing the operable linked coding region in a specific tissue type or types.

[0081] The term "rate of bone healing" as used herein is defined as the time required to repair a bone fracture.

[0082] The term "recipient-subject" as used herein refers to any species of the animal kingdom wherein modified-donor-cells can be introduced from a donor-subject.

[0083] The term "regulator protein" as used herein refers to a protein that increases or facilitates transcription of a target nucleic acid sequence.

[0084] The term "residual linear plasmid backbone" as used herein comprises any fragment of the plasmid backbone that is left at the end of the process making the nucleic acid expression plasmid linear.

[0085] The terms "subject" or "animal" as used herein refers to any species of the animal kingdom. In preferred embodiments, it refers more specifically to humans and domesticated animals used for: pets (*e.g.* cats, dogs, *etc.*); work (*e.g.* horses, *etc.*); food (cows, chicken, fish, lambs, pigs, etc); and all others known in the art.

[0086] The term "tissue" as used herein refers to a collection of similar cells and the intercellular substances surrounding them. A skilled artisan recognizes that a tissue is an aggregation of similarly specialized cells for the performance of a particular function. For the scope of the present invention, the term tissue does not refer to a cell line, a suspension of cells, or a culture of cells. In a specific embodiment, the tissue is electroporated *in vivo*. In another embodiment, the tissue is not a plant tissue. A skilled artisan recognizes that there are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue. In a specific embodiment, the methods

and compositions are directed to transfer of linear DNA into a muscle tissue by electroporation.

[0087] The term "therapeutic element" as used herein comprises nucleic acid sequences that will lead to an *in vivo* expression of an encoded gene product. One skilled in the art of molecular biology will recognize that the therapeutic element may include, but is not limited to a promoter sequence, a transgene, a poly A sequence, or a 3' or 5' UTR.

[0088] The term "transfects" as used herein refers to introduction of a nucleic acid into a eukaryotic cell. In some embodiments, the cell is not a plant tissue or a yeast cell.

[0089] The term "viral backbone" as used herein refers to a nucleic acid sequence that does not contain a promoter, a gene, and a 3' poly A signal or an untranslated region, but contain elements including, but not limited at site-specific genomic integration Rep and inverted terminal repeats ("ITRs") or the binding site for the tRNA primer for reverse transcription, or a nucleic acid sequence component that induces a viral immunogenicity response when inserted in vivo, allows integration, affects specificity and activity of tissue specific promoters, causes transcriptional silencing or poses safety risks to the subject.

[0090] The term "vascular pressure pulse" refers to a pulse of pressure from a large volume of liquid to facilitate uptake of a vector into a cell. A skilled artisan recognizes that the amount and duration of the vascular pressure pulse is dependent on the tissue, size, and overall health of the recipient animal, and furthermore knows how to determine such parameters empirically.

[0091] The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell by delivering a nucleic acid sequence into that cell. A vector may contain multiple genetic elements positionally and sequentially oriented with other necessary elements such that an included nucleic acid cassette can be transcribed and when necessary translated in the transfected cells. These elements are operatively linked. The term "expression vector" refers to a DNA plasmid that contains all of the information necessary to produce a recombinant protein in a heterologous cell.

[0092] The present invention concerns a method for decreasing the body fat proportion, increasing lean body mass ("LBM"), increasing bone density, increasing the rate

of bone healing, or a combination thereof, of an animal subject. In general the present invention can be accomplished by delivering a nucleic acid sequence encoding GHRH or functional biological equivalent thereof into the cells of the subject (*e.g.* somatic, stem, or germ cells) and allowing expression of the encoded gene to occur while the modified cells are within the living organism. The subsequent expression of the GHRH or functional biological equivalent thereof is regulated by a tissue specific promoter (*e.g.* muscle), and/or by a regulator protein that contains a modified ligand binding domain (*e.g.* molecular switch), which will only be active when the correct modified ligand (*e.g.* mifepistone) is administered to the subject. The expression and ensuing release of GHRH or functional biological equivalent thereof by the modified cells within the living organism will decrease the body fat proportion, increase the LBM, increase the bone density, and/or increase the bone healing rate of the subject.

[0093] One aspect of the current invention is a method for altering lean body mass in a subject by utilizing a nucleic acid expression vector regulated by a constitutive promoter. The method comprises delivering into cells of the subject the nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof. In a specific embodiment, the nucleic acid expression construct is delivered into the cells of the subject via electroporation, wherein the cells comprise somatic, stem or germ cells. In another specific embodiment the nucleic acid expression construct comprises SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21. Transfection of the nucleic acid expression construct can be expedited by utilizing a transfection-facilitating polypeptide (e.g. charged polypeptide or poly-L-glutamate). The encoded GHRH or functional biological equivalent thereof are expressed in tissue specific cells of the subject, which comprises muscle cells. The encoded GHRH or the encoded functional biological equivalent of GHRH are biologically active polypeptides that have been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. In a preferred embodiment the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6). Additionally, the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

[0094] A second aspect of the current invention is a method for altering lean body mass in a subject by utilizing a nucleic acid expression vector regulated by a molecular switch molecule. The method comprises steps of delivering into cells of the subject a first nucleic acid expression construct (SeqID No: 26), a second nucleic acid expression construct (SeqID No: 27), and a molecular switch; wherein the first nucleic acid expression construct encodes growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof; and wherein the second nucleic acid expression construct has an encoding region of a regulator protein; and delivering a molecular switch molecule into the subject, wherein the molecular switch molecule governs activation of the regulator protein and the regulator protein governs the activation of the first nucleic acid expression construct. In some specific embodiments, the nucleic acid expression construct further comprises a transfection-facilitating polypeptide (e.g. a charged polypeptide or poly-L-glutamate). The delivering step of the first nucleic acid and the second nucleic acid expression construct into the cells of the subject is via electroporation. A specific embodiment of this method comprises that delivering the nucleic acid expression construct into the cells of the subject initiates expression of the encoded regulatory protein, but the regulatory protein is inactive. However, upon delivering a molecular switch (e.g. mifepristone) into the subject, the regulatory protein becomes active, and the active regulatory protein initiates expression of the GHRH or functional biological equivalent encoded on the first nucleic acid sequence. The encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. The encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6). The encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject. In another specific embodiment, the first nucleic acid expression vector encodes a polypeptide of sequence SeqID No: 1, SeqID No: 2, SeqID No: 3, or SeqID No: 4.

[0095] A third aspect of the current invention is a altering lean body mass in a subject comprising the steps of: delivering into a subject a recombinant growth-hormone-releasing-hormone ("GHRH") or a biological functional equivalent thereof, wherein the recombinant GHRH is a biologically active polypeptide. In specific embodiments, the recombinant functional biological equivalent of GHRH is a polypeptide that has been

engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. In another specific embodiment, the recombinant GHRH or functional biological equivalent thereof is of formula (SEQID No: 6). The recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

[0096] A fourth aspect of the current invention is a method for altering bone properties in a subject by utilizing a nucleic acid expression vector regulated by a constitutive promoter. The method comprises delivering into cells of the subject the nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof. In a specific embodiment, the nucleic acid expression construct is delivered into the cells of the subject via electroporation, wherein the cells comprise somatic, stem or germ cells. In another specific embodiment the nucleic acid expression construct comprises SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21. Transfection of the nucleic acid expression construct can be expedited by utilizing a transfection-facilitating polypeptide (e.g. charged polypeptide or poly-L-glutamate). The encoded GHRH or functional biological equivalent thereof are expressed in tissue specific cells of the subject, which comprises muscle cells. The encoded GHRH or the encoded functional biological equivalent of GHRH are biologically active polypeptides that have been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. In a preferred embodiment the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6). Additionally, the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

[0097] A fifth aspect of the current invention is a method for altering bone properties in a subject by utilizing a nucleic acid expression vector regulated by a molecular switch molecule. The method comprises steps of delivering into cells of the subject a first nucleic acid expression construct, a second nucleic acid expression construct, and a molecular switch; wherein the first nucleic acid expression construct encodes growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof; and wherein the second nucleic acid expression construct has an encoding region of a regulator protein; and delivering a molecular switch molecule into the subject, wherein the molecular switch

molecule governs activation of the regulator protein and the regulator protein governs the activation of the first nucleic acid expression construct. In some specific embodiments, the nucleic acid expression construct further comprises a transfection-facilitating polypeptide (e.g. a charged polypeptide or poly-L-glutamate). The delivering step of the first nucleic acid and the second nucleic acid expression construct into the cells of the subject is via electroporation. A specific embodiment of this method comprises that delivering the nucleic acid expression construct into the cells of the subject initiates expression of the encoded regulatory protein, but the regulatory protein is inactive. However, upon delivering a molecular switch (e.g. mifepristone) into the subject, the regulatory protein becomes active, and the active regulatory protein initiates expression of the GHRH or functional biological equivalent encoded on the first nucleic acid sequence. The encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. The encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6). The encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject. In another specific embodiment, the first nucleic acid expression vector encodes a polypeptide of sequence SeqID No: 1, SeqID No: 2, SeqID No: 3, or SeqID No: 4.

[0098] A sixth aspect of the current invention is a method for altering bone properties in a subject comprising the steps of: delivering into a subject a recombinant growth-hormone-releasing-hormone ("GHRH") or a biological functional equivalent thereof, wherein the recombinant GHRH is a biologically active polypeptide. In specific embodiments, the recombinant functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. In another specific embodiment, the recombinant GHRH or functional biological equivalent thereof is of formula (SEQID No: 6). The recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

[0099] The plasmid-mediated supplementation of GHRH approach described herein offers advantages over the limitations of directly injecting recombinant GHRH protein. Expression of nucleic acid sequences encoding novel functional biological

equivalents of GHRH that are serum protease resistant can be directed by an expression plasmid controlled by a synthetic muscle-specific promoter. Expression of such GHRH or functional biological equivalent thereof elicited high GH and IGF-I levels in pigs following delivery by intramuscular injection and *in vivo* electroporation (Draghia-Akli et al., 1999). The process of *in vivo* electroporation may involve externally supplied electrodes, or in the case of needles, internally supplied electrodes to aid in the inclusion of desired nucleotide sequences into the cells of the subject within the living organism. Although *in vivo* electroporation is the preferred method of introducing the heterologous nucleic acid encoding system into the cells of the subject, other methods exist and are known by a person skilled in the art (e.g electroporation, lipofectamine, calcium phosphate, *ex vivo* transformation, direct injection, DEAE dextran, sonication loading, receptor mediated transfection, microprojectile bombardment, *etc.*). For example, it is also possible to introduce the nucleic acid sequence that encodes the GHRH or functional biological equivalent thereof directly into the cells of the subject by first removing the cells from the body of the subject or donor, maintaining the cells in culture, then introducing the nucleic acid encoding system by a variety of methods (e.g electroporation, lipofectamine, calcium phosphate, *ex vivo* transformation, direct injection, DEAE dextran, sonication loading, receptor mediated transfection, microprojectile bombardment, *etc.*), and finally reintroducing the modified cells into the original subject or other host subject (the *ex vivo* method). The GHRH sequence can be cloned into an adenovirus vector or an adeno-associated vector and delivered by simple intramuscular injection, or intravenous or intra-arterial injection. Plasmid DNA carrying the GHRH sequence can be complexed with cationic lipids or liposomes and delivered intramuscularly, intravenously or subcutaneously.

[0100] Administration as used herein refers to the route of introduction of a vector or carrier of DNA into the body. Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for supplementing GHRH by administration of the vector, such as a plasmid, to the body in order to establish controlled expression of the specific nucleic acid sequence within tissues at certain useful levels.

