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(54) Titre : SUPPLEMENTATION ADMINISTREE PAR PLASMIDES POUR LE TRAITEMENT DE PATIENTS A MALADIE CHRONIQUE

(54) Title: PLASMID MEDIATED SUPPLEMENTATION FOR TREATING CHRONICALLY ILL SUBJECTS

(57) Abrégé/Abstract:
The present invention pertains to compositions and methods for plasmid-mediated supplementation. The compositions and method are useful for retarding the growth of the tumor, and retarding cachexia, wasting, anemia and other effects that are commonly associated in cancer bearing animals. Overall, the embodiments of the invention can be accomplished by delivering an effective amount of a nucleic acid expression construct that encodes a GHRH or functional biological equivalent thereof into a tissue of an animal and allowing expression of the encoded gene in the animal. For example, when such a nucleic acid sequence is delivered into the specific cells of the animal 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 animal. 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 animal by the process of in vivo electroporation. In addition, a treatment for retarding the growth of the tumor, and retarding cachexia or the wasting effects that are commonly associated with tumors is achieved by the delivery of recombinant GHRH or biological equivalent into the animal.

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(54) Title: GROWTH HORMONE RELEASING HORMONE SUPLEMENTATION FOR TREATING CHRONICALLY ILL SUBJECTS

(57) Abstract: The present invention pertains to compositions and methods for plasmid-mediated supplementation. The compositions and method are useful for retarding the growth of the tumor, and retarding cachexia, wasting, anemia and other effects that are commonly associated in cancer bearing animals. Overall, the embodiments of the invention can be accomplished by delivering an effective amount of a nucleic acid expression construct that encodes a GHRH or functional biological equivalent thereof into a tissue of an animal and allowing expression of the encoded gene in the animal. For example, when such a nucleic acid sequence is delivered into the specific cells of the animal 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 animal. 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 animal by the process of in vivo electroporation. In addition, a treatment for retarding the growth of the tumor, and retarding cachexia or the wasting effects that are commonly associated with tumors is achieved by the delivery of recombinant GHRH or biological equivalent into the animal.

PLASMID MEDIATED SUPPLEMENTATION FOR TREATING CHRONICALLY ILL SUBJECTS

BACKGROUND

[0002] The present invention pertains to compositions and methods for plasmid-mediated supplementation. The present invention pertains to compositions and methods that are useful for retarding the growth rate of abnormal cells, tumor progression reduction, prevention of kidney failure, reduction in metastasis, increased survival and other conditions commonly associated with cancer-bearing animals. Some embodiments of the invention can be accomplished by delivering an effective amount of 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, a treatment for retarding tumor growth, and retarding cachexia or the wasting effects that are commonly associated with tumors is achieved by the delivery of recombinant GHRH or biological equivalent into the subject. Anemia, wasting, tumor growth, immune dysfunction, kidney failure, cancer, decreased life expectancy, and other conditions can be related to a specific cancer, tumor, disease or the effects of a disease treatment. This invention relates to a plasmid-mediated supplementation for:

1) treating anemia in a subject;

2) increasing total red blood cell mass in a subject;

3) decreasing tumor growth in a tumor bearing individual;

4) preventing or reversing the wasting of a subject;

5) reversing abnormal weight loss in a subject;

6) treating immune dysfunction;

7) preventing the onset of kidney failure

8) preventing the onset and / or development of metastasis

9) reversing the suppression of lymphopoesis in a subject; and/or

10) extending life expectancy and increasing survival for the chronically ill subject.

[0003] The present invention pertains to compositions and methods that are useful for retarding the growth rate of abnormal cells, tumor progression reduction, prevention of kidney failure, reduction of metastasis, and increased survival in cancer-bearing animals. Overall, the embodiments of the invention can be accomplished by delivering an effective amount of 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, a treatment for retarding the growth of abnormal cells and tumor growth is achieved by the delivery of recombinant GHRH or biological equivalent into the subject. Anemia, wasting, tumor growth, immune dysfunction, kidney failure, cancer, decreased life expectancy, and other conditions also can be related to a specific cancer, tumor, disease or the effects of a disease treatment

GHRH could be also delivered directly, as protein, by intravenous, subcutaneous or intranasal administration or through a slow release pump.

[0004] **Anemia:** Anemia refers to a condition in which there is a reduction of the number or volume of red blood corpuscles or of the total amount of hemoglobin in the bloodstream, resulting in paleness, generalized weakness, etc. of the subject. The production of red blood cells in mammals is known as erythropoiesis. Erythropoiesis is primarily controlled by erythropoietin ("EPO"), an acidic glycoprotein. The EPO stimulates the production of new erythrocytes to replace those lost to the aging process. Additionally, EPO production is stimulated under conditions of hypoxia, wherein the oxygen supply to the tissues is reduced below normal physiological levels despite adequate perfusion of the tissue by blood. Hypoxia may be caused by hemorrhaging, radiation-induced erythrocyte destruction, various anemia's, high altitude, or long periods of unconsciousness. In response to tissues undergoing hypoxic stress, EPO will increase red blood cell production by stimulating the conversion of primitive precursor cells in the bone marrow into proerythroblasts that subsequently mature, synthesize hemoglobin and are released into the circulation as red blood cells.

[0005] EPO is normally present in low concentrations in plasma, where it is sufficient to maintain equilibrium between normal blood cell loss (*i.e.*, through aging) and red blood cell production. Anemia is a decrease in red blood cell mass caused by decreased production or increased destruction of red blood cells. EPO supplementation is currently used for treatment of the anemia's associated with different diseases, as end-stage renal failure (Cremagnani et al., 1993; Diez et al., 1996) and acquired immunodeficiency syndrome ("AIDS") (Sowade et al., 1998), particularly in subjects who are being treated with zidovudine ("AZT"). EPO is also used for amelioration of the anemia associated with cancer chemotherapy (Vansteenkiste et al., 2002).

[0006] Another group of anemic disorders, each of which results from an inherited abnormality in globin production, is termed the hemoglobinopathies. Hemoglobinopathies include a spectrum of disorders that can be classified broadly into two types. The first types are those that result from an inherited structural alteration in one of the globin chains, for example sickle cell anemia. These disorders give rise to the production of abnormal hemoglobin molecules (Papassotiriou et al., 2000). The second

major subdivision of hemoglobinopathies, the thalassemias, results from inherited defects in the rate of synthesis of one or more of the globin chains. This causes ineffective erythropoiesis, hemolysis, and varying degrees of anemia due to the inadequate production of red blood cells. Accordingly, EPO can be used in the treatment of anemia's, for example, hemoglobinopathies that are characterized by low or defective red blood cell production and/or increased red blood cell destruction (Makis et al., 2001; Payen et al., 2001).

[0007] Additional prior art has indicated that anemic patients with panhypopituitarism, a condition in which hemoglobin ("Hb") concentration remained as low as 11.0 g/dl in spite of appropriate replacement with thyroid and adrenocortical hormones, were treated with recombinant human growth hormone ("GH") and EPO levels were increased (Sohmiya and Kato, 2000). Recombinant human GH was constantly infused subcutaneously for 12 months, which caused the plasma erythropoietin ("EPO") levels to nearly double, with a concomitant increase of Hb concentration. When the administration of human GH was interrupted, both plasma EPO levels and Hb concentrations decreased. There was a close correlation between plasma GH and EPO levels before and during the human GH administration. Plasma GH levels were well correlated with Hb concentrations before and during human GH administration. Plasma IGF-I levels were also correlated with Hb concentrations, but not with plasma EPO levels.

[0008] U.S. Patents No. 5,846,528 ("the '528 patent") and 6,274,158 (" the '158 patent") teach that conditions of anemia can be treated by deliberately increasing erythropoietin ("EPO"). In addition, the '528 patent teaches the use of recombinant adeno-associated virus ("AAV") virions for delivery of DNA molecules encoding EPO to muscle cells and tissue in the treatment of anemia. The '528 patent shows a direct *in vivo* injection of recombinant AAV virions into muscle tissue (*e.g.*, by intramuscular injection), and *in vitro* transduction of muscle cells that can be subsequently introduced into a subject for treatment. Thus, a sustained high-level expression of a delivered nucleotide sequence encoding erythropoietin results, whereby *in vivo* secretion from transduced muscle cells allows systemic delivery. The '158 patent teaches the use of the subcutaneous, intravenous or oral administration of recombinant human EPO as a hemostatic agent for the treatment or prevention of bleeding from any organ or body part involved with benign

or malignant lesions, surgical traumatic, non-healing/difficult to treat lesions, or radiation injury.

[0009] In brief, anemia can be caused by a specific disease, environmental factors, or the effects of a disease treatment. As discussed, circulating levels of EPO can be increased directly (*e.g.* injections of recombinant EPO) or indirectly (*e.g.* injections of recombinant GH). Although not wanting to be bound by theory, the related art suggests that anemic conditions can be successfully treated by methods or compounds capable of increasing the circulating levels of EPO. However, a skilled artisan recognizes that biological systems are immeasurably complex, and the ability to accurately predict what methods or compounds will elicit a specific biological response is outside the realm of a skilled artesian. Only through diligent laboratory experiments can insight to compounds or methods to treat anemia be discovered.

[0010] **Wasting:** Wasting of a subject can be defined as decreased body weight of at least 5-10% of the minimum ideal weight of the individual that is characterized by significant loss of both adipose tissue and muscle mass, which makes weight gain especially difficult for patients with a progressive disease (*e.g.* cancer, AIDS *etc.*). Wasting or cachexia is a classic clinical phenomenon that evokes historical images of sickbeds and patients with "consumption." It simply means "poor condition" in Greek. Accelerated loss of skeletal muscle can occur in setting of cancer, AIDS, or tuberculosis, as well as other chronic conditions (Barber et al., 1999; Weinroth et al., 1995). Weight loss is the most obvious manifestation of wasting associated with cancer (Nelson, 2000). Other clinical manifestations include anorexia, muscle wasting, and/or loss of adipose tissue and fatigue, which results in poor performance status (Davis and Dickerson, 2000). Because weight loss, tumor histology, and a poor performance status lead to a poor prognosis, wasting can become the direct cause of death. In contrast to simple starvation, the weight loss cannot be adequately treated with aggressive feeding. The weight loss therefore cannot be attributed entirely to poor intake, but is also a result of increased basal energy expenditure.

[0011] Wasting is present in more than one half of ambulatory cancer patients, and represents a serious problem when treating chronically ill patients. Although not wanting to be bound by theory, cytokine release and/or activation and liberation of several

tumor derived substances is postulated to be responsible for the wasting syndrome. The related art teaches that many agents have been evaluated for treatment of wasting, with only modest benefit obtained from progestational agents (Barber et al., 1999; Nelson, 2001). In contrast, recombinant growth hormone ("GH"), insulin-like growth factor-I ("IGF-I") and IGF binding protein 3 ("IGFBP-3") therapies are effective in producing a benefit in cancer cachexia (Bartlett et al., 1994). Thus, the related art suggests that wasting may be treated by methods or compounds that increase the circulating levels of GH, IGF-I or IGFBP-3. Unfortunately, the complexity of biological systems makes it impossible to accurately predict what methods or compounds will elicit a specific biological response. Thus, only through meticulous laboratory experiments can an insight to useful compounds or methods to treat wasting be elucidated by one skilled in the art.

[0012] **Cancer and tumor growth:** Cancer is one of the leading causes of morbidity and mortality in the US and around the world. The average annual incidence rate for cancer increased in the last 20 years, to reach 475 to 100,000 in 1999. Due to population growth and aging, the number of cancer patient is expected to double from 1.3 million to 2.6 million between 2000 and 2050. In addition, the number and proportion of older persons with cancer are expected to increase dramatically: from 389,000 persons aged 75 years and older with newly diagnosed malignancies in 2000, to 1,102,000 persons in 2050, an increase from 30% to 42% of the cancer population (Edwards et al., 2002). Cancer in elderly has a poor prognosis due to complicating factors as anorexia of aging, alterations in the gastrointestinal system, the effect of elevated leptin levels, especially in men, and a variety of changes in central nervous system neurotransmitters. Body mass declines after the age of about 70 years old. This includes both loss of adipose tissue and muscle mass. The loss of muscle mass in older individuals is termed sarcopenia. Illness results in an increase of cytokines that produce both anorexia and cause protein wasting. Many of the causes of cachexia in older individuals are treatable (Morley, 2001; Yeh and Schuster, 1999). Tumor growth is accelerated by increases in cytokines and other pathological changes in cancer patients, but correction of cachexia, anemia, improvement of immune function and a positive nitrogen balance can decrease tumor growth and its complications (Demetri, 2001; Koo et al., 2001). Thus, a therapy that would address most of these complications could be of important benefit for patients.

[0013] **Kidney failure:** The predicted increase in the number of people with kidney failure and end-stage renal disease places an enormous burden on healthcare providers system (Hostetter and Lising, 2002). In order to reduce this burden, strategies must be implemented to improve the detection of kidney disease, and preventative measures must be targeted at those at greatest risk of disease (Crook et al., 2002). Important risk factors include hypertension, diabetes, obesity and cancer (Al Suwaidi et al., 2002; Nampoory et al., 2002). Serum creatinine, proteinuria, and microalbuminuria as early detection markers of disease are important, but treatments that could delay or prevent kidney failure could be of significant benefit for patients and the medical system (LeBrun et al., 2000; Sakhuja et al., 2000).

[0014] **Growth Hormone ("GH") and Immune Function:** The central role of growth hormone ("GH") is controlling somatic growth in humans and other vertebrates, and the physiologically relevant pathways regulating GH secretion from the pituitary is 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.

[0015] The immune function is modulated by IGF-I, which has two major effects on B cell development: potentiation and maturation, and as a B-cell proliferation cofactor that works together with interlukin-7 ("IL-7"). These activities were identified through the use of anti-IGF-I antibodies, antisense sequences to IGF-I, and the use of recombinant IGF-I to substitute for the activity. There is evidence that macrophages are a

rich source of IGF-I. The treatment of mice with recombinant IGF-I confirmed these observations as it increased the number of pre-B and mature B cells in bone marrow (Jardieu et al., 1994). The mature B cell remained sensitive to IGF-I as immunoglobulin production was also stimulated by IGF-I *in vitro* and *in vivo* (Robbins et al., 1994).

[0016] The production of recombinant proteins in the last 2 decades provided a useful tool for the treatment of many diverse conditions. For example, GH-deficiencies in short stature children, anabolic agent in burn, sepsis, and AIDS patients. However, resistance to GH action has been reported in malnutrition and infection. Long-term studies on transgenic animals and in patients undergoing GH therapies have shown no correlation in between GH or IGF-I therapy and cancer development. GH replacement therapy is widely used clinically, with beneficial effects, but therapy is associated several disadvantages: GH must be administered subcutaneously or intramuscularly once a day to three times a week for months, or usually years; insulin resistance and impaired glucose tolerance; accelerated bone epiphysis growth and closure in pediatric patients (Blethen and MacGillivray, 1997; Blethen and Rundle, 1996).

[0017] In contrast, essentially no side effects have been reported for recombinant GHRH therapies. 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 (Esch et al., 1982; 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 recombinant 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).

[0018] 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 GHRH, and inhibited by somatostatin, and both hypothalamic hormones (Thorner et al., 1995). 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., 1990; Vance, 1990; Vance et al., 1985). Although recombinant 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.

[0019] 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).

[0020] Extracranially secreted GHRH, as processed protein species GHRH(1-40) hydroxy or GHRH(1-44) 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 nucleic acid therapy strategies and thus does not require viral genes or lipid particles (Aihara and Miyazaki, 1998; Muramatsu et al., 2001). 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).

[0021] 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 have 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.

[0022] 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 therapeutic techniques.

[0023] U.S. Patents No. 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 teach 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.

[0024] **Gene Delivery and *in vivo* Expression:** Recently, the delivery of specific genes to somatic tissue in a manner that can correct inborn or acquired deficiencies and imbalances was proved to be possible (Herzog et al., 2001; Song et al., 2001; Vilquin et al., 2001). 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 vector therapy allows for prolonged exposure to the protein in the therapeutic range, because the newly secreted protein is present continuously in the blood circulation. In a few cases, the relatively low expression levels achieved after simple plasmid injection, are sufficient to reach physiologically acceptable levels of bioactivity of secreted peptides (Danko and Wolff, 1994; Tsurumi et al., 1996).

[0025] The primary limitation of using recombinant protein is the limited availability of protein after each administration. Nucleic acid vector therapy 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.

[0026] In a plasmid-based expression system, a non-viral gene vector may be composed of a synthetic gene delivery system in addition to the nucleic acid encoding a therapeutic gene product. 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 gene 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 nucleic acid vector therapy 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.

[0027] Efforts have been made to enhance the delivery of plasmid DNA to cells by physical means including electroporation, sonoporation, and pressure. Administration 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 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.

[0028] 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).

[0029] 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).

[0030] 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. Furthermore, PLG will prevent the muscle damage associated with in vivo plasmid delivery (Draghia-Akli et al., 2002a) and will increase plasmid stability *in vitro* prior to injection.

[0031] 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).

[0032] 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.

[0033] 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.

[0034] 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 vector therapy, the entire content of each is hereby incorporated by reference. A molecular switch for regulating expression in nucleic acid vector therapy 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 vector therapy 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 or expression of the gene contained in the nucleic acid cassette.

[0035] In summary, treatments for conditions such as anemia, wasting and immune dysfunction are uneconomical and restricted in scope. The related art has shown that it is possible to treat these different disease conditions in a limited capacity utilizing recombinant protein technology, but these treatments have some significant drawbacks. It has also been taught that nucleic acid expression constructs that encode recombinant proteins are viable solutions to the problems of frequent injections and high cost of

traditional recombinant therapy. The introduction of point mutations into the encoded recombinant proteins was a significant step forward in producing proteins that are more stable *in vivo* than the wild type counterparts. Unfortunately, each amino acid alteration in a given recombinant protein must be evaluated individually, because the related art does not teach one skilled in the art to accurately predict how changes in structure *(e.g.* amino acid sequences) will lead to changed functions *(e.g.* increased or decreased stability) of a recombinant protein. Therefore, the beneficial effects of nucleic acid expression constructs that encode expressed proteins can only be ascertained through direct experimentation. There is a need in the art to expanded treatments for subjects with a disease by utilizing nucleic acid expression constructs that are delivered into a subject and express stable therapeutic proteins *in vivo.*

SUMMARY OF THE INVENTION

[0035a] In one particular embodiment there is provided use in muscle tissue cells of a subject of an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone (GHRH) of SeqID#6 to increase total red blood cell mass in the subject.

[0035b] In another particular embodiment there is provided use of a recombinant growth-hormone-releasing-hormone (GHRH) of SeqID#6 in muscle tissue cells of a subject to increase total red blood cell mass in the subject.

[0035c] Certain exemplary embodiments provide a method of increasing total red blood cell mass in a subject, comprising: delivering into cells of the subject an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone (GHRH) or functional biological equivalent thereof.

[0035d] Other exemplary embodiments provide a method of increasing total red blood cell mass in a subject, comprising: delivering into the subject a recombinant growth-hormone-releasing-hormone (GHRH) or a biological functional equivalent thereof.

[0036] The present invention pertains to compositions and methods that are useful for retarding the growth of abnormal cells, tumor progression reduction, prevention of kidney failure, reduction of metastasis, and increased survival in cancer-bearing animals. The method of this invention comprises treating a subject with plasmid mediated gene supplementation. The method comprises delivering an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof into a tissue, such as a muscle, of the subject. Specific embodiments of this invention are directed toward various types of tumors, such as adenoma; carcinoma; leukemia; lymphoma; lung tumor; mast cell tumor; melanoma; sarcoma; and solid tumors. The subsequent *in vivo* expression of the GHRH or biological equivalent in the subject is sufficient to retard tumor growth, prevent kidney failure and increase survival in cancer-bearing animals. It is also possible to enhance this method by placing a plurality of electrodes in a selected tissue, then delivering nucleic acid expression construct to the selected tissue in an area that interposes the plurality of electrodes, and applying a cell-transfecting pulse to the selected tissue in an area of the selected tissue where the nucleic acid expression construct was delivered. Electroporation, direct injection, gene gun, or gold particle bombardment are also used in specific embodiments to deliver the nucleic acid expression construct encoding the GHRH or biological equivalent into the subject. The subject in this invention comprises mammals, such as a humans, and domesticated animals.

[0037] The composition of this invention comprises an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof, wherein delivering and subsequent expression of the GHRH or biological equivalent in a tissue of the subject is sufficient to retard tumor growth and retard cachexia or the wasting effects that are commonly associated with tumor growth. Specific elements of the nucleic acid expression construct of this invention are also described. For example, the construct comprises a tissue specific promoter; a GHRH or functional biological equivalent; and a 3' untranslated region ("3' UTR") that are operatively linked. The nucleic acid expression construct of this invention comprises a construct that is substantially free of a viral backbone. Specific examples of a nucleic acid expression constructs used for this invention are also presented. The encoded

functional biological equivalent of GHRH comprises a polypeptide having similar or improved biological activity when compared to the GHRH polypeptide. The GHRH or functional biological equivalent that is encoded by the nucleic acid expression construct and useful for this invention comprises an amino acid structure with a general sequence as follows:

-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. Specific examples of amino acid sequences for GHRH or functional biological equivalents that are useful for this invention are presented. In a specific embodiment, the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in a subject that has received the nucleic acid expression construct. In specific embodiments of the invention, a transfection-facilitating polypeptide that increase the tissues ability to uptake the nucleic acid expression construct comprises a charged polypeptide, such as poly-L-glutamate.

[0038] One embodiment of the present invention pertains to a plasmid mediated supplementation method for treating anemia; increasing total red blood cell mass in a subject; reversing the wasting; reversing abnormal weight loss; treating immune dysfunction; reversing the suppression of lymphopoesis; or extending life expectancy for the chronically ill subject. This can be achieved utilizing an effective amount of a nucleic acid expression construct that contains both a constitutive promoter and an encoding sequence for growth hormone releasing hormone ("GHRH") or biological equivalent 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 and 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 biological equivalent 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.

[0039] A further embodiment of the present invention pertains to pertains to a plasmid mediated method for the treatment of anemia, wasting, immune dysfunction and life extension for the chronically ill subject by utilizing the ability to regulate the expression of GHRH or biological equivalent 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 biological equivalent 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 external regulation, expression and ensuing release of GHRH or biological equivalent thereof by the modified-cells within the subject will the conditions of anemia, wasting, immune dysfunction and life extension for the chronically ill subject. The delivery of the nucleic acid sequences that allow external regulation of GHRH or the biological equivalent thereof directly into the cells of the subject can be accomplished by the process of *in vivo* electroporation.

[0040] A further embodiment of the present invention pertains to a method of treatment for anemia, wasting, immune dysfunction and life extension for the chronically ill subject by utilizing therapy that introduces specific recombinant GHRH-biological equivalent protein into the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] 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.

[0042] Figure 1 shows the amino acid sequence of GHRH or biological equivalent thereof.

[0043] Figure 2 shows the increase in the percentage of IGF-I levels in healthy dogs that were injected with different concentrations of the pSP-HV-GHRH plasmid.

[0044] Figure 3 shows the increase in the percentage of IGF-I levels at 9-27 and 28-56 days post-injection in dogs with cancer that were injected at day 0 with 100 mcg/kg to a total of no more than 1000mcg of the pSP-HV-GHRH plasmid.

[0045] Figure 4 shows the percentage of weight gain in healthy dogs that were injected with different concentrations of the pSP-HV-GHRH plasmid.

[0046] Figure 5 shows the number of red blood cells in dogs with spontaneous malignancies treated with the GHRH plasmid therapy compared with control dogs with cancer.

[0047] Figure 6 shows hemoglobin values in dogs with spontaneous malignancies treated with the GHRH plasmid therapy compared with control dogs with cancer.

[0048] Figure 7 shows hematocrit levels in dogs with spontaneous malignancies treated with the GHRH plasmid therapy compared with control dogs with cancer.

[0049] Figure 8 shows the percentage of lymphocytes in dogs with spontaneous malignancies treated with the GHRH plasmid therapy compared with control dogs with cancer.

[0050] Figure 9 shows a schematic of the mifepristone-dependent GHRH/GeneSwitch® system. Plasmid p1633 encodes for the GeneSwitch® regulator protein, that 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.

[0051] Figure 10 shows tumor volume progression in immunocompetent C57/Bl6 mice that received $2x10^6$ Lewis lung adenocarcinoma cells in their left flank. Treated animals received at 1 day after tumor cells implantation 20 micrograms of plasmid expressing human growth hormone releasing hormone, while controls received a control beta-galactosidase plasmid. GHRH treated tumor bearing animals have significantly slower tumor development and progression.

[0052] Figure 11 shows survival time for immunocompetent C57/Bl6 mice that received $2x10^6$ Lewis lung adenocarcinoma cells in their left flank. Treated animals received at 1 day after tumor cells implantation 20 micrograms of plasmid expressing human growth hormone releasing hormone, while controls received a control beta-galactosidase plasmid. GHRH-treated tumor bearing animals have increase survival.

[0053] Figure 12 shows kidney size for immunocompetent C57/Bl6 mice that received $2x10^6$ Lewis lung adenocarcinoma cells in their left flank. Treated animals received at 1 day after tumor cells implantation 20 micrograms of plasmid expressing human growth hormone releasing hormone, while controls received a control beta-galactosidase plasmid. Control animals have significantly smaller kidney size, sign of kidney failure.

[0054] Figure 13 shows that the markers associtaed with metastasis development are increased in control animals versus GHRH-treated animals, and that the histopathology report showed considerably more metastasis in the control animals than in treated once.

[0055] Figure 14 shows that tumor growth did not increase in nude mice treated with plasmid-mediated growth hormone releasing hormone (constitutively active pGHRH or regulated Gene Switch system GHRH-IS +/ - MFP). NCI – human lung adenocarcinoma cell line. Animals treated with the constitutively active GHRH had smaller tumors than controls ($p < 0.02$) at 33 days post-treatment. No other group displayed significant differences when compared to controls.

[0056] Figure 15 shows the protein metabolism in dogs at 56 days post-injection.

[0057] Figure 16 shows the blood values in dogs at 56 days post-injection.

[0058] Figure 17 shows the bone metabolism in dogs at 56 days post-injection.

[0059] Figure 19 shows the blood values for dogs with cancer.

[0060] Figure 20 shows the blood values for old healthy dogs.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

DEFINITIONS

[0061] 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.

[0062] The term "abnormal weight loss," as used herein is defined as decreased body weight of at least 5-10% of the minimum ideal weight of the individual that is characterized by significant loss of both adipose tissue and muscle mass.

[0063] The term "AIDS therapy" as used herein refers to treatment of acquired immune deficiency syndrome ("AIDS") by any medical or physical means, including, but not limited to: antiretrovirals, nucleoside analogues, non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors, and/or other drugs used to boost the immune system.

[0064] 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# 1), TI-GHRH (SEQID# 2), TV-GHRH (SEQID# 3), 15/27/28-GHRH (SEQID# 4), (1-44)NH_2 (SEQID# 5) or (1-40)OH (SEQID# 6) forms, or any shorter form to no less than (1-29) amino acids.

[0065] The term "anemia" as used herein refers to a condition in which there is a reduction of the number and/or volume of red blood corpuscles or of the total amount of hemoglobin in the bloodstream, resulting in paleness, generalized weakness, *etc.*, of the subject.

[0066] The term "antiviral therapy" as used herein refers to a group of drugs that are of three main types, including: nucleoside analog drugs, protease (proteinase) inhibitor drugs, and non-nucleoside reverse-transcriptase inhibitor drugs (NNRTIs).

[0067] The term "bodily fat proportion" as used herein is defined as the body fat mass divided by the total body weight.

[0068] The term "cancer therapy" as used herein refers to treatment of cancer by any medical or physical means, including, but not limited to surgery, immunotherapy, chemotherapy, radiation therapy, hyperthermia and/or photodynamic therapy.

[0069] The term "cachexia" as used herein is defined as the accelerated loss of skeletal muscle.

[0070] The term "cassette" as used herein is defined as one or more transgene expression vectors.

[0071] 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.

[0072] The term "chronically ill" as used herein is defined as patients with conditions as chronic obstructive pulmonary disease, chronic heart failure, stroke, dementia, rehabilitation after hip fracture, chronic renal failure, rheumatoid arthritis, and multiple disorders in the elderly, with doctor visits and/or hospitalization once a month for at least two years.

[0073] 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.

[0074] 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.

[0075] 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.

[0076] 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.

[0077] 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.

[0078] 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.

[0079] The term "effective amount" as used herein refers to sufficient nucleic acid expression construct or encoded protein administered to humans, animals or into tissue culture to produce the adequate levels of protein, RNA, or hormone. One skilled in the art recognizes that the adequate level of protein or RNA will depend on the use of the particular nucleic acid expression construct. These levels will be different depending on the type of administration and treatment or vaccination.

[0080] The term "electroporation" as used herein refers to a method that utilized electric pulses to deliver a nucleic acid sequence into cells.

[0081] 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.

[0082] The term "encoded GHRH" as used herein is a biologically active polypeptide of growth hormone releasing hormone.

[0083] 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.

[0084] 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, as prolactin.

[0085] 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.