[0101] The preferred means for administration of vector and use of formulations for delivery are described above. The preferred embodiment is by *in vivo* electroporation.

[0102] The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the tissue specific DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

[0103] Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months. DNA uptake in muscle cells is further enhanced by utilizing *in vivo* electroporation.

[0104] Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

[0105] Delivery can also be through use of DNA transporters. DNA transporters refers to molecules which bind to DNA vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of non-covalently binding to DNA and efficiently transporting the DNA through the cell

membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, *e.g.*, the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo *et al.*, U.S. Patent No. 6,150,168 entitled: "A DNA Transporter System and Method of Use;" (2) Woo *et al.*, PCT/US93/02725, entitled "A DNA Transporter System and method of Use", filed Mar. 19, 1993; (3) Woo *et al.*, U.S. Patent No. 6,177,554 "Nucleic Acid Transporter Systems and Methods of Use;" (4) Szoka *et al.*, U.S. Patent No. 5,955,365 entitled "Self-Assembling Polynucleotide Delivery System;" and (5) Szoka *et al.*, PCT/US93/03406, entitled "Self-Assembling Polynucleotide Delivery System", filed Apr. 5, 1993.

[0106] Another method of delivery involves a DNA transporter system. The DNA transporter system consists of particles containing several elements that are independently and non-covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations which may be used are spermine, spermine derivatives, histone, cationic peptides and/or polylysine. One element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. An example of such a ligand is the nuclear targeting sequence from SV40 large T antigen or histone. A third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. Examples include inactivated virus particles such as adenovirus, peptides related to influenza virus hemagglutinin, or the GALA peptide described in the Skoka patent cited above.

[0107] Administration may also involve lipids. The lipids may form liposomes which are hollow spherical vesicles composed of lipids arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the

use of cationic lipids and complexes containing DOPE which interact with DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

[0108] Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblast genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

[0109] Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not only tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs supplementation of GHRH, and the genetically engineered cells can also be easily put back with out causing damage to the patient Is muscle. Similarly, keratinocytes may be used to delivery genes to tissues. Large numbers of keratinocytes can be generated by cultivation of a small biopsy. The cultures can be prepared as stratified sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality over many years. The keratinocytes are genetically engineered while in culture by transfecting the keratinocytes with the appropriate vector. Although keratinocytes are separated from the circulation by the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

[0110] Delivery may also involve the use of viral vectors. For example, an adenoviral vector may be constructed by replacing the E1 region of the virus genome with the vector elements described in this invention including promoter, 5'UTR, 3'UTR and nucleic acid cassette and introducing this recombinant genome into 293 cells which will package this gene into an infectious virus particle. Virus from this cell may then be used to infect tissue ex vivo or *in vivo* to introduce the vector into tissues leading to expression of the gene in the nucleic acid cassette.

[0111] Although not wanting to be bound by theory, it is believed that in order to provide an acceptable safety margin for the use of such heterologous nucleic acid sequences in humans, a regulated gene expression system is mandated to possess low

levels of basal expression of GHRH, and still retain a high inducibility. Thus, target gene expression can be regulated by incorporating molecular switch technology as schematically diagramed in FIGURE 1A. The commercially available GeneSwitch® system for ligand-dependent induction of transgene expression is based on a C-terminally truncated progesterone receptor that fails to bind to its natural agonist, progesterone, but instead is activated by antiprogestins, such as mifepristone ("MFP") (Vegeto et al., 1992; Xu et al., 1996). Thus, the heterologous nucleic acid sequence introduced into the cells of the subject requires MFP to be transcriptionally activated. The chimeric regulator protein of the GeneSwitch® system consists of the ligand binding domain of the truncated human progesterone receptor that has been fused to the DNA binding domain of the yeast GAL4 protein (which binds a specific 17 bp recognition sequence) and a transcriptional activation domain from the p65 subunit of human NF-kB (Abruzzese et al., 1999; Abruzzese et al., 2000). The gene for the GeneSwitch® regulator protein was inserted into a myogenic expression vector, designated pGS1633, which is expressed constitutively under the control of a muscle-specific skeletal alpha-actin ("SK") promoter The GHRH plasmid, designated, p6xGal4/TATA-GHRH, or pGHRH1633 contains an inducible promoter that consists of six copies of the 17-mer Gal4 binding site fused to a minimal TATA box promoter. The GHRH coding sequence is a 228-bp fragment of super-porcine mutated GHRH cDNA, termed HV-GHRH (Draghia-Akli et al., 1999). The HV-GHRH molecule displays a high degree of stability in serum, with a half-life of 6 hours, versus the natural GHRH, that has a 6-12 min half-life. The muscle-specific GeneSwitch® and inducible GHRH plasmids both have a 5' untranslated region that contains a synthetic intron, and a 3' untranslated region/poly(A) site that is from the human GH gene.

[0112] Recombinant GH replacement therapy is widely used clinically, with beneficial effects, but generally, the doses are supraphysiological. Such elevated doses of recombinant GH are associated with deleterious side-effects, for example, up to 30% of the recombinant GH treated patients report a higher frequency of insulin resistance (Blethen, 1995; Verhelst et al., 1997) or accelerated bone epiphysis growth and closure in pediatric patients (Blethen and Rundle, 1996). In addition, molecular heterogeneity of circulating GH may have important implications in growth and homeostasis, which can lead to a less potent GH that has a reduced ability to stimulate the prolactin receptor; it has

also been described that the 20kDa form of GH has less potency to cause urine retention than the 22 kDa form (Satozawa et al., 2000; Tsunekawa et al., 1999; Wada et al., 1998). These unwanted side effects result from the fact that treatment with recombinant exogenous GH protein raises basal levels of GH and abolishes the natural episodic pulses of GH. In contradistinction, no side effects have been reported for recombinant GHRH therapies. The normal levels of GHRH in the pituitary portal circulation range from about 150-to-800 pg/ml, while systemic circulating values of the hormone are up to about 100-500 pg/ml. Some patients with acromegaly caused by extracranial tumors have level that is nearly 10 times as high (*e.g.* 50 ng/ml of immunoreactive GHRH) (Thorner et al., 1984). Long-term studies using recombinant GHRH therapies (1-5 years) in children and elderly humans have shown an absence of the classical GH side-effects, such as changes in fasting glucose concentration or, in pediatric patients, the accelerated bone epiphysal growth and closure or slipping of the capital femoral epiphysis (Chevalier et al., 2000) (Duck et al., 1992; Vittone et al., 1997). Numerous studies in humans, sheep or pigs showed that continuous infusion with recombinant GHRH protein restores the normal GH pattern without desensitizing GHRH receptors or depleting GH supplies (Dubreuil et al., 1990b). As this system is capable of a degree of feed-back which is abolished in the GH therapies, GHRH recombinant protein therapy may be more physiological than GH therapy. However, due to the short half-life of GHRH *in vivo*, frequent (one to three times per day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administrations are necessary (Evans et al., 1985; Thorner et al., 1986). Thus, as a chronic therapy, recombinant GHRH protein administration is not practical. A gene transfer approach, however could overcome this limitations to GHRH use. Moreover, a wide range of doses can be therapeutic. The choice of GHRH for a gene therapeutic application is favored by the fact that the gene, cDNA and native and several mutated molecules have been characterized for the pig and other species (Bohlen et al., 1983; Guillemin et al., 1982), and the measurement of therapeutic efficacy is straightforward and unequivocal.

[0113] Among the non-viral techniques for gene transfer *in vivo*, the direct injection of plasmid DNA into muscle is simple, inexpensive, and safe. The inefficient DNA uptake into muscle fibers after simple direct injection hag led to relatively low expression levels (Prentice et al., 1994; Wells et al., 1997) In addition, the duration of the

transgene expression has been short (Wolff et al., 1990). The most successful previous clinical applications have been confined to vaccines (Danko and Wolff, 1994; Tsurumi et al., 1996). Recently, significant progress to enhance plasmid delivery *in vivo* and subsequently to achieve physiological levels of a secreted protein was obtained using the electroporation technique. Recently, significant progress has been obtained using electroporation to enhance plasmid delivery *in vivo*. Electroporation has been used very successfully to transfect tumor cells after injection of plasmid (Lucas et al., 2002; Matsubara et al., 2001) or to deliver the anti-tumor drug bleomycin to cutaneous and subcutaneous tumors in humans (Gehl et al., 1998; Heller et al., 1996). Electroporation also has been extensively used in mice (Lesbordes et al., 2002; Lucas et al., 2001; Vilquin et al., 2001), rats (Terada et al., 2001; Yasui et al., 2001), and dogs (Fewell et al., 2001) to deliver therapeutic genes that encode for a variety of hormones, cytokines or enzymes. Our previous studies using growth hormone releasing hormone (GHRH) showed that plasmid therapy with electroporation is scalable and represents a promising approach to induce production and regulated secretion of proteins in large animals and humans (Draghia-Akli et al., 1999; Draghia-Akli et al., 2002). Electroporation also has been extensively used in rodents and other small animals (Bettan et al., 2000; Yin and Tang, 2001). It has been observed that the electrode configuration affects the electric field distribution, and subsequent results (Gehl et al., 1999; Miklavcic et al., 1998). Preliminary experiments indicated that for a large animal model, needle electrodes give consistently better reproducible results than external caliper electrodes.

[0114] Combining the powerful electroporation delivery method with an improved plasmid DNA vector system produced significant changes that decreased the body fat proportion, increased lean body mass ("LBM"), or both, in an animal, such as a large animal, at very low plasmid dosage.

I. Vectors

[0115] The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell wherein, in some embodiments, it can be replicated. A nucleic acid sequence can be native to the animal, or it can be "exogenous," which means that it is foreign to the cell into which the vector is

being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), linear DNA fragments, and artificial chromosomes (*e.g.*, YACs), although in a preferred embodiment the vector contains substantially no viral sequences. One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (see, for example, (Sambrook et al., 1989).

[0116] The term "expression vector" refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operatively linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra.*

II. Plasmid Vectors

[0117] In certain embodiments, a linear DNA fragment from a plasmid vector is contemplated for use to transfect a eukaryotic cell, particularly a mammalian cell. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. In a non-limiting example, *E. coli* is often transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, for example, promoters which can be used by the microbial organism for expression of its own proteins. A skilled artisan recognizes that any plasmid in the art may be modified for use in the methods of the present invention. In a specific embodiment, for example, a GHRH vector

used for the therapeutic applications is derived from pBlueScript KS+ and has a kanamycin resistance gene.

[0118] In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEM™-11 may be utilized in making a recombinant phage vector which can be used to transform host cells, such as, for example, *E. coli* LE392.

[0119] Further useful plasmid vectors include pIN vectors (Inouye et al., 1985); and pGEX vectors, for use in generating glutathione S-transferase ("GST") soluble fusion proteins for later purification and separation or cleavage. Other suitable fusion proteins are those with β-galactosidase, ubiquitin, and the like.

[0120] Bacterial host cells, for example, *E. coli*, comprising the expression vector, are grown in any of a number of suitable media, for example, LB. The expression of the recombinant protein in certain vectors may be induced, as would be understood by those of skill in the art, by contacting a host cell with an agent specific for certain promoters, *e.g.*, by adding IPTG to the media or by switching incubation to a higher temperature. After culturing the bacteria for a further period, generally of between 2 and 24 h, the cells are collected by centrifugation and washed to remove residual media.