[0086] The term "GeneSwitch®" (a registered trademark of Valentis, Inc.; Burlingame, CA) as used herein refers to the technology of a mifepristone-inducible heterologous nucleic acid sequences encoding regulator proteins, GHRH, biological equivalent or combination thereof. Such a technology is schematically diagramed in Figure 1 and Figure 9. A skilled artisan recognizes that antiprogesterone agent alternatives to mifepristone are available, including onapristone, ZK112993, ZK98734, and 5α pregnane-3,2-dione.

[0087] 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.

[0088] 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.

[0089] The term "heterologous nucleic acid sequence" as used herein is defined as a DNA sequence comprising differing regulatory and expression elements.

[0090] The term "immune dysfunction" as used herein refers to the abnormal, impaired, or incomplete functioning of a subject's immune system, as determined indirectly or directly by immune specific markers (*e.g.* IGF-I levels, or % lymphocytes).

[0091] The term "immunotherapy" as used herein refers to any treatment that promotes or enhances the body's immune system to build protective antibodies that will reduce the symptoms of a medical condition and/or lessen the need for medications.

[0092] 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.

[0093] The term "life extension for the chronically ill" as used herein refers to an increase in the actual life expectancy for a subject that undertakes the treatment compared to a subject that did not have treatment.

[0094] The term "lymphopoiesis" as used herein is defined as the production of lymphocytes.

[0095] The term "kidney failure" as used herein is defined as the abrupt or chronic decline in glomerular filtration rate resulting from ischemic or toxic injury to the kidney, and includes a decrease of glomerular capillary permeability, back-leak of glomerular filtrate, tubular obstruction, and intrarenal vasoconstriction.

[0096] 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.

[0097] The term "modified-donor-cells" as used herein refers to any donor-cells that have had a GHRH-encoding nucleic acid sequence delivered.

[0098] The term "molecular switch" as used herein refers to a molecule that is delivered into a subject that can regulate transcription of a gene.

[0099] 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.

[00100] 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.

[0100] 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.

[0101] 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 a 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 living organism.

[0102] 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.

[0103] 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 an effective amount of a nucleic acid expression construct *in vivo*.

[0104] 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.

[0105] 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.

[0106] 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.

[0107] The term "radiation therapy" as used herein refers to radiation treatment given to cancer patients that damages the DNA in cancer cells, which often results in the death of cancer cells.

[0108] The term "replication element" as used herein comprises nucleic acid sequences that will lead to replication of a plasmid in a specified host. One skilled in the art of molecular biology will recognize that the replication element may include, but is not limited to a selectable marker gene promoter, a ribosomal binding site, a selectable marker gene sequence, and a origin of replication.

[0109] 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.

[0110] 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.

[0111] The term "red blood cell mass" ("RBC-mass") of a subject as used herein is determined using one of the three following tests: 1) Hematocrit: the percentage of red blood cells in plasma; 2) red blood cell ("RBC") count: the number of red blood cells in plasma; and 3) hemoglobin: the level of oxygen-carrying protein within the subjects' red blood cells.

[0112] The term "regulator protein" as used herein refers to any protein that can be used to control the expression of a gene.

[0113] The term "regulator protein" as used herein refers to protein that increasing the rate of transcription in response to an inducing agent.

[0114] 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.

[0115] 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.

[0116] 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.

[0117] 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.

[0118] The term "vector" as used herein refers to any vehicle that delivers a nucleic acid into a cell or organism. Examples include plasmid vectors, viral vectors, liposomes, or cationic lipids.

[0119] 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.

[0120] 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.

[0121] 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.

[0122] The term "wasting" as used herein is defined as decreased body weight characterized by significant loss of both adipose tissue and muscle mass that makes weight gain especially difficult for patients with progressive diseases, such as cancer or AIDS. Wasting can be related to the disease itself or the effects of its treatment, or both.

[0123] One aspect of the current invention pertains to a method useful for retarding the growth of abnormal cells, and promoteing tumor progression reduction in cancer-bearing animals. The method of this invention comprises treating a subject with plasmid mediated gene supplementation. The method comprises delivering an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof into a tissue, such as a muscle, of the subject. Specific embodiments of this invention are directed toward particular types of tumors (e.g. adenoma; carcinoma; leukemia; lymphoma; lung tumor; mast cell tumor; melanoma; sarcoma; and solid tumors). The subsequent *in vivo* expression of the GHRH or biological equivalent in the subject is sufficient to retard tumor growth, and retard cachexia or the wasting effects that are commonly associated with tumors. It is also possible to enhance this method by placing a plurality of electrodes in a selected tissue, then delivering nucleic acid expression construct to the selected tissue in an area that interposes the plurality of electrodes, and applying a cell-transfecting pulse (e.g. electrical) to the selected tissue in an area of the selected tissue where the nucleic acid expression construct was delivered. However, the cell-transfecting pulse need not be an electrical pulse, a vascular pressure pulse can also be utilized. Electroporation, direct

injection, gene gun, or gold particle bombardment are also used in specific embodiments to deliver the nucleic acid expression construct encoding the GHRH or biological equivalent into the subject. The subject in this invention comprises an animal (e.g. a human, a pig, a horse, a cow, a mouse, a rat, a monkey, a sheep, a goat, a dog, or a cat).

[0124] Specific elements of the nucleic acid expression construct of this invention are also described. For example, the construct comprises a tissue specific promoter; a GHRH or functional biological equivalent; and a 3' untranslated region ("3' UTR") that are operatively linked. In specific embodiments, the tissue-specific promoter comprises a muscle-specific promoter (e.g. SPc5-12 (SeqID# 7)), and the 3′ UTR of the nucleic expression construct comprises a human growth hormone 3′ UTR (SeqID# 8), bovine growth hormone 3′ UTR, skeletal alpha actin 3′ UTR, or SV40 polyadenylation signal. The nucleic acid expression construct of this invention comprises a construct that is substantially free of a viral backbone. Specific examples of a nucleic acid expression constructs used for this invention comprises plasmids with SeqID# 11, SeqID# 12, SeqID# 13, and SeqID# 14. The encoded functional biological equivalent of GHRH comprises a polypeptide having similar or improved biological activity when compared to the GHRH polypeptide. The GHRH or functional biological equivalent that is encoded by the nucleic acid expression construct and useful for this invention comprises an amino acid structure with a general sequence as follows (SeqID #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. Specific examples of amino acid sequences for GHRH or functional biological equivalents that are useful for this invention are presented in SeqID# 2; SeqID# 3; SeqID# 4; and SeqID#10. In a specific embodiment, the encoded GHRH or functional biological equivalent thereof facilitates growth hormone ("GH") secretion in a subject that has received the nucleic acid expression construct.

[0125] Although not wanting to be bound by theory, the ability of cells in a tissue to uptake the nucleic acid expression construct can be facilitated by a transfection-facilitating polypeptide. In specific embodiments of the invention, the transfection-facilitating polypeptide comprises a charged polypeptide such as poly-L-glutamate.

[0126] Another aspect of the current invention comprise compositions that are useful for retarding the growth of abnormal cells, tumor progression reduction, prevention of kidney failure, reduction of metastasis, and increased survival in cancer-bearing animals. The composition of this invention comprises an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof, wherein delivering and subsequent expression of the GHRH or biological equivalent in a tissue of the subject is sufficient to retard the growth of abnormal cells, promote tumor progression reduction, prevent kidney failure, and increase survival in cancer-bearing animals. Specific embodiments of this invention are directed toward particular types of tumors and cancers (e.g. adenoma; carcinoma; leukemia; lymphoma; lung tumor; mast cell tumor; melanoma; sarcoma; and solid tumors).

[0127] The subsequent *in vivo* expression of the GHRH or biological equivalent encoded by the composition is sufficient to retard the growth of abnormal cells, promote tumor progression reduction, prevent kidney failure, and increase survival in cancer-bearing animals. It is also possible to enhance the uptake of the composition (i.e. nucleic acid expression construct) of this invention by placing a plurality of electrodes in a selected tissue, then delivering nucleic acid expression construct to the selected tissue in an area that interposes the plurality of electrodes, and then applying a cell-transfecting pulse (e.g. electrical) to the selected tissue in an area of the selected tissue where the nucleic acid expression construct was delivered. However, the cell-transfecting pulse need not be an electrical pulse, a vascular pressure pulse can also be utilized. Electroporation, direct injection, gene gun, or gold particle bombardment are also used in specific embodiments to deliver the composition that encodes the GHRH or biological equivalent into the subject. The subject in this invention comprises a mammal (e.g. a human, a pig, a horse, a cow, a mouse, a rat, a monkey, a sheep, a goat, a dog, or a cat).

[0128] Additionally, the invention relates to a plasmid-mediated supplementation method for the treatment of anemia, wasting, tumor growth, immune dysfunction, kidney failure and/or life extension for the chronically ill subject. Anemia refers to a condition in which there is a reduction of the number, volume, or both of red blood corpuscles or of the total amount of hemoglobin in the bloodstream, resulting in paleness, generalized weakness, *etc.* of the subject. Wasting of a subject can be defined as decreased body weight that is characterized by significant loss of both adipose tissue and muscle mass, which makes weight gain especially difficult for patients with a progressive disease (*e.g.* cancer, AIDS, *etc.*). Anemia, wasting, tumor growth, immune dysfunction, kidney failure and decreased life expectancy can be related to a specific disease or the effects of a disease treatment. More specifically, this invention pertains to a method for delivering a heterologous nucleic acid sequence encoding growth hormone releasing hormone ("GHRH") or biological equivalent thereof into the cells of the subject (*e.g.* somatic, stem, or germ cells) and allowing expression of the encoded GHRH or biological equivalent gene to occur while the modified cells are within the subject. The subsequent expression of the GHRH or 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 externally administered into the subject. The extracranial expression and ensuing release of GHRH or biological equivalent thereof by the modified cells can be used to treat anemia, wasting, tumor growth, immune dysfunction, kidney failure and life extension for the chronically ill subject. The preferred means to deliver the GHRH or biological equivalent thereof is by electroporation.

[0129] 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 (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., 1990). 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.

[0130] 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.

[0131] The ability of electroporation to enhance plasmid uptake into the skeletal muscle has been well documented, as described above. 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). 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.

[0132] The plasmid supplementation approach to treat anemia, wasting, tumor growth, immune dysfunction, kidney failure and life extension for the chronically ill

subject that is described herein offers advantages over the limitations of directly injecting recombinant GH or GHRH protein. Expression of novel 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 biological equivalent thereof elicited high GH and IGF-I levels in subjects that have had the encoding sequences delivered into the cells of the subject by intramuscular injection and *in vivo* electroporation. 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 should be 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 may also be 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 intravenously or intra-arterially. Plasmid DNA carrying the GHRH sequence can be complexed with cationic lipids or liposomes and delivered intramuscularly, intravenously or subcutaneous.

[0133] 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 treating disease by administration of the vector to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for plasmid mediated supplementation. The preferred means for administration of vector and use of formulations for delivery are described above.

[0134] 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 enhance utilizing *in vivo* electroporation.

[0135] 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) determine the bioavailability of the vector within the body. Other elements of the formulation function as ligands that 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.

[0136] Delivery can also be through use of DNA transporters. DNA transporters refer 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.

[0137] 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 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.

[0138] 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.

[0139] 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.

[0140] Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs plasmid-mediated supplementation and the genetically engineered cells can also be easily put back with out causing damage to the patient's 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.

[0141] 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.

[0142] 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 ability to induce. Thus, target gene expression can be regulated by incorporating molecular switch technology as schematically diagramed in Figure 9 and further discussed in Example 1. The HV-GHRH or biological equivalent 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 minutes half-life. Thus, by combining the powerful electroporation DNA delivery method with stable and regulable GHRH or biological equivalent encoded nucleic acid sequences, a therapy can be utilized that will reverse chronic wasting, allow the subject to gain weight, and extend the subject's life expectancy.

I. Vectors

[0143] 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, Maniatis *et al.*, 1988 and Ausubel *et al.,* 1994, both incorporated herein by reference).

[0144] 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

[0145] 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.

[0146] 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.

[0147] 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.

[0148] 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

[0149] 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.

[0150] 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.

[0151] 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.

[0152] 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.

[0153] 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, incorporated herein by reference). 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.

[0154] 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.

[0155] 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	

TABLE 1 Promoter and/or Enhancer	
Promoter/Enhancer	Relevant References
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	
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

[0156] 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).

[0157] 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

[0158] 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.

[0159] 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

[0160] 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

[0161] 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

[0162] 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.

[0163] 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.

[0164] 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

[0165] 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

[0166] 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

[0167] 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.

[0168] 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

[0169] 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.

[0170] 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.

[0171] 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.

[0172] 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.

[0173] 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.

[0174] 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.

[0175] 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.

[0176] 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.

[0177] Although not wanting to be bound by theory, the nature of the voltage pulse to be generated is determine 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

[0178] 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

[0179] 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

[0180] 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

[0181] In order to treat anemia; increase total red blood cell mass; reverse the wasting; reverse abnormal weight loss; treat immune dysfunction; reverse the suppression of lymphopoesis; or extend life expectancy for the chronically ill subject, 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 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.

[0182] **DNA Constructs:** Plasmid vectors containing the muscle specific synthetic promoter SPc5-12 (SEQID# 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# 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# 15), or pSP-HV-GHRH (SEQID# 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 treating anemia; increasing total red blood cell mass in a subject; reversing the wasting; reversing abnormal weight loss; decreasing tumor growth; preventing kidney failure; treating immune dysfunction; reversing the suppression of lymphopoesis; or extending life expectancy for the chronically ill subject 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#10)
HV-GHRH	HVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SEQID#1)
TI-GHRH	YIDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SEQID#2)
TV-GHRH	YVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SEQID#3)
15/27/28-GHRH	YADAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH (SEQID#4)

[0183] 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#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").

[0184] Another plasmid that was utilized included the pSP-SEAP construct (SEQID# 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).

[0185] 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.

[0186] 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 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) 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.

[0187] **Large Animal Studies:** *Healthy Dogs:* A group of 4 dogs (2 males and 2 females) were used as controls and 3 groups of 8 dogs (4 males and 4 females) were injected with the pSP-HV-GHRH system. The dogs were injected with vehicle alone (control), or 200mcg, or 600mcg or 1000mcg of pSP-HV-GHRH followed by caliper electroporation.

[0188] **Cancer Dogs:** Fifteen dogs with spontaneous malignancies were used in GHRH studies. The dogs were injected with 100 mcg/kg to a total of no more than 1000 mcg pSP-HV-GHRH. Four dogs died or were euthanized at owner's request within the first three days after the plasmid injection from unrelated complications of their advanced disease. The condition of inclusion in our study was a survival of at least 14 days post-injection (in order to allow for plasmid activation and expression of GHRH from the skeletal muscle), when a second blood draw could be made. Eleven dogs were analyzed in this study. The animals were under specific treatment using either chemotherapy, radiotherapy or combination therapy (see Figure 18). The animals were weighed and bled before the treatment and at 9-27 and 28-56 days post-injection. At each time point complete CBC and metabolic profile was assessed by the same independent laboratory (Antech Diagnostics, Irvine, CA). Wellness forms were completed by owners at each visit. Nineteen non-injected dogs with spontaneous malignancies, in treatment in the clinic in the same time window, were used as contemporary controls. The quality of life in the treated patients increased. No adverse effects linked to the therapy were noted by owners. Three owners noticed a dramatic improvement in the general well-being of the treated dog compared to pre-injection status.

[0189] **Electroporation devices:** A BTX T820 generator (BTX, division of Genetronics Inc., CA) was used to deliver square wave pulses in all experiments. We used voltage conditions of 100V/cm, 6 pulses, 60 milliseconds per pulse. Two- needle electrodes (BTX, a division of Genetronics Inc., CA) were used to deliver *in vivo* electric pulses. In all injections the needles were completely inserted into the muscle.

[0190] **Intramuscular injection of plasmid DNA in Canine subjects:** Four groups of healthy Canines ("dogs") subjects, 8-12 kg in weight, were used for biodistribution -toxicology studies. Three groups of 8 dogs (4 males and 4 females) were injected with 200 mcg, 600mcg and 1000 mcg of pSP-HV-GHRH, respectively. A group of 4 dogs (2 males and 2 females) were used as controls. Animals were continuously monitored for side effects. In addition, two groups of dogs with cancer (spontaneous malignancies) were used. Animals were maintained in accordance with NIH Guide, USDA and Animal Welfare Act guidelines, and approved by the Baylor College of Medicine IACUC.

[0191] Endotoxin-free plasmid (Qiagen Inc., Chatsworth, CA, USA) preparation of pSPc-5 12-HV-GHRH were diluted in PBS pH=7.4 or water to 5 mg/mL. Dogs were injected before their regular treatment administration. For dogs on chemotherapy, the injection was administered at no less than 5 days before/ after the medication. The dogs were anesthetized with Propafol (Abbott Laboratories, IL) 4-8 mg/kg. While anesthetized, 100 μg/kg to a maximum of 1 mg of plasmid was injected directly into the semitendinosus muscle of dogs, using an 3/10 cc insulin syringe and 29G1/2" needle (Becton-Dickinson, Franklin Lacks, NJ). Two minutes after injection, the injected muscle was electroporated, 6 pulses, 100V/cm, 60 milliseconds/pulse, using a BTX T820 electroporator and two-needle electrodes (BTX, a division of Genetronics Inc., CA), as described (Miklavcic et al., 2000). In all injections the needles were completely inserted into the muscle. Animals were allowed to recover before rejoining their owners.

[0192] 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); including microinjection (Harland and Weintraub, 1985; U.S. Patent No. 5,789,215); by electroporation (U.S. Patent No. 5,384,253; 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); by agitation with silicon carbide fibers (Johnson et al., 1992; U.S. Patent Nos. 5,302,523 and 5,464,765); by *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,591,616 and 5,563,055); by PEG-mediated transformation of protoplasts (Omirulleh et al., 1993; U.S. Patent Nos. 4,684,611 and 4,952,500); 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.

[0193] **Body weight data:** Animals were weighted before the plasmid injection and at 14, 28 and 56 days post-injection using the same calibrated scale.

[0194] **Protein Metabolism, Bone metabolism and Blood values:** Blood and urine samples were collected before plasmid injection, and at 14, 28 and 56 days post-injection, and analyzed for biochemistry, metabolisms and hormone. At each time point complete CBC and metabolic profile was assessed by the same independent laboratory (Antech Diagnostics, Irvine, CA).

[0195] **Plasma IGF-I:** IGF-I levels were 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.

[0196] **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

LOW VOLTAGE ELECTROPORATION FOR DNA UPTAKE

AND EXPRESSION IN AN ANIMAL SUBJECT

[0197] 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), 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. Although not wanting to be bound by theory, 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 or dog muscle fibers are quite large and consequently more suitable for electropermeabilization than rodent muscle. In this report, we show that a single injection various dosages of GHRH or analog nucleic acid sequences followed by electroporation with intramuscular applicators, in a large mammal is sufficient to produce therapeutic plasma hormone levels, with biologically significant effects that can treat anemia, reverse wasting, allow the subject to gain weight, and extend life expectancy of the chronically ill.

[0198] The pSP-HV-GHRH system was delivered to the left tibialis anterior muscle of healthy dogs via *in vivo* electroporation. A group of 4 dogs (2 males and 2 females) were used as controls and 3 groups of 8 dogs (4 males and 4 females) were injected with the pSP-HV-GHRH system. The dogs were injected with vehicle alone (control), or 200mcg, or 600mcg or 1000mcg of pSP-HV-GHRH followed by needle electroporation. An indication of increased systemic levels of GHRH and GH is an increase in serum IGF-I concentration. Therefore, following 28 days post injection blood

serum was collected from the dogs were injected with vehicle alone (control), or 200mcg, or 600mcg or 1000mcg of pSP-HV-GHRH and IGF-I levels were determined. The IGF-I levels for dogs injected with 600 mcg were 3-fold higher than the control (vehicle alone) treated animals (Figure 2). The increase in IGF-I levels was statistically significant ($p<0.046$). Although animals injected with 200 mcg and 1000 mcg of plasmid showed higher IGF-I levels than controls, the IGF-I levels were lower than animals injected with 600 mcg. Increased IGF-I levels corresponding to higher GHRH levels are in agreement with other studies that utilized recombinant porcine GH ("pGH") in dogs. For example, there were dose-related increased serum IGF-I levels (approximately 2-10-fold) that correlated with the elevated serum GH levels in pGH-treated dogs.

[0199] Although 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. Thus, an indication of increased systemic levels of GHRH and GH is an increase in serum IGF-I concentration. The level of serum IGF-I in healthy dogs injected with 200, 600 and 1000 mcg of pSP-HV-GHRH were all higher 28 days post-injection when compared to the pre-injection values. Dogs injected with 600 mcg pSP-HV-GHRH showed the highest statistically significant increase (*e.g.* greater than 90%, $p<0.046$) in IGF-I levels, which indicates that 600 mcg may be the optimal concentration for healthy dogs.

EXAMPLE 3

INCREASED SURVIVAL IN ANIMAL SUBJECTS WITH CANCER

[0200] Eleven injected dogs with cancer had survived for at least 56 days after the injection, and complete data was collected in all cases. The dogs enrolled in the study were in a relatively advanced stage of their disease (206 days since the beginning of the therapy). The average survival post-injection is listed in Figure 18. At the time this application was prepared, 8 out of the eleven treated dogs were still alive (average survival post injection 150.6 days). The 19 control dogs were in a less advanced stage of disease and had an average survival of 56 days after the initial diagnosis. In contrast, after the enrollment into the present study the average survival post enrolment was 162.5 days. Five

control animals died during this period. The quality of life in the treated patients increased. No adverse effects linked to the therapy were noted by owners. Three owners noticed a dramatic improvement in the general well-being of the treated dog compared to pre-injection status.

EXAMPLE 4

INCREASED WEIGHT GAIN IN HEALTHY ANIMAL SUBJECTS

[0201] In order to show that increased levels of GHRH or biological equivalent thereof could alter metabolism in large healthy animals, body weight was determined. As shown in Figure 4, the treated dogs had increased weight gain compared with controls fifty-six days post-injection. Although animals injected with 200mcg and 1000mcg of plasmid showed higher body weights than controls, the weights were lower than animals injected with 600mcg. These results are a good indicator that the metabolism of the dogs injected with pSP-HV-GHRH was altered in a dose dependent manner. In addition, the additional weight gain associated with increased production of GHRH also indicates that the levels of GH were increased. This observation is in agreement with other studies that utilized recombinant porcine GH (pGH) in dogs. In one of these studies, recombinant pGH was administered for 14-weeks in dogs. Porcine GH caused increased body weight gain in mid - and high-dose groups (2.8 kg and 4.7 kg, respectively), compared to 0.4 kg and 0.8 kg in control and low-dose groups, respectively.

EXAMPLE 5

GHRH OR BIOLOGICAL EQUIVALENT TREATMENT IMPROVES PROTEIN UTILIZATION IN HEALTHY DOGS

[0202] Figure 15 shows the changes in values associated with protein metabolism in healthy dogs injected with different concentrations of the pSP-HV-GHRH plasmid. Many values that indicate protein metabolism were monitored for 56 days including: AST, ALT, T. bilirubin, Alk Phos, GGT, total protein, albumin, globulin, A/G ratio, Cholesterol, BUN and creatinine. Groups of 8 dogs (4 males and 4 females) were injected with 200 mcg, 600mcg and 1000 mcg of pSP-HV-GHRH. A group of 4 dogs (2 males and 2 females) were used as controls. Dogs injected with 200, and 600mcg of

plasmid had increased total protein levels. All injected groups have slightly decreased urea compared with controls, which is a sign of improved protein utilization.

EXAMPLE 6

GHRH OR BIOLOGICAL EQUIVALENT TREATMENT DOES NOT AFFECT GLUCOSE METABOLISM IN HEALTHY ANIMAL SUBJECTS.

[0203] Figure 16 shows the changes in values associated with blood components in healthy dogs injected with different concentrations of the pSP-HV-GHRH plasmid. Abbreviations are as follows: WBC – White Blood Cell; RBC – Red blood cell; HGB - hemoglobin; PCV – hematocrit, or packed cell volume; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; n% - % of neutrophils; lym% - % of lymphocytes; mono% - % of monocytes; eos % - % of eosinophils; Bas % - % of basophils; LDH – lactate dehydrogenase ; Prothom - prothrombine; Qnt - quantitative; Plat - platelets; BUN – blood urea nitrogen / urea.

[0204] Many blood component values were monitored for 56 days including: WBC, RBC, HGB, PCV, MCB, MCH, MCHC, n%, lym% mono% eos %, Bas %, LDH, Prothom, Qnt. Plat. Groups of 8 dogs (4 males and 4 females) were injected with 200 mcg, 600mcg and 1000 mcg of pSP-HV-GHRH. A group of 4 dogs (2 males and 2 females) were used as controls. No statistical differences were found between experimental and control groups. However, circulation lymphocytes decreased with the increase in the plasmid dosage, sign of lymphocyte sequestration in the lymphatic organs. Importantly, glucose levels in all experimental groups and controls are within the normal range, which indicates that our therapy does not impair glucose metabolism.

EXAMPLE 7

GHRH OR BIOLOGICAL EQUIVALENT TREATMENT EFFECTS BONE REMODELING

[0205] Figure 17 shows the changes in values associated with bone metabolism in healthy dogs injected with different concentrations of the pSP-HV-GHRH plasmid. The

phosphorus, calcium and calcium/phosphorous ratio was monitored for 56 days post injection. Groups of 8 dogs (4 males and 4 females) were injected with 200 mcg, 600mcg and 1000 mcg of pSP-HV-GHRH. A group of 4 dogs (2 males and 2 females) were used as controls. Dogs injected plasmid had an increased Ca/PO_4 ratio that was proportional with the dosage of the treatment, which is a sign of bone remodeling.

EXAMPLE 8

GHRH OR BIOLOGICAL EQUIVALENT TREATMENT EXTENDS LIFE EXPECTANCY IN CHRONICALLY ILL SUBJECTS

[0206] Figure 18 shows the diagnosis, specific therapy chart and survival for dogs with spontaneous cancer that were injected with different concentrations of the pSP-HV-GHRH plasmid. The study, group, treatment, dose, # of dogs, cancer type, and days survived post-treatment are indicated. Groups of dogs with spontaneous cancer were injected with 100 mcg/Kg body weight to a total of no more than 1000 mcg of pSP-HV-GHRH. In addition, dogs treated with pSP-HV-GHRH had an improved quality of life.

EXAMPLE 9

GHRH OR BIOLOGICAL EQUIVALENT TREATMENT POSITIVELY AFFECTS IMMUNE FUNCTION IN CANCER SUBJECTS

[0207] Figure 19 shows the changes in values associated with blood components in dogs with spontaneous cancer injected with different concentrations of the pSP-HV-GHRH plasmid. Many blood component values were monitored post injection including: WBC/HPF, RBC/HBF, HGB, %PCV, MCV, MCH, MCHC, n%, lym% mono% eos %, Bas %. Groups of dogs with spontaneous cancer were injected with 100-1000 mcg/Kg body weight of pSP-HV-GHRH. Overall dogs treated with the pSV-HV-GHRH plasmid therapy, had increased RBC hemoglobin and hematocrit levels two weeks post-injection compared with un-injected controls. In addition, treated dogs have a significant decrease in the levels of circulation white blood cells (usually associated with increase of white blood cells (WBC) in the lymphatic organs but an increase in lymphocyte percentage. Treated animals showed a significant increase in the circulating lymphocytes

at the early time points post-injection (15.11 ± 2.81 vs. 12.5 ± 2.41 %, $p < 0.046$ pst/pri). Control animals had lymphocyte values at the time points tested ($p = 0.32$).

EXAMPLE 10

GHRH OR BIOLOGICAL EQUIVALENT TREATMENT SHOWS BENEFICIAL EFFECTS IN OLD HEALTHY DOGS

[0208] Figure 20 shows the changes in values associated with blood components in old healthy dogs injected with 1000mcg of pSP-HV-GHRH plasmid. Many blood component values were monitored two-weeks post injection including: WBC, RBC, HGB, lym%, total protein, albumin, globulin, A/G ratio, cholesterol, BUN, Creatinine, phosphorous, Calcium, glucose. Old dogs treated with the pSP-HV-GHRH plasmid therapy, had increased RBC hemoglobin and hematocrit levels two weeks post-injection compared with per-injected values. In addition, treated dogs have a significant decrease in urea levels, increased total protein levels and normal glucose levels. An increase in Ca/P ratio is an indication of bone remodeling.

EXAMPLE 11

TREATMENT OF ANEMIA

[0209] It is well known that erythroid cell number is primarily regulated by erythropoietin ("EPO") but is impacted by many growth factors. For example, hypophysectomized rats show low blood cell counts for erythroid, myeloid, and lymphoid cells, and there is extensive literature showing effects of both GH, and IGF-I on all hematopoietic lineages (Kurtz et al., 1990; Kurtz et al., 1982; Claustres et al., 1987). In polycytemia vera, patients present increased sensitivity of erythroid progenitor cells to IGF-I, elevated level of IGFBP-1 and consequently overproduction of red blood cells (Mirza et al., 1997; Correa et al., 1994). There is evidence to support the concept that IGF-I rather than EPO modulates erythropoiesis during accelerated growth or catabolism and thus manages a proportional increase in body mass and oxygen transport capacity (Kurtz et al., 1990). IGF-I is important factor regulating erythropoiesis in uremic patients (Urena et al., 1992). Moreover, the effect of treatment with recombinant human GH in anemic patients with panhypopituitarism is known. After the treatment with human GH plasma

EPO levels double, with a concomitant increase of Hb concentration to normal levels. When the administration of human GH is interrupted, both plasma EPO levels and Hb concentrations decrease.