III. Promoters and Enhancers

[0121] A promoter is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription of a gene product are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

[0122] A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5′ end of the transcription initiation site of the transcriptional reading frame "downstream" of (*i.e.*, 3′ of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

[0123] The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[0124] A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant, synthetic or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant, synthetic or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or

enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.,* containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the β-lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent Nos. 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

[0125] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (see, for example (Sambrook et al., 1989)). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

[0126] Additionally any promoter/enhancer combination (as per, for example, the Eukaryotic Promoter Data Base EPDB, http://www.epd.isb-sib.ch/) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

[0127] Tables 1 and 2 list non-limiting examples of elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a

RNA. Table 2 provides non-limiting examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 1
Promoter and/or Enhancer

Promoter/Enhancer	Relevant References
Immunoglobulin Heavy Chain	
Immunoglobulin Light Chain	
T-Cell Receptor	
HLA DQ a and/or DQ β	
β-Interferon	
Interleukin-2	
Interleukin-2 Receptor	
MHC Class II 5	
MHC Class II HLA-Dra	
β-Actin	(Kawamoto et al., 1988; Kawamoto et al., 1989)
Muscle Creatine Kinase (MCK)	(Horlick and Benfield, 1989; Jaynes et al., 1988)
Prealbumin (Transthyretin)	
Elastase I	
Metallothionein (MTII)	(Inouye et al., 1994; Narum et al., 2001; Skroch et al., 1993)
Collagenase	
Albumin	(Pinkert et al., 1987; Tronche et al., 1989)
α-Fetoprotein	
γ-Globin	
β-Globin	(Tronche et al., 1990; Trudel and Costantini, 1987)
c-fos	
c-HA-*ras*	
Insulin	(German et al., 1995; Ohlsson et al., 1991)
Neural Cell Adhesion Molecule (NCAM)	
α_1-Antitrypsin	
H2B (TH2B) Histone	
Mouse and/or Type I Collagen	
Glucose-Regulated Proteins (GRP94 and GRP78)	
Rat Growth Hormone	(Larsen et al., 1986)
Human Serum Amyloid A (SAA)	
Troponin I (TN I)	(Lin et al., 1991; Yutzey and Konieczny, 1992)
Platelet-Derived Growth Factor (PDGF)	(Pech et al., 1989)
Duchenne Muscular Dystrophy	(Klamut et al., 1990; Klamut et al., 1996)
SV40	
Polyoma	
Retroviruses	
Papilloma Virus	
Hepatitis B Virus	

TABLE 1 Promoter and/or Enhancer	
Promoter/Enhancer	Relevant References
Human Immunodeficiency Virus	
Cytomegalovirus (CMV)	(Boshart et al., 1985; Dorsch-Hasler et al., 1985)
Gibbon Ape Leukemia Virus	
Synthetic muscle specific promoters (c5-12, c1-28)	(Draghia-Akli et al., 1999; Draghia-Akli et al., 2002; Li et al., 1999)

TABLE 2 Element/Inducer	
Element	Inducer
MT II	Phorbol Ester (TFA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β-Interferon	Poly(rI)x / Poly(rc)
Adenovirus 5 E2	ElA
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA)
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2κb	Interferon
HSP70	ElA, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor α	PMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

[0128] The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Nonlimiting examples of such regions include the human LIMK2 gene (Nomoto et al., 1999), the somatostatin receptor 2 gene (Kraus et al., 1998), murine epididymal retinoic acid-binding gene (Lareyre et al., 1999), human CD4 (Zhao-Emonet et al., 1998), mouse alpha2 (XI) collagen (Liu et al., 2000; Tsumaki et al., 1998), D1A dopamine receptor gene (Lee et al., 1997), insulin-like growth factor II (Dai et al., 2001; Wu et al., 1997), and human platelet endothelial cell adhesion molecule-1 (Almendro et al., 1996).

[0129] In a preferred embodiment, a synthetic muscle promoter is utilized, such as SPc5-12 (Li et al., 1999), which contains a proximal serum response element ("SRE") from skeletal α-actin, multiple MEF-2 sites, MEF-1 sites, and TEF-1 binding sites, and greatly exceeds the transcriptional potencies of natural myogenic promoters.

The uniqueness of such a synthetic promoter is a significant improvement over, for instance, issued patents concerning a myogenic promoter and its use (*e.g.* U.S. Pat. No. 5,374,544) or systems for myogenic expression of a nucleic acid sequence (*e.g.* U.S. Pat. No. 5,298,422). In a preferred embodiment, the promoter utilized in the invention does not get shut off or reduced in activity significantly by endogenous cellular machinery or factors. Other elements, including *trans*-acting factor binding sites and enhancers may be used in accordance with this embodiment of the invention. In an alternative embodiment, a natural myogenic promoter is utilized, and a skilled artisan is aware how to obtain such promoter sequences from databases including the National Center for Biotechnology Information ("NCBI") GenBank database or the NCBI PubMed site. A skilled artisan is aware that these databases may be utilized to obtain sequences or relevant literature related to the present invention.

IV. Initiation Signals and Internal Ribosome Binding Sites

[0130] A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

[0131] In certain embodiments of the invention, the use of internal ribosome entry sites ("IRES") elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be

transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent Nos. 5,925,565 and 5,935,819, each herein incorporated by reference).

V. Multiple Cloning Sites

[0132] Vectors can include a MCS, which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, (Carbonelli et al., 1999; Cocea, 1997; Levenson et al., 1998) incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

VI. Splicing Sites

[0133] Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, (Chandler et al., 1997), herein incorporated by reference.)

VII. Termination Signals

[0134] The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by

an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

[0135] In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues ("polyA") to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

[0136] Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

VIII. Polyadenylation Signals

[0137] In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal, skeletal alpha actin 3'UTR or the human or bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

IX. Origins of Replication

[0138] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence ("ARS") can be employed if the host cell is yeast.

X. Selectable and Screenable Markers

[0139] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

[0140] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase ("tk") or chloramphenicol acetyltransferase ("CAT") may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

XI. Electroporation

[0141] In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent No.5,384,253, incorporated herein by reference). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding and other methods known in the art.

[0142] Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter et al., 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa et al., 1986) in this manner.

[0143] The underlying phenomenon of electroporation is believed to be the same in all cases, but the exact mechanism responsible for the observed effects has not been elucidated. Although not wanting to be bound by theory, the overt manifestation of the electroporative effect is that cell membranes become transiently permeable to large molecules, after the cells have been exposed to electric pulses. There are conduits through cell walls, which under normal circumstances, maintain a resting transmembrane potential of ca. 90 mV by allowing bi-directional ionic migration.

[0144] Although not wanting to be bound by theory, electroporation makes use of the same structures, by forcing a high ionic flux through these structures and opening or enlarging the conduits. In prior art, metallic electrodes are placed in contact with tissues and predetermined voltages, proportional to the distance between the electrodes are imposed on them. The protocols used for electroporation are defined in terms of the resulting field intensities, according to the formula $E=V/d$, where ("E") is the field, ("V") is the imposed voltage and ("d") is the distance between the electrodes.

[0145] The electric field intensity E has been a very important value in prior art when formulating electroporation protocols for the delivery of a drug or macromolecule into the cell of the subject. Accordingly, it is possible to calculate any electric field intensity for a variety of protocols by applying a pulse of predetermined voltage that is proportional to the distance between electrodes. However, a caveat is that an electric field can be generated in a tissue with insulated electrodes (i.e. flow of ions is not necessary to create an electric field). Although not wanting to be bound by theory, it is the current that is necessary for successful electroporation not electric field per se.

[0146] During electroporation, the heat produced is the product of the interelectrode impedance, the square of the current, and the pulse duration. Heat is produced during electroporation in tissues and can be derived as the product of the inter-electrode current, voltage and pulse duration. The protocols currently described for electroporation are defined in terms of the resulting field intensities E, which are dependent on short voltage pulses of unknown current. Accordingly, the resistance or heat generated in a tissue cannot be determined, which leads to varied success with different pulsed voltage electroporation protocols with predetermined voltages. The ability to limit heating of cells across electrodes can increase the effectiveness of any given electroporation voltage pulsing protocol. For example, prior art teaches the utilization of an array of six needle electrodes utilizing a predetermined voltage pulse across opposing electrode pairs. This situation sets up a centralized pattern during an electroporation event in an area where congruent and intersecting overlap points develop. Excessive heating of cells and tissue along electroporation path will kill the cells, and limit the effectiveness of the protocol. However, symmetrically arranged needle electrodes without opposing pairs can produce a decentralized pattern during an electroporation event in an area where no congruent electroporation overlap points can develop.

[0147] Controlling the current flow between electrodes allows one to determine the relative heating of cells. Thus, it is the current that determines the subsequent effectiveness of any given pulsing protocol, and not the voltage across the electrodes. Predetermined voltages do not produce predetermined currents, and prior art does not provide a means to determine the exact dosage of current, which limits the usefulness of the technique. Thus, controlling an maintaining the current in the tissue

between two electrodes under a threshold will allow one to vary the pulse conditions, reduce cell heating, create less cell death, and incorporate macromolecules into cells more efficiently when compared to predetermined voltage pulses.

[0148] One embodiment of the present invention to overcome the above problem by providing a means to effectively control the dosage of electricity delivered to the cells in the inter-electrode space by precisely controlling the ionic flux that impinges on the conduits in the cell membranes. The precise dosage of electricity to tissues can be calculated as the product of the current level, the pulse length and the number of pulses delivered. Thus, a specific embodiment of the present invention can deliver the electroporative current to a volume of tissue along a plurality of paths without, causing excessive concentration of cumulative current in any one location, thereby avoiding cell death owing to overheating of the tissue.

[0149] Although not wanting to be bound by theory, the nature of the voltage pulse to be generated is determined by the nature of tissue, the size of the selected tissue and distance between electrodes. It is desirable that the voltage pulse be as homogenous as possible and of the correct amplitude. Excessive field strength results in the lysing of cells, whereas a low field strength results in reduced efficacy of electroporation. Some electroporation devices utilize the distance between electrodes to calculate the electric field strength and predetermined voltage pulses for electroporation. This reliance on knowing the distance between electrodes is a limitation to the design of electrodes. Because the programmable current pulse controller will determine the impedance in a volume of tissue between two electrodes, the distance between electrodes is not a critical factor for determining the appropriate electrical current pulse. Therefore, an alternative embodiment of a needle electrode array design would be one that is non-symmetrical. In addition, one skilled in the art can imagine any number of suitable symmetrical and non-symmetrical needle electrode arrays that do not deviate from the spirit and scope of the invention. The depth of each individual electrode within an array and in the desired tissue could be varied with comparable results. In addition, multiple injection sites for the macromolecules could be added to the needle electrode array.

XII. Restriction Enzymes

[0150] In some embodiments of the present invention, a linear DNA fragment is generated by restriction enzyme digestion of a parent DNA molecule. Examples of restriction enzymes are provided below.