[0210] In injected dogs, a rapid correction of the anemia was obtained, as early as two weeks after the plasmid injection. Red blood cells (RBC) increased by 8.9%, 9-27 days and the normal values were maintained to 56 days post-injection ("pti"), compared with pre-injection ("pri") values (6.27 ± 0.33, vs. 5.75 ± 0.39, $p < 0.027$), while the control group had a 6% decrease in their RBC levels (6.00 ± 0.2 vs. 5.5 ± 0.2) ($p < 0.006$ compared to post-injection controls) in the same period of time (Figure 5). Hemoglobin levels (Figure 6) increased by 6.8% (14.68 ± 0.78 vs. 13.74 ± 0.97 g/l pti/pri), while the control group had a 5.7% decrease in the same period of time (12.9 ± 0.4 vs. 13.7 ± 0.4 g/l), $p < 0.01$ compared to post-injection controls. Hematocrit levels (Figure 7) increased significantly, by 8.26% (42.22 ± 2.16 vs. 39 ± 2.62 %, $p < 0.032$ pst/pri). In the same period, control values decreased by 7.6% (36.9 ± 1.4 vs. 39.9 ± 1.6 %, $p < 0.012$ compared with pst).

[0211] In a pre-clinical study on dog cancer patients, a rapid correction of the anemia was obtained, as early as two weeks after the plasmid injection. At the beginning of the study, the patients were in a catabolic state, with hemoglobin (Hb), hematocrit ("PVC") and red blood cell ("RBC") values significantly lower than normal dogs. After the plasmid injection, the dogs entered a rapid reverse stage, and became biochemically anabolic, mimicking a rapid growth process, as in the study described previously ("Growth Hormone Axis and the immune function") on young rats in the growth phase. Hb, PVC and RBC values increased with 10-25%, values significant statistically, and normalized two weeks after the beginning of the therapy. All values were within the normal limits throughout the experiment. Although not wanting to be bound by theory, our hypothesis is that stimulation of the GHRH – GH - IGF-I axis in a catabolic state is stimulating erytropoiesis most probably through stimulation of erythropoietin. When the patients are reversed to a normal anabolic state, the natural GH effect is to induce a slight degree of anemia. Nevertheless, these patients have cancer, and the natural course of the disease is towards catabolism. Patients will be maintained in balance by these

contradictory mechanisms, thus the Hb, PVC and RBC values will be corrected to normal, but never exceed the upper normal limits, as shown in our studies.

[0212] Target gene expression also can be regulated by incorporating molecular switch technology as schematically diagramed in Figure 9. A commercially available system for ligand-dependent induction of transgene expression has a registered trademark name of GeneSwitch®. The GeneSwitch® technology is based on a C-terminally truncated progesterone receptor that actually synthesized, but fails to bind to its natural agonist, progesterone. Instead the truncated progesterone receptor is only activated by antiprogestins, such as mifepristone ("MFP") (Vegeto et al., 1992; Xu et al., 1996). A similar system is used for the chimeric regulator protein of the GeneSwitch™ system, which 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). 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 minutes 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.

EXAMPLE 12

PHARMACOLOGICAL AND TOXICOLOGICAL EFFECTS OF EXOGENOUS GH ADMINISTRATION IN NORMAL ANIMAL SUBJECTS

[0213] Because porcine GH (pGH) is structurally identical to canine GH, pGH was used in different studies on dogs. In one of these studies, pGH was administered for a

14-weeks in dogs. Porcine GH caused increased body weight gain in mid - and high-dose groups (2.8 kg and 4.7 kg, respectively), compared to 0.4 kg and 0.8 kg in control and low-dose groups, respectively. In pGH-treated dogs, increased skin thickness seen grossly correlated histologically with increased dermal collagen. There was no gross or histomorphological evidence of edema. There were dose-related increased serum IGF-I levels (approximately 2-10-fold) that correlated with the elevated serum GH levels in pGH-treated dogs. Also, increased serum insulin levels through the mid dose were seen throughout the study. In high-dose dogs, the insulin levels remained elevated over 24 hr postdose. The serum glucose levels in fasted dogs remained within the control range and there was no chronic hyperglycemia based on glycosylated hemoglobin levels. Renal glomerular changes, significant polyuria with decreased urine specific gravity, and increased serum insulin levels suggested that the dogs had early insulin-resistant diabetes. There was minimal or no biologically significant effect of pGH on serum T3, T4, and cortisol levels in dogs. Other serum biochemical changes in pGH-treated dogs included decreased urea nitrogen and creatinine, and increased potassium, cholesterol, and triglycerides. Significant increases in serum calcium and phosphorous levels and alkaline phosphatase activity (bone isozyme) correlated with the histological changes in bone. In pGH-treated dogs, there was a dose-related *normochromic, normocytic, nonregenerative anemia*. The changes described above, except for the anemia, are related to catabolic effects of high doses of GH (Prahalada et al., 1998)

EXAMPLE 13

PLASMID MEDIATED GHRH DELIVERY SLOWS TUMOR GROWTH, PREVENTS KIDNEY FAILURE AND INCREASES SURVIVABILITY IN TUMOR-BEARING ANIMALS

[0214] Immunocompetent C57/Bl6 mice or immunocompromised nude mice were injected with tumor lines (C57/Bl6 mice were implanted with a Lewis lung rat adenocarcinoma line, while the nude mice were implanted with a human lung small cell adenocarcinoma line). At 1 day after the tumor implantation, mice were treated with either constitutively active GHRH construct, an inducible GHRH construct or with a β-galactosidase expressing construct (as negative control). Tumor established slower (Figure

10 and Figure 14), and developed less rapidly in the GHRH treated animals. Consequently, GHRH treated animals survived longer (Figure 11), and were less likely to develop kidney failure (Figure 12), than controls. Metastases are less likely to develop (Figure 13).

[0215] The invention described herein involves the utilization of several distinctive GHRH or biological equivalent 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. However, based on the known art and the teachings of this specification, one skilled in the art would know how to perform the plasmid mediated supplementation experimentation(s), characterizing variations and permutations on any unique nucleic acid sequence in a specific tissue to accurately evaluate the *in vivo* effect on normal or chronic conditions within a living organism. Therefore, the utilization of the distinctive nucleic acid sequence encoding GHRH or biological equivalent thereof or corresponding recombinant protein as a plasmid-mediated method to treat anemia; increase total red blood cell mass; reverse the wasting; reverse abnormal weight loss; treat immune dysfunction; reverse the suppression of lymphopoesis; and/or extend life expectancy for a chronically ill subject could not have been predicted based on speculation.

[0216] Although not wanting to be bound by theory, it is believed that an increase in GHRH will increase the GH levels sufficient to treat anemia; increase total red blood cell mass; reverse the wasting; reverse abnormal weight loss; treat immune dysfunction; reverse the suppression of lymphopoesis; or extend life expectancy for the chronically ill subject. Hormones (*e.g.* GHRH and GH) often contain a complex feedback-regulated pathway, which are further complicated by chronic conditions such as cancer or AIDS. Without direct experimentation of GHRH or biological equivalents used in plasmid-mediated supplementation or the teachings provided herein, beneficial therapy 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 biological equivalent thereof is further complicated by the tissue used for plasmid mediated supplementation, and would not have been obvious to

one skilled in the art without actual experimentations 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 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 nucleic acid-based 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 0.2 mg plasmid delivered under the proper electroporation conditions had an important biological impact that reversed wasting, increase weight gain, and extend life in an ailing canine 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).

[0217] The treatment of anemia, wasting, or immune dysfunction; the increase in total red blood cell mass; the reverse of abnormal weight loss; the reverse in the suppression of lymphopoesis; and/or the extension of life expectancy for a chronically ill subject 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 the appropriate quantities of GHRH or an analog thereof by classical recombinant protein therapy or nucleic acid transfer. Accordingly, successful plasmid-mediated supplementation requires accurate delivery of encoded sequences to the cells of a subject that results 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 the teachings of this specification.

SEQUENCE LISTING

<110> Advisys, Inc. and Baylor College of Medicine

<120> GROWTH HORMONE RELEASING HORMONE SUPPLEMENTATION FOR TREATING CHRONICALLY ILL SUBJECTS

<130> 57849-NP

<140> CA 2,469,310
<141> 2002-12-10

<150> PCT/US02/39509
<151> 2002-12-10

<150> US 60/339,610
<151> 2001-12-11

<160> 25

<170> PatentIn version 3.1

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<223> This is a growth hormone releasing hormone ("GHRH") with a His1 and Val2 substituting the Try1 and Ala2, Gly15 substituted with Ala15, and Met27, Ser28 with Leu27 and Asn28.

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1				5					10					15	
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Leu	Asn	Arg	Gln	Gln	Gly
			20					25					30		
Glu	Arg	Asn	Gln	Glu	Gln	Gly	Ala								
		35					40								

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<223> This is a modified amino acid sequence for growth hormone releasing hormone (GHRH). Alpha-helical conformation was increased by substituting Gly15 to Ala15.

<400> 2

Tyr	Ile	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Ala	Gln
1				5					10					15	
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Leu	Asn	Arg	Gln	Gln	Gly
			20					25					30		
Glu	Arg	Asn	Gln	Glu	Gln	Gly	Ala								
		35					40								

<210> 3
<211> 40
<212> PRT
<213> artificial sequence

<220>
<223> This is a growth hormone releasing hormone that has a Val2 substitution for a Ala2, Gly15 substituted with Ala15, and Met27, Ser28 with Leu27 and Asn28.

<400> 3

Tyr	Val	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Ala	Gln
1				5					10					15	
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Leu	Asn	Arg	Gln	Gln	Gly
			20					25					30		
Glu	Arg	Asn	Gln	Glu	Gln	Gly	Ala								
		35					40								

<210> 4
<211> 40
<212> PRT
<213> artificial sequence

<220>

<223> This is a porcine growth hormone releasing hormone ("GHRH") that has the following substitutions: Gly15 substituted with Ala15, Met27, Ser28 with Leu27 and Asn28.

<400> 4

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Ala Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Leu Asn Arg Gln Gln Gly
20 25 30

Glu Arg Asn Gln Glu Gln Gly Ala
35 40

<210> 5

<211> 44

<212> PRT

<213> artificial sequence

<220>

<223> This is the artificial sequence for the (1-44)NH2

<400> 5

Thr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
20 25 30

Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
35 40

<210> 6

<211> 40

<212> PRT

<213> artificial sequence

<220>

<223> This is the artificial sequence for GHRH (1-40)OH.

<220>

<221> MISC_FEATURE

<222> (1)..(1)
<223> Xaa at position 1 may be tyrosine, or histidine

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> Xaa at position 2 may be alanine, valine, or isoleucine.

<220>
<221> MISC_FEATURE
<222> (15)..(15)
<223> Xaa at position 15 may be alanine, valine, or isoleucine.

<220>
<221> MISC_FEATURE
<222> (27)..(27)
<223> Xaa at position 27 may be methionine, or leucine.

<220>
<221> MISC_FEATURE
<222> (28)..(28)
<223> Xaa at position 28 may be serine or asparagine.

<220>
<221> MISC_FEATURE
<222> (34)..(34)
<223> Xaa at position 34 may be arginine or serine

<220>
<221> MISC_FEATURE
<222> (38)..(38)
<223> Xaa at position 38 may be glutamine or arginine

<400> 6

Xaa Xaa Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Xaa Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Xaa Xaa Arg Gln Gln Gly
20 25 30

Glu Xaa Asn Gln Glu Xaa Gly Ala
35 40

<210> 7
<211> 323
<212> DNA
<213> artificial sequence

<220>
<223> This is a nucleic acid sequence of a eukaryotic promoter c5-12.

<400> 7
cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg gtgaggaatg 60
gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt tggcgctcta 120
aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca aatatggcga 180
cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg cattcctggg 240
ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg cggcccacga 300
gctacccgga ggagcgggag gcg 323

<210> 8
<211> 190
<212> DNA
<213> artificial sequence

<220>
<223> This is a nucleic acid sequence of a human growth hormone ("hGH") poly A tail.

<400> 8
gggtggcatc cctgtgaccc ctccccagtg cctctcctgg ccctggaagt tgccactcca 60
gtgcccacca gccttgtcct aataaaatta agttgcatca ttttgtctga ctaggtgtcc 120
ttctataata ttatggggtg gaggggggtg gtatggagca aggggcaagt tgggaagaca 180
acctgtaggg 190

<210> 9
<211> 219
<212> DNA
<213> artificial sequence

<220>
<223> This is the cDNA for Porcine growth hormone releasing hormone

<400> 9

atggtgctct gggtgttctt ctttgtgatc ctcaccctca gcaacagctc ccactgctcc 60

ccacctcccc ctttgaccct caggatgcgg cggcacgtag atgccatctt caccaacagc 120

taccggaagg tgctggccca gctgtccgcc cgcaagctgc tccaggacat cctgaacagg 180

cagcagggag agaggaacca agagcaagga gcataatga 219

<210> 10

<211> 40

<212> PRT

<213> artificial sequence

<220>

<223> This is the amino acid sequence for porcine growth hormone releasing hormone.

<400> 10

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
20 25 30

Glu Arg Asn Gln Glu Gln Gly Ala
35 40

<210> 11

<211> 3534

<212> DNA

<213> artificial sequence

<220>

<223> This is the nucleic acid sequence for the operatively linked components of the HV-GHRH plasmid.

<400> 11

gttgtaaaac gacggccagt gaattgtaat acgactcact atagggcgaa ttggagctcc 60

accgcggtgg cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg 120

gtgaggaatg gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt 180

tggcgctcta aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca 240

aatatggcga cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg 300

cattcctggg ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg 360

cggcccacga gctacccgga ggagcgggag gcgccaagct ctagaactag tggatcccaa 420
ggcccaactc cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct 480
ctgggtgttc ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc 540
ccctttgacc ctcaggatgc ggcggcacgt agatgccatc ttcaccaaca gctaccggaa 600
ggtgctggcc cagctgtccg cccgcaagct gctccaggac atcctgaaca ggcagcaggg 660
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actgttggga agggcgatcg gtgcgggcct cttcgctatt acgccagctg gcgaaagggg 3480
gatgtgctgc aaggcgatta agttgggtaa cgccagggtt ttcccagtca cgac 3534

<210> 12
<211> 3534
<212> DNA
<213> artificial sequence

<220>
<223> This is the nucleic acid sequence for the operatively linked components of the TI-GHRH plasmid.