Name	Recognition Sequence
AatII	GACGTC
Acc65 I	GGTACC
Acc I	GTMKAC
Aci I	CCGC
Acl I	AACGTT
Afe I	AGCGCT
Afl II	CTTAAG
Afl III	ACRYGT
Age I	ACCGGT
Ahd I	GACNNNNNGTC
Alu I	AGCT
Alw I	GGATC
AlwN I	CAGNNNCTG
Apa I	GGGCCC
ApaL I	GTGCAC
Apo I	RAATTY
Asc I	GGCGCGCC
Ase I	ATTAAT
Ava I	CYCGRG
Ava II	GGWCC
Avr II	CCTAGG
Bae I	NACNNNNGTAPyCN
BamH I	GGATCC
Ban I	GGYRCC
Ban II	GRGCYC
Bbs I	GAAGAC
Bbv I	GCAGC
BbvC I	CCTCAGC
Bcg I	CGANNNNNNTGC
BciV I	GTATCC
Bcl I	TGATCA
Bfa I	CTAG
Bgl I	GCCNNNNNGGC
Bgl II	AGATCT
Blp I	GCTNAGC
Bmr I	ACTGGG
Bpm I	CTGGAG
BsaA I	YACGTR
BsaB I	GATNNNNATC

BsaH I	GRCGYC
Bsa I	GGTCTC
BsaJ I	CCNNGG
BsaW I	WCCGGW
BseR I	GAGGAG
Bsg I	GTGCAG
BsiE I	CGRYCG
BsiHKA I	GWGCWC
BsiW I	CGTACG
Bsl I	CCNNNNNNNGG
BsmA I	GTCTC
BsmB I	CGTCTC
BsmF I	GGGAC
Bsm I	GAATGC
BsoB I	CYCGRG
Bsp1286 I	GDGCHC
BspD I	ATCGAT
BspE I	TCCGGA
BspH I	TCATGA
BspM I	ACCTGC
BsrB I	CCGCTC
BsrD I	GCAATG
BsrF I	RCCGGY
BsrG I	TGTACA
Bsr I	ACTGG
BssH II	GCGCGC
BssK I	CCNGG
Bst4C I	ACNGT
BssS I	CACGAG
BstAP I	GCANNNNNTGC
BstB I	TTCGAA
BstE II	GGTNACC
BstF5 I	GGATGNN
BstN I	CCWGG
BstU I	CGCG
BstX I	CCANNNNNNTGG
BstY I	RGATCY
BstZ17 I	GTATAC
Bsu36 I	CCTNAGG
Btg I	CCPuPyGG
Btr I	CACGTG
Cac8 I	GCNNGC
Cla I	ATCGAT
Dde I	CTNAG
Dpn I	GATC
Dpn II	GATC
Dra I	TTTAAA
Dra III	CACNNNGTG

Drd I	GACNNNNNNGTC
Eae I	YGGCCR
Eag I	CGGCCG
Ear I	CTCTTC
Eci I	GGCGGA
EcoN I	CCTNNNNNAGG
EcoO109 I	RGGNCCY
EcoR I	GAATTC
EcoR V	GATATC
Fau I	CCCGCNNNN
Fnu4H I	GCNGC
Fok I	GGATG
Fse I	GGCCGGCC
Fsp I	TGCGCA
Hae II	RGCGCY
Hae III	GGCC
Hga I	GACGC
Hha I	GCGC
Hinc II	GTYRAC
Hind III	AAGCTT
Hinf I	GANTC
HinP1 I	GCGC
Hpa I	GTTAAC
Hpa II	CCGG
Hph I	GGTGA
Kas I	GGCGCC
Kpn I	GGTACC
Mbo I	GATC
Mbo II	GAAGA
Mfe I	CAATTG
Mlu I	ACGCGT
Mly I	GAGTCNNNNN
Mnl I	CCTC
Msc I	TGGCCA
Mse I	TTAA
Msl I	CAYNNNNRTG
MspA1 I	CMGCKG
Msp I	CCGG
Mwo I	GCNNNNNNNGC
Nae I	GCCGGC
Nar I	GGCGCC
Nci I	CCSGG
Nco I	CCATGG
Nde I	CATATG
NgoMI V	GCCGGC
Nhe I	GCTAGC
Nla III	CATG
Nla IV	GGNNCC

Not I	GCGGCCGC
Nru I	TCGCGA
Nsi I	ATGCAT
Nsp I	RCATGY
Pac I	TTAATTAA
PaeR7 I	CTCGAG
Pci I	ACATGT
PflF I	GACNNNGTC
PflM I	CCANNNNNTGG
PleI	GAGTC
Pme I	GTTTAAAC
Pml I	CACGTG
PpuM I	RGGWCCY
PshA I	GACNNNNGTC
Psi I	TTATAA
PspG I	CCWGG
PspOM I	GGGCCC
Pst I	CTGCAG
Pvu I	CGATCG
Pvu II	CAGCTG
Rsa I	GTAC
Rsr II	CGGWCCG
Sac I	GAGCTC
Sac II	CCGCGG
Sal I	GTCGAC
Sap I	GCTCTTC
Sau3A I	GATC
Sau96 I	GGNCC
Sbf I	CCTGCAGG
Sca I	AGTACT
ScrF I	CCNGG
SexA I	ACCWGGT
SfaN I	GCATC
Sfc I	CTRYAG
Sfi I	GGCCNNNNNGGCC
Sfo I	GGCGCC
SgrA I	CRCCGGYG
Sma I	CCCGGG
Sml I	CTYRAG
SnaB I	TACGTA
Spe I	ACTAGT
Sph I	GCATGC
Ssp I	AATATT
Stu I	AGGCCT
Sty I	CCWWGG
Swa I	ATTTAAAT
Taq I	TCGA
Tfi I	GAWTC

Tli I	CTCGAG
Tse I	GCWGC
Tsp45 I	GTSAC
Tsp509 I	AATT
TspR I	CAGTG
Tth111 I	GACNNNGTC
Xba I	TCTAGA
Xcm I	CCANNNNNNNNNTG G
Xho I	CTCGAG
Xma I	CCCGGG
Xmn I	GAANNNNTTC

[0151] The term "restriction enzyme digestion" of DNA as used herein refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction endonucleases, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 μg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5′ phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5′ terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as described in the art.

EXAMPLES

[0152] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

CONSTRUCTION OF DNA VECTORS AND METHODS IN ANIMAL SUBJECT

[0153] In order to increase lean body mass, decrease body fat proportions, increase bone density, and improve bone healing rate, it was first necessary to design several GHRH expression constructs. Briefly, the plasmid vectors contained the muscle specific synthetic promoter SPc5-12 (Li et al., 1999) attached to a wild type or analog porcine GHRH. The analog GHRH sequences were generated by site directed mutagenesis as described in methods section. Nucleic acid sequences encoding GHRH or analog were cloned into the BamHI/ HindIII sites of pSPc5-12 plasmid, to generate pSP-GHRH. Other elements contained in the plasmids include a 3' untranslated region ("3'UTR") (SEQID No: 8) of growth hormone and an SV40 3'UTR from pSEAP-2 Basic Vector as described in the methods section. The unique nucleic acid sequences for the constructs used are shown in Figure 1.

[0154] **DNA constructs:** Plasmid vectors containing the muscle specific synthetic promoter SPc5-12 (SeqID No: 7) were previously described (Li et al., 1999). Wild type and mutated porcine GHRH cDNAs were generated by site directed mutagenesis of GHRH cDNA (SeqID No: 9) (Altered Sites II *in vitro* Mutagenesis System, Promega, Madison, WI), and cloned into the BamHI/ Hind III sites of pSPc5-12, to generate pSP-wt-GHRH (SeqID No: 15), or pSP-HV-GHRH (SeqID No: 11), respectively. The 3' untranslated region (3'UTR) of growth hormone was cloned downstream of GHRH cDNA. The resultant plasmids contained mutated coding region

for GHRH, and the resultant amino acid sequences were not naturally present in mammals. Although not wanting to be bound by theory, the effects on increased bone density, and increased healing rate of bone in the animals are determined ultimately by the circulating levels of mutated hormones. Several different plasmids that encoded different mutated amino acid sequences of GHRH or functional biological equivalent thereof are as follows:

Plasmid	Encoded Amino Acid Sequence
wt-GHRH	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGA-OH (SeqID No: 10)
HV-GHRH	HVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SeqID No: 1)
TI-GHRH	YIDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SeqID No: 2)
TV-GHRH	YVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SeqID No: 3)
15/27/28-GHRH	YADAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SeqID No: 4)

[0155] In general, the encoded GHRH or functional biological equivalent thereof is of formula:

$-X_1-X_2-$DAIFTNSYRKVL$-X_3-$QLSARKLLQDI$-X_4-X_5-$RQQGERNQEQGA-OH
(SeqID No: 6)

wherein: X_1 is a D-or L-isomer of an amino acid selected from the group consisting of tyrosine ("Y"), or histidine ("H"); X_2 is a D-or L-isomer of an amino acid selected from the group consisting of alanine ("A"), valine ("V"), or isoleucine ("I"); X_3 is a D-or L-isomer of an amino acid selected from the group consisting of alanine ("A") or glycine ("G"); X_4 is a D-or L-isomer of an amino acid selected from the group consisting of methionine ("M"), or leucine ("L"); X_5 is a D-or L-isomer of an amino acid selected from the group consisting of serine ("S") or asparagine ("N").

[0156] Another plasmid that was utilized included the pSP-SEAP construct (SeqID No: 16) that contains the SacI/ HindIII SPc5-12 fragment, SEAP gene and SV40 3'UTR from pSEAP-2 Basic Vector (Clontech Laboratories, Inc.; Palo Alto, CA).

[0157] The plasmids described above do not contain polylinker, IGF-I gene, a skeletal alpha-actin promoter or a skeletal alpha actin 3' UTR /NCR. Furthermore, these plasmids were introduced by muscle injection, followed by *in vivo* electroporation, as described below.

[0158] In terms of "functional biological equivalents", it is well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent" protein, polypeptide, and/or polynucleotide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule while retaining a molecule with an acceptable level of equivalent biological activity. Functional biological equivalents are thus defined herein as those proteins (and polynucleotides) in selected amino acids (or codons) that may be substituted. A peptide comprising a functional biological equivalent of GHRH is a polypeptide that has been engineered to contain distinct amino acid sequences while simultaneously having similar or improved biologically activity when compared to GHRH. For example, one biological activity of GHRH is to facilitate growth hormone ("GH") secretion in the subject.

[0159] **Electroporation devices.** A BTX T820 generator (BTX, division of Genetronics Inc., CA) was used to deliver square wave pulses in all experiments. Voltage conditions of 100-200 V/cm, 6 pulses, 60 milliseconds per pulse were used. Caliper and needle electrodes (BTX, division of Genetronics Inc., CA) were used to deliver *in vivo* electric pulses. The plate (caliper) electrodes consisted of 1.5 cm square metallic blocks mounted on a ruler, so the distance between the plates could be easily assessed; the 6-needle device consists of a circular array (1 cm diameter) of six equally spaced filled 21-gauge needles mounted on a non-conductive material. The 3-needle device consists of two filled and one cannular needle, the last one being used both as an electrode and to deliver the plasmid. All needles were 2 cm in length. In all injections the needles were completely inserted into the muscle.

[0160] A skilled artisan recognizes that any similar electroporation device and parameters may be used in the present invention so long as the device delivers the nucleic acid sequence to the cell, tissue, or organism.

[0161] **Intramuscular injection of plasmid DNA in porcine.** Two- to three-week-old hybrid barrows (Yorkshire x Landrace x Hampshire x Duroc)(Huntsville, TX), 4-5 kg in weight, or Yorkshire x Landrace pigs were used in the secreted embryonic alkaline phosphatase studies (n = 3 /group). For the GHRH plasmid studies, time-pregnant sows (Yorkshire x Landrace) were brought three weeks before the scheduled parturition date to the Children Nutrition Research Center at Baylor College of Medicine. Piglets were born in the facility. Piglets were assigned randomly to one of the experimental (n = 2 pigs/ group/ series) or controls (n = 3) groups. All experiments were repeated three times. The animals were suckled for the first 21 days and then individually housed with ad-lib access to water. For GHRH studies, after weaning, pigs were fed a 24% protein diet (Producers Cooperative Association, Bryan, TX) at 6% of their body weight daily. The animals were weighed twice a week, at the same time of day, and the amount of feed was subsequently determined. Animals were maintained in accordance with NIH Guide, USDA and Animal Welfare Act guidelines, and approved by the Baylor College of Medicine IACUC.

[0162] Endotoxin-free plasmid (Qiagen Inc., Chatsworth, CA, USA) preparations were diluted in PBS, pH 7.4 to 1 mg/ml. Plasmid DNA was injected through the intact skin into the semitendinosus or the longissimus dorsi muscle using a 21 g needle. Two minutes later, external caliper electrodes or injectable electrodes (6-needle array or 3-needle array) were applied to the injected muscle, and 6 pulses of 200V/cm, 60 millisecond/pulse were applied. The polarity of the pulses was either constant or inverted between the needles.

[0163] Blood was collected by jugular puncture before plasmid injection, and at 3, 7, 14, 21, 35 and 45 days post-injection. At 50 days post-injection, animals were sacrificed and internal organs and the injected muscle were collected, weighed, frozen in liquid nitrogen, and stored at -80°C, or placed in 10% buffered formalin for histology.

[0164] Although *in vivo* electroporation is the preferred method for delivering the nucleic acid constructs into the cells of the subject, suitable methods for nucleic acid delivery for transformation of an organelle, a cell, a tissue or an organism for use with the current invention are believed to include virtually any method by which a nucleic acid

(*e.g.*, DNA) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by *ex vivo* transfection (Nabel et al., 1989; Wilson et al., 1989), by injection (U.S. Patent Nos. 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harland and Weintraub, 1985) U.S. Patent No. 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent No. 5,384,253, incorporated herein by reference; (Potter et al., 1984; Tur-Kaspa et al., 1986); by calcium phosphate precipitation (Chen and Okayama, 1987; Graham and van der Eb, 1973; Rippe et al., 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer et al., 1987); by liposome mediated transfection (Hafez et al., 2001; Hamm et al., 2002; Madry et al., 2001; Raghavachari and Fahl, 2002; Wiethoff et al., 2001) and receptor-mediated transfection (Wu and Wu, 1988a; Wu and Wu, 1988b); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patent Nos. 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers ((Johnson et al., 1992); U.S. Patent Nos. 5,302,523 and 5,464,765, each incorporated herein by reference); by *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,591,616 and 5,563,055, each incorporated herein by reference); by PEG-mediated transformation of protoplasts (Omirulleh et al., 1993); U.S. Patent Nos. 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus et al., 1985), and any combination of such methods. Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

[0165] **Porcine plasma IGF-I and insulin concentrations.** Porcine IGF-I was measured by heterologous human radioimmunometric assay (Diagnostic System Lab., Webster, TX). The sensitivity of the assay was 0.8 ng/ml; intra-assay and inter-assay variation was 3.4% and 4.5%, respectively. Porcine insulin was measured using a heterologous human radioimmunoassay (Linco Research Inc., St. Charles, MO). The

sensitivity level of the assay was 2 μU/ml; intra-assay and inter-assay variation was 3.2% and 3.9% respectively.

[0166] **Body composition data.** Weights were measured on the same calibrated scales (certified to have an accuracy to ± 0.2 kg and a coefficient of variation of 0.3%) throughout the study, twice a week. Body composition measurements were performed *in vivo*, 50 days after birth. The piglets were anesthetized using a combination of xylazine (15 mg/kg) and ketamine (2 mg/kg) and the total body content of fat, percent of fat, non-bone lean tissue mass and bone mineral content was measured by dual-energy x-ray absorptiometry (Hologic QDR-2000, Waltham, Massachusetts) ("DEXA") (Toner et al., 1996). Total body potassium was measured in a potassium chamber ("K40") using a whole body detector (Cohn et al., 1984).

[0167] **Statistics** Data are analyzed using STATISTICA analysis package (StatSoft, Inc. Tulsa, OK). Values shown in the figures are the mean ± s.e.m. Specific P values were obtained by comparison using ANOVA. A $P < 0.05$ was set as the level of statistical significance.

EXAMPLE 2

CONSTITUTIVE GHRH SYSTEM IN VIVO

[0168] To test the constitutive GHRH system *in vivo*, 7.5 micrograms of pSP-GHRH or functional biological equivalents (FIGURE 1) were delivered into SCID mice. All GHRH analog sequences were obtained by site directed mutagenesis of the porcine wild type sequence. Groups of five mice were injected with 7.5 micrograms of plasmid expressing either one of the GHRH analogs, or a pSP-beta-galactosidase as control. At 45 days post-injection, animals were analyzed by PIXImus (Draghia-Akli et al., 2002)(DEXA for mice), sacrificed, blood and organs were collected and weighed. At the end of the experiment, the TI-GHRH and HV-GHRH animals were significantly bigger than controls (FIGURE 2). The body composition of the injected SCID mice was also altered. At 45 days post-injection, animals that were injected with the TI mutant had a significant increase in lean body mass of 11% versus controls, $p < 0.036$. The HV-GHRH injected animals had a significant increase of the lean body mass of almost 5% (FIGURE 3). All

GHRH injected groups had larger bone areas than the control animals, up to 10.7%, in the TI-GHRH injected group, $p < 0.027$. (FIGURE 4). At 14 and 28 days post-injection, blood was collected and IGF-I levels were measured (FIGURE 5). All GHRH injected groups had significantly increased IGF-I levels compared with control animals, up to $p < 0.005$. Some groups developed neutralizing antibodies, and in these cases the IGF-I levels dropped at the second time point. The animals injected with TI-GHRH did not develop any antibodies, and their GHRH expression continued to 45 days, correlating with significant changes in their body composition.

EXAMPLE 3

INDUCIBLE GHRH SYSTEM IN VITRO

[0169] To test the inducible GHRH system *in vitro*, primary chicken myoblasts were transfected as described previously (Bergsma et al., 1986; Draghia-Akli et al., 1997) with 4 micrograms of a mixture of the GHRH/GeneSwitch® plasmids, pGR1774 (inducible GHRH)/pGS1633 (Gene Switch®) in a 10:1 w/w ratio, which gave the best overall expression in skeletal muscle cells, and cells were allowed to differentiate into post-mitotic myotubes. At 24 and 48 hours after transfection, cells were washed in PBS, and MFP was added, where indicated, to the culture media. Media and cells were harvested 72 hours post-differentiation. 20μg of total RNA was DNase I treated, size separated in 1.5% agarose-formaldehyde gel and transferred to nylon membrane. The membranes were hybridized with a specific GHRH cDNA probe ^{32}P-labeled by random priming. Negative controls were cells transfected by the GeneSwitch® and GHRH plasmids, but not treated with MFP, or cells transfected by the inducible GHRH plasmid alone. The positive control was cells transfected by a constitutively expressed GHRH plasmid that was driven by a synthetic muscle-specific promoter ("SP-GHRH"). GHRH transcripts of the expected size of 0.35kb were only observed in cells transfected with the GeneSwitch®/inducible GHRH plasmids and treated with MFP, and in cells transfected with the positive control (FIGURE 7). No GHRH transcripts were detected in cells not treated with MFP or in cells transfected by the inducible GHRH plasmid alone.

EXAMPLE 4

GHRH/GENESWITCH® SYSTEM IN VIVO—IMPROVED BODY COMPOSITION AND FAT BODY MASS/TOTAL WEIGHT

[0170] For the *in vivo* experiments, the plasmids for the GHRH/GeneSwitch® system were delivered to the muscles of SCID mice. The left tibialis anterior muscle was injected with 10 μg of a 10:1 mixture of pGR1774/pGS1633, followed by caliper electroporation (Draghia-Akli et al., 1999). At twenty-one days post-injection, animals were injected inter perineum ("i.p.") with 250 micrograms/kg of MFP for 3 days. On the fourth day, the animals were bled and serum was used to measure IGF-I levels. Mouse IGF-I was measured by heterologous, 100% cross-reacting rat radioimmunoassay. The sensitivity of the assay was 0.8 ng/ml; intra-assay and inter-assay variation was 3.4% and 4.5% respectively. Following administration of MFP for 4 consecutive days, IGF-I levels increased from 1100.86 ± 33.67 ng/ml to 1797.28 ± 164.96 ng/ml ($p < 0.0005$). Significant changes in the IGF-I levels were seen when the MFP group was compared with the control group 1086.78 ± 65.34 ng/ml, $p < 0.0006$ (animals that received a control beta-galactosidase plasmid), 1171.79 ± 42 ng/ml, $p < 0.001$ (animals that received the GHRH/GeneSwitch® plasmids but were not dosed with MFP). Upon repeated administration of MFP to the animals using the same protocol followed by recovery to background 7 days over 149 days, serum IGF-I levels rose repeatedly 1.1-1.7 fold over the uninjected controls (FIGURE 8). Animals induced with MFP had statistically significant higher IGF-I levels.

[0171] Body weight was similar for all of the groups during the first 125 days of the study (FIGURE 9). However, from day 125 to day 149, mice were dosed with MFP every day. A 7.5% increased body weight was observed in the chronically MFP-induced GHRH/GeneSwitch® animals, averaging 31.84 ± 0.12g ($p < 0.027$), compared with β-gal controls, 29.62 ± 0.98g, and with animals that were not induced with MFP, 30.53 ± 0.59g. All values are average ± SEM. Organs (lungs, heart, liver, kidney, stomach, intestine, adrenals, gonads, brain) were collected and weighed. No organomegaly or associated pathology was observed in any of the animals. Pituitary glands were dissected within the first minutes post-mortem, and weighed. Pituitary weight/ total body weight (FIGURE 10) increased upon chronic stimulation of the GHRH/GeneSwitch® by 20% ($7.35 \pm 0.31 \times 10^{-5}$), compared with β-gal controls ($6.13 \pm 0.46 \times 10^{-5}$), and animals not dosed with MFP

($6.3 \pm 0.22 \times 10^{-5}$), $p < 0.035$. There was no significantly statistical difference between the β-gal injected animals and animals that were injected with the GHRH/GeneSwitch® system, but not given MFP. Although not to be bound by theory, the increase in pituitary weight was probably due to somatotrophs hypertrophy, as it is known that GHRH is capable of stimulating the synthesis/secretion of GH from the anterior pituitary and has a specific hypertrophic effect on somatotrophs (Morel et al., 1999; Murray et al., 2000). At the end of the experiment, body composition was analyzed *in vivo*, by dual-energy x-ray absorptiometry ("DEXA"), using a high resolution PIXImus scanner. Body composition studies by PIXImus (total body fat, non-bone lean tissue mass and bone mineral area, content and density) showed significant changes in chronically MFP induced animals injected with the GHRH/GeneSwitch® system. Lean body mass (non-bone) (FIGURE 11) increased by 2.5% in GHRH/GeneSwitch® animals + MFP (87.44 ± 0.65%, versus β-gal 84.94 ± 0.6%, and no MFP animals 84.88 ± 0.3%), $p < 0.022$. Fat mass (FIGURE 12) decreased by 2 % in GHRH/GeneSwitch® animals (12.59 ± 0.62%, versus β-gal 14.57 ± 0.75%, and no MFP animals 15.09 ± 0.3%), $p < 0.05$.