<400> 12
gttgtaaaac gacggccagt gaattgtaat acgactcact atagggcgaa ttggagctcc 60
accgcggtgg cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg 120
gtgaggaatg gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt 180
tggcgctcta aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca 240
aatatggcga cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg 300
cattcctggg ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg 360
cggcccacga gctacccgga ggagcgggag gcgccaagct ctagaactag tggatcccaa 420
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ccctttgacc ctcaggatgc ggcggtatat cgatgccatc ttcaccaaca gctaccggaa 600
ggtgctggcc cagctgtccg cccgcaagct gctccaggac atcctgaaca ggcagcaggg 660
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gatgtgctgc aaggcgatta agttgggtaa cgccagggtt ttcccagtca cgac 3534

<210> 13
<211> 3534
<212> DNA
<213> artificial sequence

<220>
<223> This is the nucleic acid sequence for the operatively linked components of the TV-GHRH plasmid.

<400> 13
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accgcggtgg cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg 120
gtgaggaatg gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt 180
tggcgctcta aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca 240
aatatggcga cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg 300
cattcctggg ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg 360
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cctgcgctga cagccggaac acggcggcat cagagcagcc gattgtctgt tgtgcccagt 3180
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actgttggga agggcgatcg gtgcgggcct cttcgctatt acgccagctg gcgaaagggg 3480
gatgtgctgc aaggcgatta agttgggtaa cgccagggtt ttcccagtca cgac 3534

<210> 14
<211> 3534
<212> DNA
<213> artificial sequence

<220>

<223> This is the nucleic acid sequence for the operatively linked components of the 15/27/28 GHRH plasmid.

<400> 14

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accgcggtgg cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg 120
gtgaggaatg gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt 180
tggcgctcta aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca 240
aatatggcga cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg 300
cattcctggg ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg 360
cggcccacga gctacccgga ggagcgggag gcgccaagct ctagaactag tggatcccaa 420
ggcccaactc cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct 480
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ccctttgacc ctcaggatgc ggcggtatat cgatgccatc ttcaccaaca gctaccggaa 600
ggtgctggcc cagctgtccg cccgcaagct gctccaggac atcctgaaca ggcagcaggg 660
agagaggaac caagagcaag gagcataatg actgcaggaa ttcgatatca agcttatcgg 720
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<210> 15
<211> 3534
<212> DNA
<213> artificial sequence

<220>
<223> This is the entire plasmid sequence for wildtype GHRH.

<400> 15
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accgcggtgg cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg 120
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tggcgctcta aaaataactc ccgggagtta ttttagagc ggaggaatgg tggacaccca 240
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gatgtgctgc aaggcgatta agttgggtaa cgccagggtt ttcccagtca cgac 3534

<210> 16
<211> 4260
<212> DNA
<213> Artificial sequence

<220>
<223> This is the sequence for the pSP-SEAP cDNA construct

<400> 16
ggccgtccgc cttcggcacc atcctcacga cacccaaata tggcgacggg tgaggaatgg 60
tggggagtta tttttagagc ggtgaggaag gtgggcaggc agcaggtgtt ggcgctctaa 120
aaataactcc cgggagttat ttttagagcg gaggaatggt ggacacccaa atatggcgac 180
ggttcctcac ccgtcgccat atttgggtgt ccgccctcgg ccggggccgc attcctgggg 240
gccgggcggt gctcccgccc gcctcgataa aaggctccgg ggccggcggc ggcccacgag 300
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<210> 17
<211> 2710
<212> DNA
<213> artificial sequence

<220>
<223> This is a plasmid vector with an analog growth hormone releasing hormone ("GHRH") sequence codon optimized for mouse.

<400> 17
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cggcaccatc ctcacgacac ccaaatatgg cgacgggtga ggaatggtgg ggagttattt 120
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cggtgtaggt cgttcgctcc aagctgggct gtgtgcacga accccccgtt cagcccgacc 1380
gctgcgcctt atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc 1440
cactggcagc agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag 1500
agttcttgaa gtggtggcct aactacggct acactagaag aacagtattt ggtatctgcg 1560
ctctgctgaa gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaacaaa 1620
ccaccgctgg tagcggtggt ttttttgttt gcaagcagca gattacgcgc agaaaaaaag 1680
gatctcaaga agatcctttg atcttttcta cggggctagc gcttagaaga actcatccag 1740
cagacggtag aatgcaatac gttgagagtc tggagctgca ataccataca gaaccaggaa 1800
acggtcagcc cattcaccac ccagttcctc tgcaatgtca cgggtagcca gtgcaatgtc 1860
ctggtaacgg tctgcaacac ccagacgacc acagtcaatg aaaccagaga aacgaccatt 1920
ctcaaccatg atgttcggca ggcatgcatc accatgagta actaccaggt cctcaccatc 1980
cggcatacga gctttcagac gtgcaaacag ttcagccggt gccagaccct gatgttcctc 2040
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atgttttgcc tggtggtcaa acggacaggt agctgggtcc agggtgtgca gacgacgcat 2160
tgcatcagcc atgatagaaa ctttctctgc cggagccagg tgagaagaca gcaggtcctg 2220
acccggaact tcacccagca gcagccagtc acgaccagct tcagtaacta catccagaac 2280
tgcagcacac ggaacaccag tggttgccag ccaagacaga cgagctgctt catcctgcag 2340
ttcattcaga gcaccagaca ggtcagtttt aacaaacaga actggacgac cctgtgcaga 2400
cagacggaaa acagctgcat cagagcaacc aatggtctgc tgtgcccagt cataaccaaa 2460
cagacgttca acccaggctg ccggagaacc tgcatgcaga ccatcctgtt caatcatgcg 2520
aaacgatcct catcctgtct cttgatcaga tcttgatccc ctgcgccatc agatccttgg 2580
cggcaagaaa gccatccagt ttactttgca gggcttccca accttaccag agggcgcccc 2640
agctggcaat tccggttcgc ttgctgtcca taaaaccgcc cagtctagca actgttggga 2700
agggcgatcg 2710

<210> 18
<211> 2713
<212> DNA
<213> artificial sequence

<220>
<223> This is a plasmid vector with an analog growth hormone releasing hormone ("GHRH") sequence codon optimized for rat.

<400> 18

tgtaatacga ctcactatag ggcgaattgg agctccaccg cggtggcggc cgtccgccct 60
cggcaccatc ctcacgacac ccaaatatgg cgacgggtga ggaatggtgg ggagttattt 120
ttagagcggt gaggaaggtg ggcaggcagc aggtgttggc gctctaaaaa taactcccgg 180
gagttatttt tagagcggag gaatggtgga cacccaaata tggcgacggt tcctcacccg 240
tcgccatatt tgggtgtccg ccctcggccg gggccgcatt cctgggggcc gggcggtgct 300
cccgcccgcc tcgataaaag gctccggggc cggcggcggc ccacgagcta cccggaggag 360
cgggaggcgc caagcggatc ccaaggccca actccccgaa ccactcaggg tcctgtggac 420
agctcaccta gctgccatgg ccctgtgggt gttcttcgtg ctgctgaccc tgaccagcgg 480
aagccactgc agcctgcctc ccagccctcc cttcagggtg cgccggcacg ccgacgccat 540
cttcaccagc agctacagga ggatcctggg ccagctgtac gctaggaagc tcctgcacga 600
gatcatgaac aggcagcagg gcgagaggaa ccaggagcag aggagcaggt tcaactgata 660
agcttatcgg ggtggcatcc ctgtgacccc tccccagtgc ctctcctggc cctggaagtt 720
gccactccag tgcccaccag ccttgtccta ataaaattaa gttgcatcat tttgtctgac 780
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agggttaatt tcgagcttgg tcttccgctt cctcgctcac tgactcgctg cgctcggtcg 960
ttcggctgcg gcgagcggta tcagctcact caaaggcggt aatacggtta tccacagaat 1020
caggggataa cgcaggaaag aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta 1080
aaaaggccgc gttgctggcg tttttccata ggctccgccc ccctgacgag catcacaaaa 1140
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cccctggaag ctccctcgtg cgctctcctg ttccgaccct gccgcttacc ggatacctgt 1260
ccgcctttct cccttcggga agcgtggcgc tttctcatag ctcacgctgt aggtatctca 1320
gttcggtgta ggtcgttcgc tccaagctgg gctgtgtgca cgaaccccccc gttcagcccg 1380
accgctgcgc cttatccggt aactatcgtc ttgagtccaa cccggtaaga cacgacttat 1440
cgccactggc agcagccact ggtaacagga ttagcagagc gaggtatgta ggcggtgcta 1500
cagagttctt gaagtggtgg cctaactacg gctacactag aagaacagta tttggtatct 1560
gcgctctgct gaagccagtt accttcggaa aaagagttgg tagctcttga tccggcaaac 1620
aaaccaccgc tggtagcggt ggtttttttg tttgcaagca gcagattacg cgcagaaaaa 1680
aaggatctca agaagatcct ttgatctttt ctacggggct agcgcttaga agaactcatc 1740
cagcagacgg tagaatgcaa tacgttgaga gtctggagct gcaataccat acagaaccag 1800
gaaacggtca gcccattcac cacccagttc ctctgcaatg tcacgggtag ccagtgcaat 1860
gtcctggtaa cggtctgcaa cacccagacg accacagtca atgaaaccag agaaacgacc 1920
attctcaacc atgatgttcg gcaggcatgc atcaccatga gtaactacca ggtcctcacc 1980
atccggcata cgagctttca gacgtgcaaa cagttcagcc ggtgccagac cctgatgttc 2040
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acgatgtttt gcctggtggt caaacggaca ggtagctggg tccagggtgt gcagacgacg 2160
cattgcatca gccatgatag aaactttctc tgccggagcc aggtgagaag acagcaggtc 2220
ctgacccgga acttcaccca gcagcagcca gtcacgacca gcttcagtaa ctacatccag 2280
aactgcagca cacggaacac cagtggttgc cagccaagac agacgagctg cttcatcctg 2340

cagttcattc agagcaccag acaggtcagt tttaacaaac agaactggac gaccctgtgc 2400
agacagacgg aaaacagctg catcagagca accaatggtc tgctgtgccc agtcataacc 2460
aaacagacgt tcaacccagg ctgccggaga acctgcatgc agaccatcct gttcaatcat 2520
gcgaaacgat cctcatcctg tctcttgatc agatcttgat cccctgcgcc atcagatcct 2580
tggcggcaag aaagccatcc agtttacttt gcagggcttc ccaaccttac cagagggcgc 2640
cccagctggc aattccggtt cgcttgctgt ccataaaacc gcccagtcta gcaactgttg 2700
ggaagggcga tcg 2713

<210> 19
<211> 2704
<212> DNA
<213> artificial sequence

<220>
<223> This is a plasmid vector with an analog growth hormone releasing hormone ("GHRH") sequence codon optimized for bovine.

<400> 19
tgtaatacga ctcactatag ggcgaattgg agctccaccg cggtggcggc cgtccgccct 60
cggcaccatc ctcacgacac ccaaatatgg cgacgggtga ggaatggtgg ggagttattt 120
ttagagcggt gaggaaggtg ggcaggcagc aggtgttggc gctctaaaaa taactcccgg 180
gagttatttt tagagcggag gaatggtgga cacccaaata tggcgacggt tcctcacccg 240
tcgccatatt tgggtgtccg ccctcggccg gggccgcatt cctgggggcc gggcggtgct 300
cccgcccgcc tcgataaaag gctccggggc cggcggcggc ccacgagcta cccggaggag 360
cgggaggcgc caagcggatc ccaaggccca actccccgaa ccactcaggg tcctgtggac 420
agctcaccta gctgccatgg tgctgtgggt gttcttcctg gtgaccctga ccctgagcag 480
cggctcccac ggctccctgc cctcccagcc tctgcgcatc cctcgctacg ccgacgccat 540
cttcaccaac agctaccgca aggtgctcgg ccagctcagc gcccgcaagc tcctgcagga 600
catcatgaac cggcagcagg gcgagcgcaa ccaggagcag ggagcctgat aagcttatcg 660
gggtggcatc cctgtgaccc ctccccagtg cctctcctgg ccctggaagt tgccactcca 720
gtgcccacca gccttgtcct aataaaatta agttgcatca ttttgtctga ctaggtgtcc 780
ttctataata ttatggggtg gaggggggtg gtatggagca aggggcaagt tgggaagaca 840
acctgtaggg ctcgaggggg ggcccggtac cagcttttgt tcccttttagt gagggttaat 900
ttcgagcttg gtcttccgct tcctcgctca ctgactcgct gcgctcggtc gttcggctgc 960
ggcgagcggt atcagctcac tcaaaggcgg taatacggtt atccacagaa tcaggggata 1020
acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc caggaaccgt aaaaaggccg 1080
cgttgctggc gtttttccat aggctccgcc cccctgacga gcatcacaaa aatcgacgct 1140
caagtcagag gtggcgaaac ccgacaggac tataaagata ccaggcgttt ccccctggaa 1200
gctccctcgt gcgctctcct gttccgaccc tgccgcttac cggatacctg tccgcctttc 1260
tcccttcggg aagcgtggcg ctttctcata gctcacgctg taggtatctc agttcggtgt 1320

aggtcgttcg ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg 1380
ccttatccgg taactatcgt cttgagtcca acccggtaag acacgactta tcgccactgg 1440
cagcagccac tggtaacagg attagcagag cgaggtatgt aggcggtgct acagagttct 1500
tgaagtggtg gcctaactac ggctacacta gaagaacagt atttggtatc tgcgctctgc 1560
tgaagccagt taccttcgga aaaagagttg gtagctcttg atccggcaaa caaaccaccg 1620
ctggtagcgg tggtttttt gtttgcaagc agcagattac gcgcagaaaa aaaggatctc 1680
aagaagatcc tttgatcttt tctacggggc tagcgcttag aagaactcat ccagcagacg 1740
gtagaatgca atacgttgag agtctggagc tgcaatacca tacagaacca ggaaacggtc 1800
agcccattca ccacccagtt cctctgcaat gtcacgggta gccagtgcaa tgtcctggta 1860
acggtctgca acacccagac gaccacagtc aatgaaacca gagaaacgac cattctcaac 1920
catgatgttc ggcaggcatg catcaccatg agtaactacc aggtcctcac catccggcat 1980
acgagctttc agacgtgcaa acagttcagc cggtgccaga ccctgatgtt cctcatccag 2040
gtcatcctgg tcaaccagac ctgcttccat acgggtacga gcacgttcaa tacgatgttt 2100
tgcctggtgg tcaaacggac aggtagctgg gtccagggtg tgcagacgac gcattgcatc 2160
agccatgata gaaactttct ctgccggagc caggtgagaa gacagcaggt cctgacccgg 2220
aacttcaccc agcagcagcc agtcacgacc agcttcagta actacatcca gaactgcagc 2280
acacggaaca ccagtggttg ccagccaaga cagacgagct gcttcatcct gcagttcatt 2340
cagagcacca gacaggtcag ttttaacaaa cagaactgga cgaccctgtg cagacagacg 2400
gaaaacagct gcatcagagc aaccaatggt ctgctgtgcc cagtcataac caaacagacg 2460
ttcaacccag gctgccggag aacctgcatg cagaccatcc tgttcaatca tgcgaaacga 2520
tcctcatcct gtctcttgat cagatcttga tcccctgcgc catcagatcc ttggcggcaa 2580
gaaagccatc cagtttactt tgcagggctt cccaacctta ccagagggcg ccccagctgg 2640
caattccggt tcgcttgctg tccataaaac cgcccagtct agcaactgtt gggaagggcg 2700
atcg 2704

<210> 20
<211> 2704
<212> DNA
<213> artificial sequence

<220>
<223> This is a plasmid vector with an analog growth hormone releasing hormone ("GHRH") sequence codon optimized for ovine.

<400> 20
tgtaatacga ctcactatag ggcgaattgg agctccaccg cggtggcggc cgtccgccct 60
cggcaccatc ctcacgacac ccaaatatgg cgacgggtga ggaatggtgg ggagttattt 120
ttagagcggt gaggaaggtg ggcaggcagc aggtgttggc gctctaaaaa taactcccgg 180
gagttatttt tagagcggag gaatggtgga cacccaaata tggcgacggt tcctcacccg 240
tcgccatatt tgggtgtccg ccctcggccg gggccgcatt cctgggggcc gggcggtgct 300

cccgcccgcc tcgataaaag gctccggggc cggcggcggc ccacgagcta cccggaggag 360
cgggaggcgc caagcggatc ccaaggccca actccccgaa ccactcaggg tcctgtggac 420
agctcaccta gctgccatgg tgctgtgggt gttcttcctg gtgaccctga ccctgagcag 480
cggaagccac ggcagcctgc ccagccagcc cctgaggatc cctaggtacg ccgacgccat 540
cttcaccaac agctacagga agatcctggg ccagctgagc gctaggaagc tcctgcagga 600
catcatgaac aggcagcagg gcgagaggaa ccaggagcag ggcgcctgat aagcttatcg 660
gggtggcatc cctgtgaccc ctccccagtg cctctcctgg ccctggaagt tgccactcca 720
gtgcccacca gccttgtcct aataaaatta agttgcatca ttttgtctga ctaggtgtcc 780
ttctataata ttatggggtg gaggggggtg gtatggagca aggggcaagt tgggaagaca 840
acctgtaggg ctcgaggggg ggcccggtac cagcttttgt tcccctttagt gagggttaat 900
ttcgagcttg gtcttccgct tcctcgctca ctgactcgct gcgctcggtc gttcggctgc 960
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acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc caggaaccgt aaaaaggccg 1080
cgttgctggc gtttttccat aggctccgcc cccctgacga gcatcacaaa aatcgacgct 1140
caagtcagag gtggcgaaac ccgacaggac tataaagata ccaggcgttt cccccctggaa 1200
gctccctcgt gcgctctcct gttccgaccc tgccgcttac cggatacctg tccgcctttc 1260
tcccttcggg aagcgtggcg ctttctcata gctcacgctg taggtatctc agttcggtgt 1320
aggtcgttcg ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg 1380
ccttatccgg taactatcgt cttgagtcca acccggtaag acacgactta tcgccactgg 1440
cagcagccac tggtaacagg attagcagag cgaggtatgt aggcggtgct acagagttct 1500
tgaagtggtg gcctaactac ggctacacta gaagaacagt atttggtatc tgcgctctgc 1560
tgaagccagt taccttcgga aaaagagttg gtagctcttg atccggcaaa caaaccaccg 1620
ctggtagcgg tggtttttt gtttgcaagc agcagattac gcgcagaaaa aaaggatctc 1680
aagaagatcc tttgatcttt tctacggggc tagcgcttag aagaactcat ccagcagacg 1740
gtagaatgca atacgttgag agtctggagc tgcaatacca tacagaacca ggaaacggtc 1800
agcccattca ccacccagtt cctctgcaat gtcacgggta gccagtgcaa tgtcctggta 1860
acggtctgca acacccagac gaccacagtc aatgaaacca gagaaacgac cattctcaac 1920
catgatgttc ggcaggcatg catcaccatg agtaactacc aggtcctcac catccggcat 1980
acgagctttc agacgtgcaa acagttcagc cggtgccaga ccctgatgtt cctcatccag 2040
gtcatcctgg tcaaccagac ctgcttccat acgggtacga gcacgttcaa tacgatgttt 2100
tgcctggtgg tcaaacggac aggtagctgg gtccagggtg tgcagacgac gcattgcatc 2160
agccatgata gaaactttct ctgccggagc caggtgagaa gacagcaggt cctgacccgg 2220
aacttcaccc agcagcagcc agtcacgacc agcttcagta actacatcca gaactgcagc 2280
acacggaaca ccagtggttg ccagccaaga cagacgagct gcttcatcct gcagttcatt 2340
cagagcacca gacaggtcag ttttaacaaa cagaactgga cgaccctgtg cagacagacg 2400
gaaaacagct gcatcagagc aaccaatggt ctgctgtgcc cagtcataac caaacagacg 2460
ttcaacccag gctgccggag aacctgcatg cagaccatcc tgttcaatca tgcgaaacga 2520
tcctcatcct gtctcttgat cagatcttga tcccctgcgc catcagatcc ttggcggcaa 2580
gaaagccatc cagtttactt tgcagggctt cccaacctta ccagagggcg ccccagctgg 2640

caattccggt tcgcttgctg tccataaaac cgcccagtct agcaactgtt gggaagggcg 2700
atcg 2704

<210> 21
<211> 2713
<212> DNA
<213> artificial sequence

<220>
<223> This is a plasmid vector with an analog growth hormone releasing hormone ("GHRH") sequence codon optimized for chicken.

<400> 21
tgtaatacga ctcactatag ggcgaattgg agctccaccg cggtggcggc cgtccgccct 60
cggcaccatc ctcacgacac ccaaatatgg cgacgggtga ggaatggtgg ggagttattt 120
ttagagcggt gaggaaggtg ggcaggcagc aggtgttggc gctctaaaaa taactcccgg 180
gagttatttt tagagcggag gaatggtgga cacccaaata tggcgacggt tcctcacccg 240
tcgccatatt tgggtgtccg ccctcggccg gggccgcatt cctgggggcc gggcggtgct 300
cccgcccgcc tcgataaaag gctccggggc cggcggcggc ccacgagcta cccggaggag 360
cgggaggcgc caagcggatc ccaaggccca actccccgaa ccactcaggg tcctgtggac 420
agctcaccta gctgccatgg ccctgtgggt gttctttgtg ctgctgaccc tgacctccgg 480
aagccactgc agcctgccac ccagcccacc cttccgcgtc aggcgccacg ccgacggcat 540
cttcagcaag gcctaccgca agctcctggg ccagctgagc gcacgcaact acctgcacag 600
cctgatggcc aagcgcgtgg gcagcggact gggagacgag gccgagcccc tgagctgata 660
agcttatcgg ggtggcatcc ctgtgacccc tccccagtgc ctctcctggc cctggaagtt 720
gccactccag tgcccaccag ccttgtccta ataaaattaa gttgcatcat tttgtctgac 780
taggtgtcct tctataatat tatggggtgg aggggggtgg tatggagcaa ggggcaagtt 840
gggaagacaa cctgtagggc tcgaggggggg gcccggtacc agcttttgtt ccctttagtg 900
agggttaatt tcgagcttgg tcttccgctt cctcgctcac tgactcgctg cgctcggtcg 960
ttcggctgcg gcgagcggta tcagctcact caaaggcggt aatacggtta tccacagaat 1020
caggggataa cgcaggaaag aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta 1080
aaaaggccgc gttgctggcg tttttccata ggctccgccc ccctgacgag catcacaaaa 1140
atcgacgctc aagtcagagg tggcgaaacc cgacaggact ataaagatac caggcgtttc 1200
cccctggaag ctccctcgtg cgctctcctg ttccgaccct gccgcttacc ggatacctgt 1260
ccgcctttct cccttcggga agcgtggcgc tttctcatag ctcacgctgt aggtatctca 1320
gttcggtgta ggtcgttcgc tccaagctgg gctgtgtgca cgaaccccccc gttcagcccg 1380
accgctgcgc cttatccggt aactatcgtc ttgagtccaa cccggtaaga cacgacttat 1440
cgccactggc agcagccact ggtaacagga ttagcagagc gaggtatgta ggcggtgcta 1500
cagagttctt gaagtggtgg cctaactacg gctacactag aagaacagta tttggtatct 1560
gcgctctgct gaagccagtt accttcggaa aaagagttgg tagctcttga tccggcaaac 1620

aaaccaccgc tggtagcggt ggtttttttg tttgcaagca gcagattacg cgcagaaaaa 1680
aaggatctca agaagatcct ttgatctttt ctacggggct agcgcttaga agaactcatc 1740
cagcagacgg tagaatgcaa tacgttgaga gtctggagct gcaataccat acagaaccag 1800
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gtcctggtaa cggtctgcaa cacccagacg accacagtca atgaaaccag agaaacgacc 1920
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atccggcata cgagctttca gacgtgcaaa cagttcagcc ggtgccagac cctgatgttc 2040
ctcatccagg tcatcctggt caaccagacc tgcttccata cgggtacgag cacgttcaat 2100
acgatgtttt gcctggtggt caaacggaca ggtagctggg tccagggtgt gcagacgacg 2160
cattgcatca gccatgatag aaactttctc tgccggagcc aggtgagaag acagcaggtc 2220
ctgacccgga acttcaccca gcagcagcca gtcacgacca gcttcagtaa ctacatccag 2280
aactgcagca cacggaacac cagtggttgc cagccaagac agacgagctg cttcatcctg 2340
cagttcattc agagcaccag acaggtcagt tttaacaaac agaactggac gaccctgtgc 2400
agacagacgg aaaacagctg catcagagca accaatggtc tgctgtgccc agtcataacc 2460
aaacagacgt tcaacccagg ctgccggaga acctgcatgc agaccatcct gttcaatcat 2520
gcgaaacgat cctcatcctg tctcttgatc agatcttgat cccctgcgcc atcagatcct 2580
tggcggcaag aaagccatcc agtttacttt gcagggcttc ccaaccttac cagagggcgc 2640
cccagctggc aattccggtt cgcttgctgt ccataaaacc gcccagtcta gcaactgttg 2700
ggaagggcga tcg 2713

<210> 22
<211> 55
<212> DNA
<213> artificial sequence

<220>
<223> This is a nucleic acid sequence of a human growth hormone ("hGH") 5' untranslated region ("5' UTR).

<400> 22
caaggcccaa ctccccgaac cactcagggt cctgtggaca gctcacctag ctgcc 55

<210> 23
<211> 782
<212> DNA
<213> artificial sequence

<220>
<223> This is a nucleic acid sequence of a plasmid pUC-18 origin of replication

<400> 23

tcttccgctt cctcgctcac tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta 60

tcagctcact caaaggcggt aatacggtta tccacagaat caggggataa cgcaggaaag 120

aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg 180

tttttccata ggctccgccc ccctgacgag catcacaaaa atcgacgctc aagtcagagg 240

tggcgaaacc cgacaggact ataaagatac caggcgtttc cccctggaag ctccctcgtg 300

cgctctcctg ttccgaccct gccgcttacc ggatacctgt ccgcctttct cccttcggga 360

agcgtggcgc tttctcatag ctcacgctgt aggtatctca gttcggtgta ggtcgttcgc 420

tccaagctgg gctgtgtgca cgaaccccc gttcagcccg accgctgcgc cttatccggt 480

aactatcgtc ttgagtccaa cccggtaaga cacgacttat cgccactggc agcagccact 540

ggtaacagga ttagcagagc gaggtatgta ggcggtgcta cagagttctt gaagtggtgg 600

cctaactacg gctacactag aaggacagta tttggtatct gcgctctgct gaagccagtt 660

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<223> This is a nucleic acid sequence of a prokaryotic PNEO promoter.

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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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CLAIMS

1. Use in muscle tissue cells of a subject of an effective amount of a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone (GHRH) of SeqID#6 to increase total red blood cell mass in the subject.

2. The use of claim 1, wherein the construct is in the muscle tissue cells of the subject by electroporation.

3. The use of claim 1, wherein the nucleic acid expression construct comprises SeqID#11, SeqID#12, SeqID#13, SeqID#14, SeqID#17, SeqID#18, SeqID#19, SeqID#20, or SeqID#21.

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

5. The use of claim 4, wherein the transfection-facilitating polypeptide comprises a charged polypeptide.

6. The use of claim 4, wherein the transfection-facilitating polypeptide comprises poly-L-glutamate.

7. The use of claim 1, wherein delivery into the cells of the subject of the nucleic acid expression construct initiates expression of the encoded GHRH.

8. The use of claim 7, wherein the encoded GHRH is a biologically active polypeptide with an increased level of GHRH activity in the subject when compared to the GHRH polypeptide.

9. The use of claim 1, wherein the nucleic acid expression construct encodes a polypeptide of a sequence consisting of SeqID#1, SeqID#2, SeqID#3, or SeqID#4.

10. The use of claim 1, wherein the encoded GHRH facilitates growth hormone ("GH") secretion in the subject.

11. Use of a recombinant growth-hormone-releasing-hormone (GHRH) of SeqID#6 in muscle tissue cells of a subject to increase total red blood cell mass in the subject.

12. The use of claim 11, wherein the recombinant GHRH is a biologically active polypeptide with an increased level of GHRH activity in the subject when compared to the GHRH polypeptide.

13. The use of claim 11, wherein the recombinant GHRH is of a formula consisting of SeqID#1, SeqID#2, SeqID#3, or SeqID#4.