EXAMPLE 5

GHRH/GENESWITCH® IN VIVO—INCREASED BONE AREA AND MINERAL CONTENT

[0172] One aspect of the present invention involves demonstrating that the introduction of mifepristone-inducible heterologous nucleic acid sequences encoding GHRH or functional biological equivalent thereof into the cells of subjects can lead to high levels of circulating hormones (Mir et al., 1999), without the disadvantages (*e.g.* high production costs, safety concerns with the virus backbone, or *ex vivo* manipulation) associated with viral vector delivery or organoids (Barr and Leiden, 1991; Dhawan et al., 1991; Draghia-Akli et al., 1999). In addition, the invention must demonstrate that animal growth and body composition can be efficiently regulated by mifepristone following *in vivo* electroporation of the GeneSwitch® technology (*i.e.* mifepristone-inducible heterologous nucleic acid sequences encoding GHRH or functional biological equivalent thereof) into skeletal muscle of the subject, as schematically diagrammed in FIGURE 6. Enhanced biological potency, delivery and proper gene expression regulation was observed over 149 days post-injection, and effectively reduced the theoretical quantity of

GHRH needed to achieve physiological levels of GH secretion when compared to the recombinant GHRH therapies. Post-injected subjects did not experience any side effects from the GeneSwitch® technology therapy. For example, mice had normal biochemical profiles, and no associated pathology or organomegaly. From a functional standpoint, the IGF-I levels increased, growth was enhanced by 7.5%, and changes in body composition (*e.g.* with increased lean body mass by 2.5% and decreased fat by 2%) were observed following chronic induction of the GHRH/Gene Switch system. In addition, bone mineral density increased by 6%, and the stimulation of GHRH on bone metabolism were even more remarkable. Although not to be bound by theory, the observed pituitary hypertrophy was indicative that ectopic expression of myogenic GHRH plasmids operates through the natural GH axis (stimulation of GH synthesis and secretion at the pituitary level). This long-lasting regulated therapy has the potential to replace classical GH therapy regimens and may stimulate the GH axis in a more physiologically appropriate manner. It is known that GHRH stimulates bone formation (Dubreuil et al., 1996), and the described GeneSwitch® therapy may be used to promote post-fracture bone growth.

[0173] Upon chronic stimulation of the GHRH/ GeneSwitch® system, significant changes occurred in bone area (FIGURE 13), that increased by 7 %, (12.81 ± 0.14 cm^2, versus B=Beta β-gal injected controls 11.98 ± 0.3 cm^2, or no MFP animals 12.07 ± 0.26 cm^2), $p < 0.0006$, bone mineral content (FIGURE 14) increased by 14.6% (0.755 ± 0.012g, versus β-gal injected controls 0.659 ± 0.019g, or no MFP animals 0.694 ± 0.023 cm^2), $p < 0.002$, and bone mineral density increased by 6% (0.059 ± 0.0007 g/cm^2, versus β-gal injected controls 0.056 ± 0.0009 g/cm^2, or no MFP animals 0.057 ± 0.0007 g/cm^2), $p < 0.012$. Practically, there is no overall difference between the β-gal injected animals and animals that were injected with the GHRH/ GeneSwitch®, but were not given MFP, which supports the absence of GHRH expression by the GHRH/GeneSwitch® plasmids in the absence of MFP dosing.

EXAMPLE 6

LOW VOLTAGE ELECTROPORATION INCREASES PLASMID UPTAKE AND EXPRESSION IN ADULT PIGS

[0174] Direct intra-muscular plasmid DNA injection followed by electroporation is a method for the local and controlled delivery of plasmid DNA into skeletal muscle. It has the advantage that is uses low plasmid quantities (as low as 0.1 mg in pigs), rather than the high quantities typically used with passive delivery modalities. Although not wanting to be bound by theory, the mechanism of the increased plasmid uptake by electroporation probably occurs through newly created membrane pores with or without protein active transport. It has been shown that the degree of permeabilization of the muscle cells is dependent on the electric field intensity, length of pulses, shape and type of electrodes (Bureau et al., 2000; Gilbert et al., 1997), and cell size (Somiari et al., 2000). Classical electrode configuration, plates or a pair of wire electrodes placed 4 mm apart were shown to be effective in rodents, but in large mammals as pigs or humans the increased resistance of the skin, the thickness of the subcutaneous fat tissue, and the concern for tissue damage if the intensity of the electric field would be proportionally increased, make these types of electrodes unpractical. The porcine muscle fibers are quite large and consequently more suitable for electropermeabilization than rodent muscle. Data provided herein indicate that a single injection of an optimum dosage of plasmid followed by electroporation with intramuscular applicators is sufficient to produce therapeutic plasma hormone levels in a large mammal with biologically significant effects on the body fat distribution and lean body mass of the subject.

[0175] External caliper electrodes and injectable electrodes were evaluated to determine the type of electrode needed to achieve a physiologically relevant level of a secreted reporter protein in 4-5 kg hybrid pigs. Reporter vectors expressing secreted embryonic alkaline phosphatase ("SEAP") were used in these studies at a dose of 2 mg pSP-SEAP/animal. Six-needle and 3-needle array electrodes were compared with standard caliper electrodes (FIGURE 15). Conditions of 6 pulses, 200V/cm, 60 milliseconds/pulse, previously tested as being the most effective in pigs (Draghia-Akli et al., 1999) were applied in all tests. For the three-needle electrode, three pulses were applied in one direction, then the polarity was changed and the next three pulses were delivered in the opposite direction. SEAP values were measured at day 0, day 3 and day 7 post-injection. Seven days post-injection, the SEAP levels were 9.33 ± 2.26 ng/(ml·kg) in plasmid-injected and caliper electroporated animals, compared to 0.02 ± 0.005 ng/(ml·kg)

in vehicle-injected animals. Using the 3-needle and 6-needle arrays, a 12.4 and 19 fold increase in SEAP values was obtained compared to caliper delivery (116.07 ± 44.36 ng/(ml·kg), and 177.41 ± 18.44 ng/(ml·kg), respectively). When using the same number of pulses, but lower voltage (100V/cm), and the 6-needle electrodes, the average SEAP increased to 144.64 ± 11.82 ng/(ml·kg) after seven days. When longissimus dorsi and semitendinosus muscles were injected using similar conditions, expression in the semitendinosus muscle was slightly higher. Skin and muscle from the injected pigs were collected at the end of the experiment (at 50 days post-injection) and histologically analyzed. At 100-200 V/cm used in the injectable electrodes experiments, no skin or muscle damage was seen for any of the needle-type electrodes used.

EXAMPLE 7

INCREASED EFFICIENCY USING NEEDLE-TYPE ELECTROPORATION DELIVERY FOR THERAPEUTIC PROTEINS

[0176] Not wanting to be bound by theory, growth hormone releasing hormone ("GHRH") stimulates the production and release from the anterior pituitary of growth hormone ("GH"), which in turn stimulates the production of IGF-I from the liver and other target organs (Frohman et al., 1968). In previous studies (Draghia-Akli et al., 1999), young pigs weighing 4-5 kg, were injected with 10 mg myogenic vector expressing a mutated form of GHRH, stable to proteases ("pSP-HV-GHRH") and electroporated using a caliper electrode.

[0177] The present invention involves determination of the best age for treatment of young pigs. Groups of 2 piglets were injected with 2 mg pSP-HV-GHRH using the 6-needle array electrodes at different time points: birth, 7, 14 and 21 days of age (FIGURE 16). Each animal received one injection. The group injected at 14 days of age demonstrated the best weight gain, (statistically significant and different from PBS controls (n=3) at every time point (final weights: 25.8 ± 1.5 kg versus 19.7 ± 0.03 kg, $p < 0.013$)). The next best group was injected at 7 days of age, and weighed 21.9 ± 1.5 kg at age 50 days, $p < 0.02$.

[0178] In a parallel study, the reduction in the plasmid quantity needed to achieve improved growth and changes in the metabolic and hormonal profile of pigs was

explored. Groups of two piglets each (Yorkshire x Landrace) were injected at 10 days of age with pSP-HV-GHRH (3 mg, 1 mg, 0.1 mg), and electroporated using a 6-needle array electrode (FIGURE 17). The group injected with 0.1 mg of plasmid had the greater weight gain, with statistically significant differences to controls (n=3) to 50 days of age (22.4 ± 0.8 kg versus 19.7 ± 0.03 kg, $p < 0.012$). One animal in the group injected at 21 days and one animal injected with the highest plasmid dose (3 mg) developed neutralizing antibodies against the mutated HV-GHRH and showed significant reduced rates of weight gain (at 50 days post-injection 15.6 kg and 15.95 kg, respectively, versus more than 21 kg for the paired animal in the same treatment group). No other group developed neutralizing antibodies. Thus, the minimal plasmid dosage (0.1 mg) and injection at optimum age using the 6-needle electrodes resulted in the best growth performances. It is noteworthy that in previous studies the inventors used 100-fold less, *i.e.*, 10 mg pSP-HV-GHRH with the caliper electrodes to produce similar changes.

[0179] An indication of increased systemic levels of GHRH and GH is an increase in serum IGF-I concentration. The level of serum IGF-I started to rise at 3 days post-injection in pigs that received the 0.1 and 1 mg doses of pSP-HV-GHRH (FIGURE 18). By 35 days after the injection (age of animals: 45 days), serum IGF-I concentrations were approximately 10-fold higher in pigs injected with 0.1 mg and 7-fold higher in pigs injected with 1 mg plasmid compared with controls ($p < 0.007$ and $p < 0.04$ respectively).

[0180] In pSP-HV-GHRH injected pigs, under optimum conditions (Table I) serum urea decreased (8.36±1.33 to 9.67±1.27 mg/ml in pSP-HV-GHRH injected pigs versus 11.14±1.9 mg/ml in controls, respectively ($p<0.05$), indicating decreased amino acid catabolism. Serum glucose levels were similar between the plasmid pSP-HV-GHRH injected pigs and controls; insulin levels were normal and within the control range.

Table 1
The plasma metabolic profile of pSP-HV-GHRH injected and control pigs.

Group Age	Glucose (mg/ml)	Urea (mg/ml)	Creatine (mg/ml)	Total Protein (g/dl)
Day 0	125.82 ± 5.64	8.36 ± 1.33 *p < 0.01*	0.85 ± 0.05	4.81 ± 0.11
Day 7	122.43 ± 5.05	9.43 ± 1.67 *p< 0.03*	0.87 ± 0.04	5.21 ± 0.19
Day 14	129.54 ± 6.39	9.62 ± 1.72 *p < 0.02*	1.00 ± 0.04	5.22 ± 0.20
Day 21	110.25 ± 5.02	13.83 ± 1.2	0.93 ± 0.05	4.81 ± 0.21
Dose				
3 mg	111.07 ± 3.88	10.50 ± 1.87 *p < 0.05*	0.9 ± 0.09	3.89 ± 0.16
1 mg	121.63 ± 2.93	9.44 ± 1.07 *p < 0.02*	0.81 ± 0.06	4.11 ± 0.11
0.1 mg	120.73 ± 2.53	9.67 ± 1.27 *p < 0.02*	0.95 ± 0.05	4.05 ± 0.19
Control	119.77 ± 3.67	12.81 ± 2.01	0.98 ± 0.08	4.00 ± 0.11

[0181] The fact that these animals have a normal carbohydrate metabolism is very important, as most livestock and/or patients under recombinant GH therapy develop impaired glucose metabolism and insulin resistance. Creatinine concentration (a measure of kidney function) was normal in all animals. Pigs that developed antibodies to GHRH showed a tendency to increased urea levels and decreased glucose levels.

[0182] Body composition studies by dual-energy x-ray absorptiometry (total body fat, non-bone lean tissue mass and bone mineral content), K40 potassium (lean body mass) and carcass neutron activation analysis (nitrogen) showed a proportional increase of all internal organs in GHRH injected animals (heart, lung, liver, spleen, brain, adrenals, stomach, kidney, pancreas, intestine). Nevertheless, the final body composition was different: animals injected with pSP-HV-GHRH at different ages gained proportionally less fat than controls and were leaner at the end of the study (4.34 ± 0.04 g of fat gained/ kg of fat free mass gained per day for injection at birth, 4.4 ± 0.04 g for injection at 7 days,

versus controls 5.63 ± 0.34 g, $p < 0.05$). Bone mineral density was higher in animals injected at 14 days after birth, and correlates with increased efficacy of the treatment: 0.363 ± 0.005 g/cm^2 versus 0.329 ± 0.003 g/cm^2 in controls, $p < 0.004$.

[0183] Treated pigs did not experience any side effects from the therapy, had normal biochemical profiles, and had no associated pathology or organomegaly. From a functional standpoint, the increases in IGF-I levels and enhancement in growth and changes in body composition (with decreased fat deposition by 22%) were dramatic in extent. The effects of the stimulation of GHRH on bone metabolism were even more remarkable, with an increase in bone mineral density by 10%. These results indicate that ectopic expression of myogenic HV-GHRH vectors has the potential to replace classical GH therapy regimens and may stimulate the GH axis in a more physiologically appropriate manner. The HV-GHRH molecule, which displays a high degree of stability and GH secretory activity in pigs, may also be useful in human clinical medicine. However, a skilled artisan is aware that a minimal plasmid dose should be determined on a pertinent model and used in order to avoid the unwanted pathology associated with antibody development, and routine methods in the art and/or described herein teach how to achieve this goal.

[0184] The molecular techniques used to produce alterations in any conceivable encoded nucleic acid sequences are well established, and exemplified by the large number of scientific publications and patents in the field of molecular biology. Despite the accuracy of the molecular techniques used to create distinctive nucleic acid sequences, a skilled artisan recognizes that the expression of any given nucleic acid will influence the complex biochemistry of an entire organism. Thus, the highly predictable nature of constructing unique nucleic acid sequences must not be confused with unknown facts of an associated biological effect.

[0185] The invention described herein involves the utilization of several distinctive GHRH or analog nucleic acid sequences. Based upon the current understanding of protein-protein interactions, it is neither obvious nor possible to accurately speculate upon the *in vivo* parameters (*e.g.* half life, efficacy, post-translational modifications, *etc.*) of a GHRH sequence that contains a point mutation which alters a

single amino acid in the polypeptide chain. As seen in the Examples provided herein, mutation of a few base pairs gave rise to GHRH mutants with significantly longer bio-availability. The endogenous GHRH has a half-life of 6-12 minutes in different species. The HV-GHRH has a half-life of 6 hours. In further analysis, the TI-GHRH (that has only two base pair difference with the HV-GHRH) has been shown to have a much higher effect *in vivo* on lean body mass than the HV-GHRH (from simple to double). This property was not evident in extensive *in vitro* studies on pituitary cell. Correspondingly, one skilled in the art would know how to perform the plasmid-mediated supplementation of GHRH or the related recombinant protein experimentation(s), characterizing variations and permutations on a unique nucleic acid sequence in a specific tissue to accurately evaluate the *in vivo* effect within a living organism. Therefore, the utilization of the distinctive nucleic acid sequence encoding GHRH or functional biological equivalent thereof or corresponding recombinant protein as a method to decrease body fat proportion and increase lean body mass could not have been predicted based on speculation.

[0186] Although not wanting to be bound by theory, it is believed that an increase in GHRH or functional biological equivalent will increase the GH levels to decrease body fat proportion and increase lean body mass. Hormones (*e.g.* GHRH and GH) often contain a complex feedback-regulated pathway. Without direct experimentation of GHRH or analogs used in gene or recombinant protein therapy, it could not have been predicted by one skilled in the art to determine which concentrations of non-native encoded sequences will yield desired results. Ideal regulation of a nucleic acid sequence encoding GHRH or functional biological equivalent thereof is further complicated by the tissue used for polynucleotide delivery, and would not have been obvious to one skilled in the art without actual experimentation with the distinctive sequence in a particular tissue. The invention described herein contains the descriptions and results of essential experimentation that explored tissue specific and inducible regulation of distinctive nucleic acid sequences that encoded GHRH or functional biological equivalent thereof, which was not obvious based upon prior art. The present invention is a significant step forward in developing non-viral therapy for large animals, including humans. In order for gene therapies to be transferred from rodents to large mammals, and ultimately to humans, it was surprising that extremely low quantities of

plasmid were effective. It is shown herein that as little as 0.1 mg plasmid delivered under the proper electroporation conditions had an important biological impact that decreases the body fat proportion, increases lean body mass ("LBM"), or both of a subject. This plasmid quantity was 100 fold lower than the theoretical one, and could not have been predicted from the relative doses used in rodents (in average 1mg/kg). Although not wanting to be bound by theory, unlike other therapies using growth factors (GH and/ or IGF-I), GHRH is stimulating the endogenous secretion of hormones, and enhancing the own bio-potential of the animal, with no adverse effects. This experimental finding cannot be theoretically predicted, as the three hormones are members of the same growth axis.

[0187] The increase in lean body mass, decrease in body fat proportions, increase in bone density, and/or increase in bone rate of healing are a consequence of the GHRH molecules present in the subjects circulation, regardless of the means of the delivery. For example, one would obtain the same effect by delivering appropriate quantities of GHRH or analog thereof, outlined in FIGURE 1, by classical recombinant protein therapy or nucleic acid transfer. Accordingly, successful plasmid-mediated supplementation of GHRH requires accurate delivery of the encoded sequences to the cells of a subject, resulting in expression of the gene product at levels appropriate to produce a biological effect. The duration of treatment will extend through the course of the disease symptoms, and possibly continuously. Since the method to deliver nucleic acid sequences to the cells of a subject is highly dependent on specific diseases and the encoded gene, it could not have been predicted by one skilled in the art which method and conditions are appropriate without laborious and failed experimentations. Thus, the preferred method of outlined for this invention is *in vivo* electroporation.

[0188] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned as well as those inherent therein. Methods, procedures, techniques, and kits described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the invention.

[0189] All of the methods and compositions disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the methods and compositions of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the systems and compositions without departing from the concept, spirit, and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically, structurally and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as defined by the appended claims.

WHAT IS CLAIMED:

1. A method of decreasing body fat proportion in a subject, comprising: delivering into cells of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof.

2. The method of claim 1, wherein delivering into the cells of the subject the nucleic acid expression construct is via electroporation.

3. The method of claim 1, wherein the cells of the subject are somatic cells, stem cells, or germ cells.

4. The method of claim 1, wherein the nucleic acid expression construct comprises SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21.

5. The method of claim 1, wherein the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

6. The method of claim 5, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

7. The method of claim 5, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

8. The method of claim 1, wherein the delivering into the cells of the subject the nucleic acid expression construct initiates expression of the encoded GHRH or functional biological equivalent thereof.

9. The method of claim 8, wherein the encoded GHRH or functional biological equivalent thereof is expressed in tissue specific cells of the subject.

10. The method of claim 9, wherein the tissue specific cells of the subject comprises muscle cells.

11. The method of claim 8, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

12. The method of claim 8, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

13. The method of claim 12, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

14. The method of claim 1, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

15. A method of decreasing body fat proportion in a subject, comprising the steps of:

(a) delivering into cells of the subject a first nucleic acid expression construct, a second nucleic acid expression construct, and a molecular switch; wherein the first nucleic acid expression construct encodes growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof; and wherein the second nucleic acid expression construct has an encoding region of a regulator protein; and

(b) delivering a molecular switch molecule into the subject, wherein the molecular switch molecule governs activation of the regulator protein.

16. The method of claim 15, wherein delivering step of the first nucleic acid expression construct into the cells of the subject is via electroporation.

17. The method of claim 15, wherein delivering step of the second nucleic acid expression construct into the cells of the subject is via electroporation.

18. The method of claim 15, wherein the first nucleic acid expression construct encodes a polypeptide of a sequence comprising SeqID No: 1, SeqID No: 2, SeqID No: 3, or SeqID No: 4.

19. The method of claim 15, wherein the first nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

20. The method of claim 19, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

21. The method of claim 19, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

22. The method of claim 15, wherein the delivering into the cells of the subject the second nucleic acid expression construct initiates expression of the encoded regulatory protein.

23. The method of claim 22, wherein the encoded regulatory protein is expressed in the cells of the subject, but is inactive.

24. The method of claim 23, wherein the delivering of the molecular switch into the subject activates the regulatory protein, and the molecular switch comprises mifepristone or its functional equivalent.

25. The method of claim 24, wherein the active regulatory protein initiates expression of the GHRH or functional biological equivalent encoded on the first nucleic acid sequence.

26. The method of claim 25, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

27. The method of claim 25, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

28. The method of claim 27, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

29. The method of claim 15, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

30. The method of claim 15 wherein the first nucleic acid expression construct comprises SeqID No: 26.

31. The method of claim 15 wherein the second nucleic acid expression construct comprises SeqID No: 27.

32. A method of decreasing body fat proportion in a subject, comprising the steps of: delivering into a subject a recombinant growth-hormone-releasing-hormone ("GHRH") or a biological functional equivalent thereof.

33. The method of claim 32, wherein the recombinant GHRH is a biologically active polypeptide; and the recombinant functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

34. The method of claim 32, wherein the recombinant GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

35. The method of claim 32, wherein the recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

36. The method of claim 32, wherein the recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

37. A method for increasing lean body mass a subject, comprising the step of: delivering into cells of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof.

38. The method of claim 37, wherein delivering into the cells of the subject the nucleic acid expression construct is via electroporation.

39. The method of claim 37, wherein the cells of the subject are somatic cells, stem cells, or germ cells.

40. The method of claim 37, wherein the nucleic acid expression construct comprises SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21.

41. The method of claim 37, wherein the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

42. The method of claim 41, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

43. The method of claim 41, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

44. The method of claim 37, wherein the delivering into the cells of the subject the nucleic acid expression construct initiates expression of the encoded GHRH or functional biological equivalent thereof.

45. The method of claim 44, wherein the encoded GHRH or functional biological equivalent thereof is expressed in tissue specific cells of the subject.

46. The method of claim 45, wherein the tissue specific cells of the subject comprises muscle cells.

47. The method of claim 44, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

48. The method of claim 44, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

49. The method of claim 48, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

50. The method of claim 37, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

51. A method for increasing lean body mass a subject, comprising the steps of:

(a) delivering into cells of the subject a first nucleic acid expression construct, a second nucleic acid expression construct, and a molecular switch;

wherein the first nucleic acid expression construct encodes growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof; and wherein the second nucleic acid expression construct has an encoding region of a regulator protein; and

(b) delivering a molecular switch molecule into the subject, wherein the molecular switch molecule governs activation of the regulator protein.

52. The method of claim 51, wherein delivering step of the first nucleic acid expression construct into the cells of the subject is via electroporation.

53. The method of claim 51, wherein delivering step of the second nucleic acid expression construct into the cells of the subject is via electroporation.

54. The method of claim 51, wherein the first nucleic acid expression construct encodes a polypeptide of a sequence comprising SeqID No: 1, SeqID No: 2, SeqID No: 3, or SeqID No: 4.

55. The method of claim 51, wherein the first nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

56. The method of claim 55, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

57. The method of claim 55, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

58. The method of claim 51, wherein the delivering into the cells of the subject the second nucleic acid expression construct initiates expression of the encoded regulatory protein.

59. The method of claim 58, wherein the encoded regulatory protein is expressed in the cells of the subject, but is inactive.

60. The method of claim 59, wherein the delivering of the molecular switch into the subject activates the regulatory protein, and the molecular switch comprises mifepristone or its functional equivalent.

61. The method of claim 60, wherein the active regulatory protein initiates expression of the GHRH or functional biological equivalent encoded on the first nucleic acid sequence.

62. The method of claim 51, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

63. The method of claim 51, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

64. The method of claim 63 wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

65. The method of claim 51, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

66. The method of claim 51 wherein the first nucleic acid expression construct comprises SeqID No: 26.

67. The method of claim 51 wherein the second nucleic acid expression construct comprises SeqID No: 27.

68. A method for increasing lean body mass a subject, comprising the steps of: delivering into a subject a recombinant growth-hormone-releasing-hormone ("GHRH") or a biological functional equivalent thereof.

69. The method of claim 68, wherein the recombinant GHRH is a biologically active polypeptide; and the recombinant functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

70. The method of claim 68, wherein the recombinant GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

71. The method of claim 70, wherein the recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

72. The method of claim 68, wherein the recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

73. A method for increasing bone density in a subject, comprising: delivering into cells of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof.

74. The method of claim 73, wherein delivering into the cells of the subject the nucleic acid expression construct is via electroporation.

75. The method of claim 73, wherein the cells of the subject are somatic cells, stem cells, or germ cells.

76. The method of claim 73, wherein the nucleic acid expression construct comprises SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21.

77. The method of claim 73, wherein the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

78. The method of claim 73, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

79. The method of claim 73, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

80. The method of claim 73, wherein the delivering into the cells of the subject the nucleic acid expression construct initiates expression of the encoded GHRH or functional biological equivalent thereof.

81. The method of claim 80, wherein the encoded GHRH or functional biological equivalent thereof is expressed in tissue specific cells of the subject.

82. The method of claim 81, wherein the tissue specific cells of the subject comprises muscle cells.

83. The method of claim 80, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

84. The method of claim 80, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

85. The method of claim 84, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

86. The method of claim 73, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

87. A method for increasing bone density in a subject, comprising the steps of:

(a) delivering into cells of the subject a first nucleic acid expression construct, a second nucleic acid expression construct, and a molecular switch; wherein the first nucleic acid expression construct encodes growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent

thereof; and wherein the second nucleic acid expression construct has an encoding region of a regulator protein; and

(b) delivering a molecular switch molecule into the subject, wherein the molecular switch molecule governs activation of the regulator protein.

88. The method of claim 87, wherein delivering step of the first nucleic acid expression construct into the cells of the subject is via electroporation.

89. The method of claim 87, wherein delivering step of the second nucleic acid expression construct into the cells of the subject is via electroporation.

90. The method of claim 87, wherein the first nucleic acid expression construct encodes a polypeptide of a sequence comprising SeqID No: 1, SeqID No: 2, SeqID No: 3, or SeqID No: 4.

91. The method of claim 87, wherein the first nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

92. The method of claim 87, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

93. The method of claim 87, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

94. The method of claim 87, wherein the delivering into the cells of the subject the second nucleic acid expression construct initiates expression of the encoded regulatory protein.

95. The method of claim 94, wherein the encoded regulatory protein is expressed in the cells of the subject, but is inactive.

96. The method of claim 95, wherein the delivering of the molecular switch into the subject activates the regulatory protein, and the molecular switch comprises mifepristone or its functional equivalent.

97. The method of claim 96, wherein the active regulatory protein initiates expression of the GHRH or functional biological equivalent encoded on the first nucleic acid sequence.

98. The method of claim 97, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

99. The method of claim 97, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

100. The method of claim 99 wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

101. The method of claim 87, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

102. The method of claim 87 wherein the first nucleic acid expression construct comprises SeqID No: 26.

103. The method of claim 87 wherein the second nucleic acid expression construct comprises SeqID No: 27.

104. A method for increasing bone density in a subject, comprising the steps of: delivering into a subject a recombinant growth-hormone-releasing-hormone ("GHRH") or a biological functional equivalent thereof.

105. The method of claim 104, wherein the recombinant GHRH is a biologically active polypeptide; and the recombinant functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

106. The method of claim 104, wherein the recombinant GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

107. The method of claim 106, wherein the recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

108. The method of claim 104, wherein the recombinant GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

109. A method for increasing a bone healing rate in a subject, comprising: delivering into cells of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof.

110. The method of claim 109, wherein delivering into the cells of the subject the nucleic acid expression construct is via electroporation.

111. The method of claim 109, wherein the cells of the subject are somatic cells, stem cells, or germ cells.

112. The method of claim 109, wherein the nucleic acid expression construct comprises SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21.

113. The method of claim 109, wherein the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

114. The method of claim 113, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

115. The method of claim 113, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

116. The method of claim 115, wherein the delivering into the cells of the subject the nucleic acid expression construct initiates expression of the encoded GHRH or functional biological equivalent thereof.

117. The method of claim 109, wherein the encoded GHRH or functional biological equivalent thereof is expressed in tissue specific cells of the subject.

118. The method of claim 117, wherein the tissue specific cells of the subject comprises muscle cells.

119. The method of claim 109, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

120. The method of claim 119, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_{-1}-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

121. The method of claim 120, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

122. The method of claim 109, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

123. A method for increasing a bone healing rate in a subject, comprising the steps of:

(a) delivering into cells of the subject a first nucleic acid expression construct, a second nucleic acid expression construct, and a molecular switch; wherein the first nucleic acid expression construct encodes growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent

thereof; and wherein the second nucleic acid expression construct has an encoding region of a regulator protein; and

(b) delivering a molecular switch molecule into the subject, wherein the molecular switch molecule governs activation of the regulator protein.

124. The method of claim 123, wherein delivering step of the first nucleic acid expression construct into the cells of the subject is via electroporation.

125. The method of claim 123, wherein delivering step of the second nucleic acid expression construct into the cells of the subject is via electroporation.

126. The method of claim 123, wherein the first nucleic acid expression construct encodes a polypeptide of a sequence comprising SeqID No: 1, SeqID No: 2, SeqID No: 3, or SeqID No: 4.

127. The method of claim 123, wherein the first nucleic acid expression construct further comprises, a transfection-facilitating polypeptide.

128. The method of claim 127, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

129. The method of claim 127, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

130. The method of claim 123, wherein the delivering into the cells of the subject the second nucleic acid expression construct initiates expression of the encoded regulatory protein.

131. The method of claim 130, wherein the encoded regulatory protein is expressed in the cells of the subject, but is inactive.

132. The method of claim 131, wherein the delivering of the molecular switch into the subject activates the regulatory protein, and the molecular switch comprises mifepristone or its functional equivalent.

133. The method of claim 132, wherein the active regulatory protein initiates expression of the GHRH or functional biological equivalent encoded on the first nucleic acid sequence.

134. The method of claim 133, wherein the encoded GHRH is a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide.

135. The method of claim 133, wherein the encoded GHRH or functional biological equivalent thereof is of formula (SEQID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

136. The method of claim 135 wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

137. The method of claim 123, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

138. The method of claim 123 wherein the first nucleic acid expression construct comprises SeqID No: 26.

139. The method of claim 123 wherein the second nucleic acid expression construct comprises SeqID No: 27.

140. A method for altering lean body mass in an subject, the method comprising:

delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof;

wherein subsequent expression of the GHRH or biological equivalent is sufficient to increase lean body mass or decrease body fat proportion.

141. The method of claim 140, further comprising:

applying a cell-transfecting pulse to the tissue;

wherein, the cell-transfecting pulse is delivered to an area in the tissue comprising the delivered nucleic acid expression construct.

142. The method of claim 141, further comprising:

placing a plurality of electrodes in the tissue before applying the cell-transfecting pulse to the tissue;

wherein the nucleic acid expression construct is delivered to the tissue in an area that interposes the plurality of electrodes.

143. The method of claim 141, wherein the cell-transfecting pulse is an electrical pulse or a vascular pressure pulse.

144. The method of claim 140, wherein delivering into a tissue of the subject the nucleic acid expression construct comprises electroporation.

145. The method of claim 140, wherein the delivering step is by injection, gene gun, or gold particle bombardment.

146. The method of claim 140, wherein the subject is a domesticated animal; a food animal; or a work animal.

147. The method of claim 140, wherein the subject is a human.

148. The method of claim 140, wherein the nucleic acid expression construct is substantially free of a viral backbone.

149. The method of claim 140, wherein a promoter of the nucleic acid expression construct comprises a tissue-specific promoter.

150. The method of claim 149, wherein the tissue-specific promoter comprises a muscle-specific promoter.

151. The construct of claim 149, wherein the promoter comprises SPc5-12 (SeqID No: 7).

152. The method of claim 140, wherein a 3′ untranslated region of the nucleic expression construct is a human growth hormone 3′ UTR, bovine growth hormone 3′ UTR, skeletal alpha actin 3′ UTR, or SV40 polyadenylation signal.

153. The method of claim 140, wherein the tissue comprises muscle.

154. The method of claim 140, wherein the nucleic acid expression construct is SeqID No: 11, SeqID No: 12, SeqID No: 13, SeqID No: 14, SeqID No: 17, SeqID No: 18, SeqID No: 19, SeqID No: 20, or SeqID No: 21.

155. The method of claim 140, wherein the encoded GHRH or functional biological equivalent comprises SeqID No: 2

156. The method of claim 140, wherein the encoded GHRH or functional biological equivalent comprises SeqID No: 3

157. The method of claim 140, wherein the encoded GHRH or functional biological equivalent comprises SeqID No: 4

158. The method of claim 140, wherein the encoded GHRH or functional biological equivalent comprises SeqID No: 10

159. The method of claim 140, wherein the encoded GHRH or functional biological equivalent thereof comprises the amino acid formula (SeqID No: 6):

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

160. The method of claim 140, wherein the nucleic acid expression construct further comprises a transfection-facilitating polypeptide.

161. The method of claim 160, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

162. The method of claim 160, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

163. The method of claim 140, wherein the encoded functional biological equivalent of GHRH is a polypeptide having similar or improved biological activity when compared to the GHRH polypeptide.

164. The method of claim 140, wherein the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in the subject.

165. A peptide for altering lean body mass comprising a functional biological equivalent of growth hormone releasing hormone ("GHRH"), wherein the GHRH is a polypeptide that is biologically active in a subject; and the functional biological equivalent of GHRH is a polypeptide that has been engineered to contain distinct amino acid sequences while simultaneously having similar or improved biologically activity when compared to GHRH.

166. The peptide of claim 165 comprising the formula:

$-X_1-X_2$–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

167. The peptide of claim 165 comprising SeqID No: 1; SeqID No: 2; SeqID No: 3; or SeqID No: 4.

168. A peptide for altering bone density comprising a functional biological equivalent of growth hormone releasing hormone ("GHRH"), wherein the GHRH is a polypeptide that is biologically active in a subject; and the functional biological equivalent of GHRH is a polypeptide that has been engineered to contain distinct amino acid sequences while simultaneously having similar or improved biologically activity when compared to GHRH.

169. The peptide of claim 168 comprising the formula:

-X_1-X_2–DAIFTNSYRKVL-X_3–QLSARKLLQDI-X_4-X_5–RQQGERNQEQGA-OH

wherein the formula has the following characteristics:

X_1 is a D-or L-isomer of the amino acid tyrosine ("Y"), or histidine ("H");

X_2 is a D-or L-isomer of the amino acid alanine ("A"), valine ("V"), or isoleucine ("I");

X_3 is a D-or L-isomer of the amino acid alanine ("A") or glycine ("G");

X_4 is a D-or L-isomer of the amino acid methionine ("M"), or leucine ("L");

X_5 is a D-or L-isomer of the amino acid serine ("S") or asparagine ("N");

or a combination thereof.

170. The peptide of claim 168 comprising SeqID No: 1; SeqID No: 2; SeqID No: 3; or SeqID No: 4.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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