14. The use of claim 11, wherein the recombinant GHRH facilitates growth hormone ("GH") secretion in the subject.

Figure 1

porcine wild-type YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGA-OH

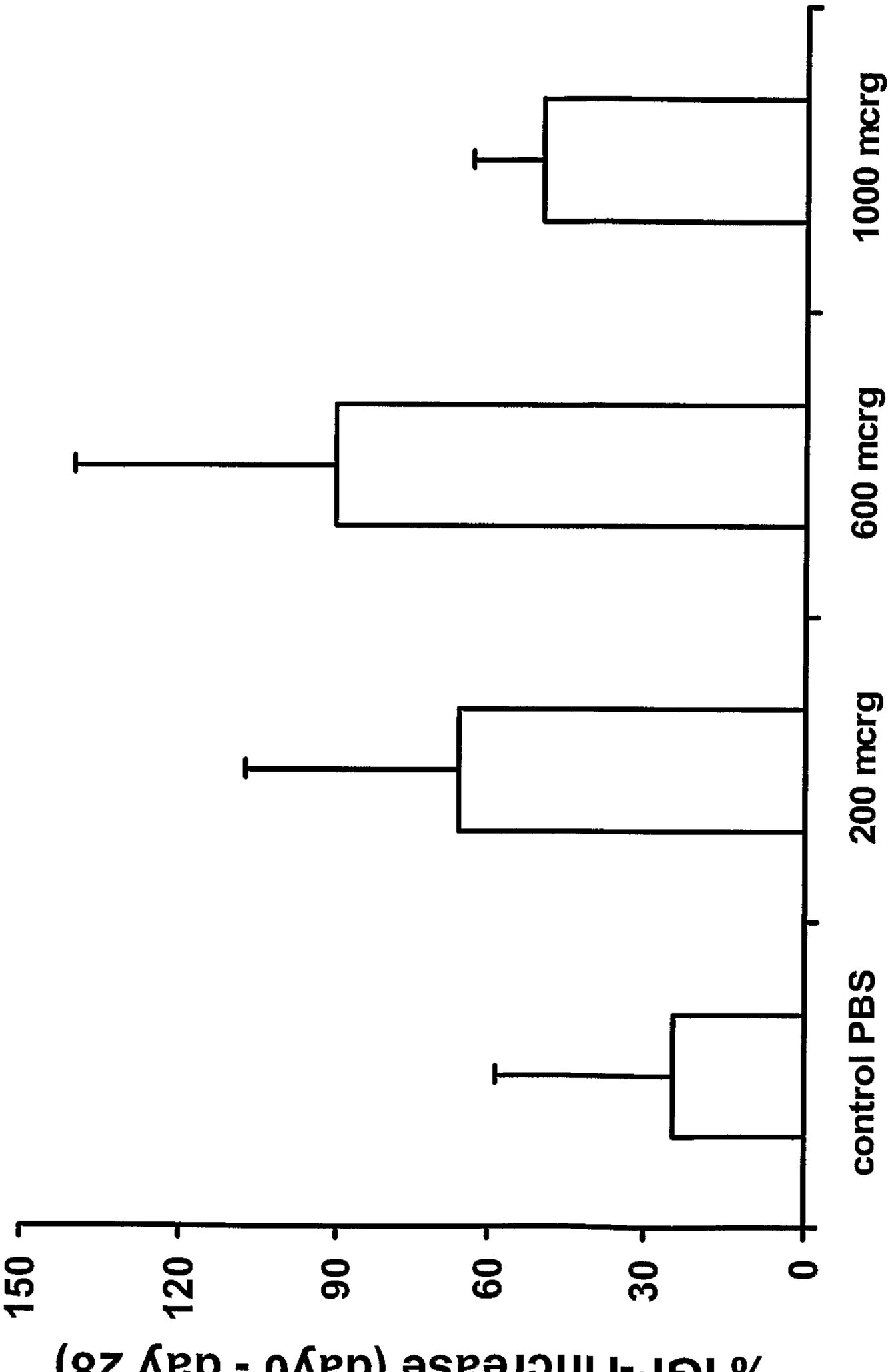
HV-GHRH YHVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

TI-GHRH YIDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

TV-GHRH YVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

15/27/28-GHRH YADAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA-OH

Figure 2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50
20
90
60
30
0
control PBS
200 mcrg
600 mcrg
1000 mcrg

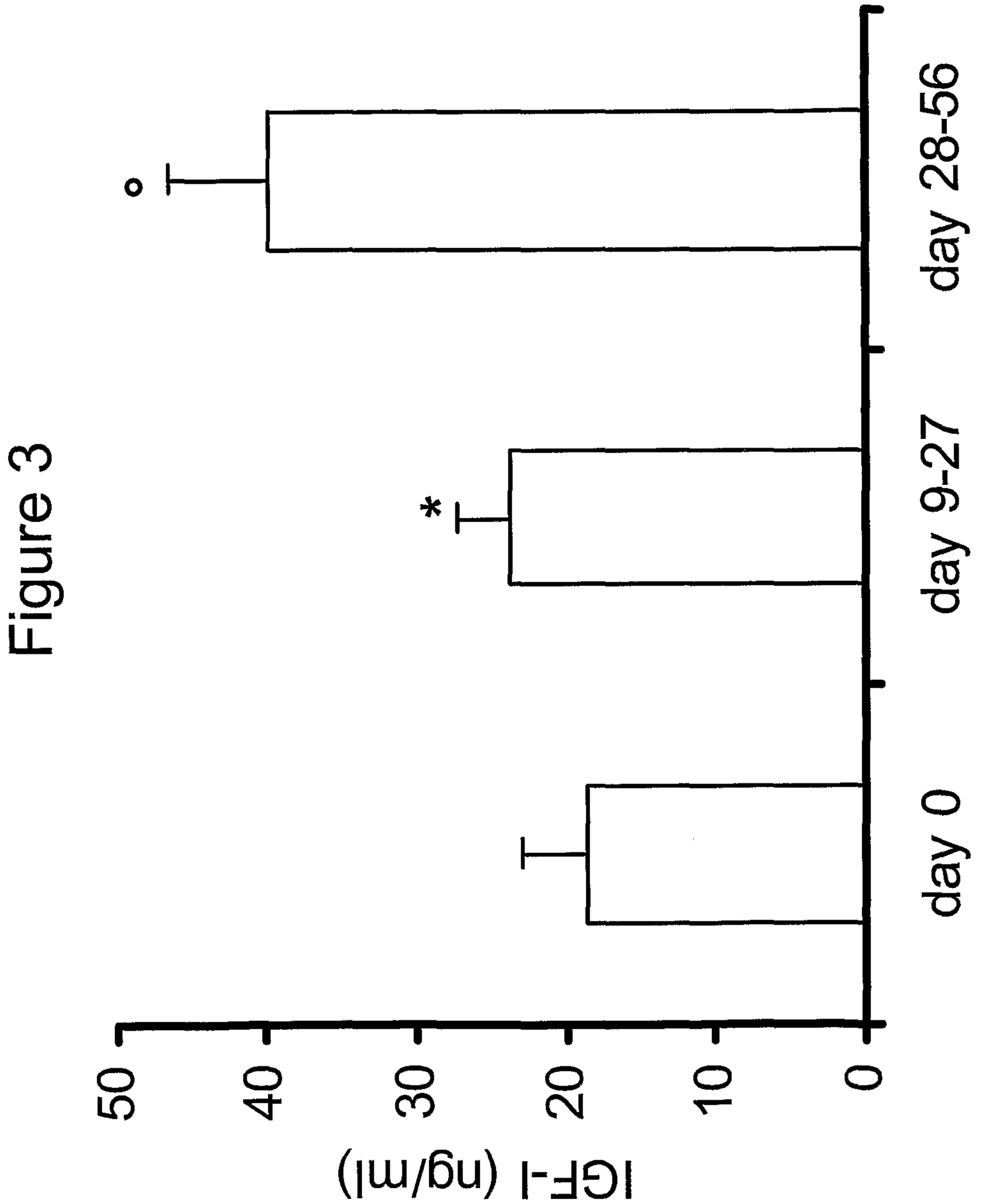


Figure 3

Figure 4

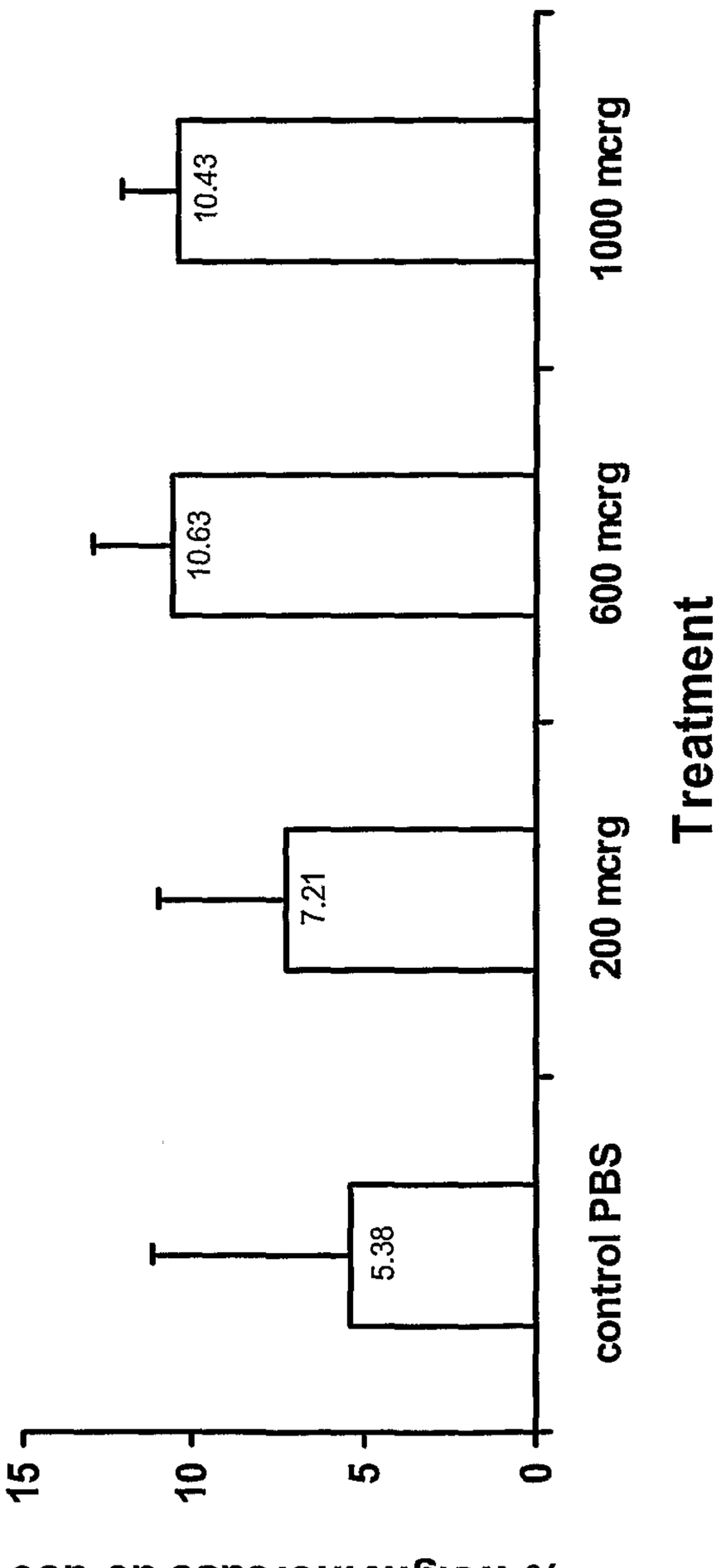
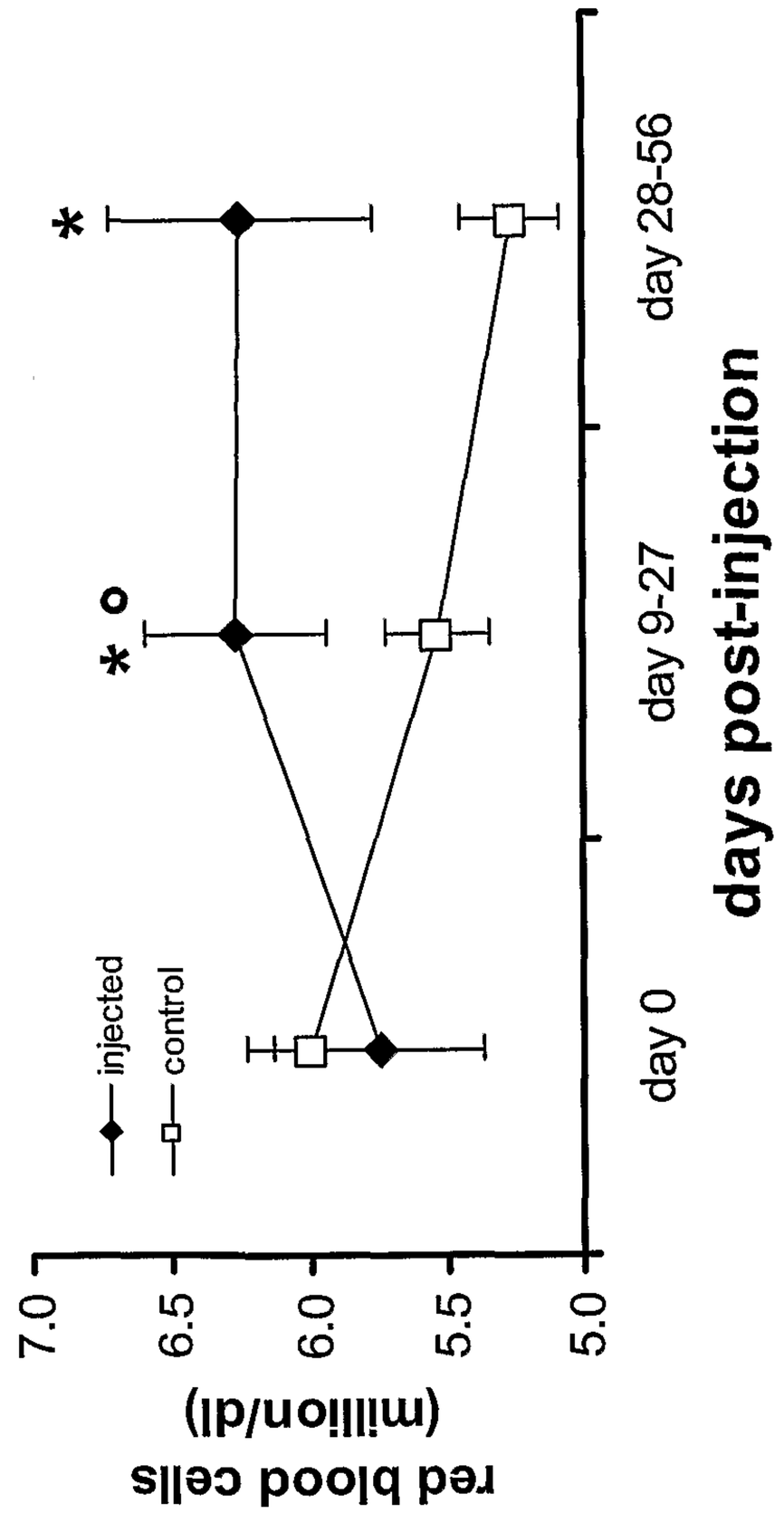
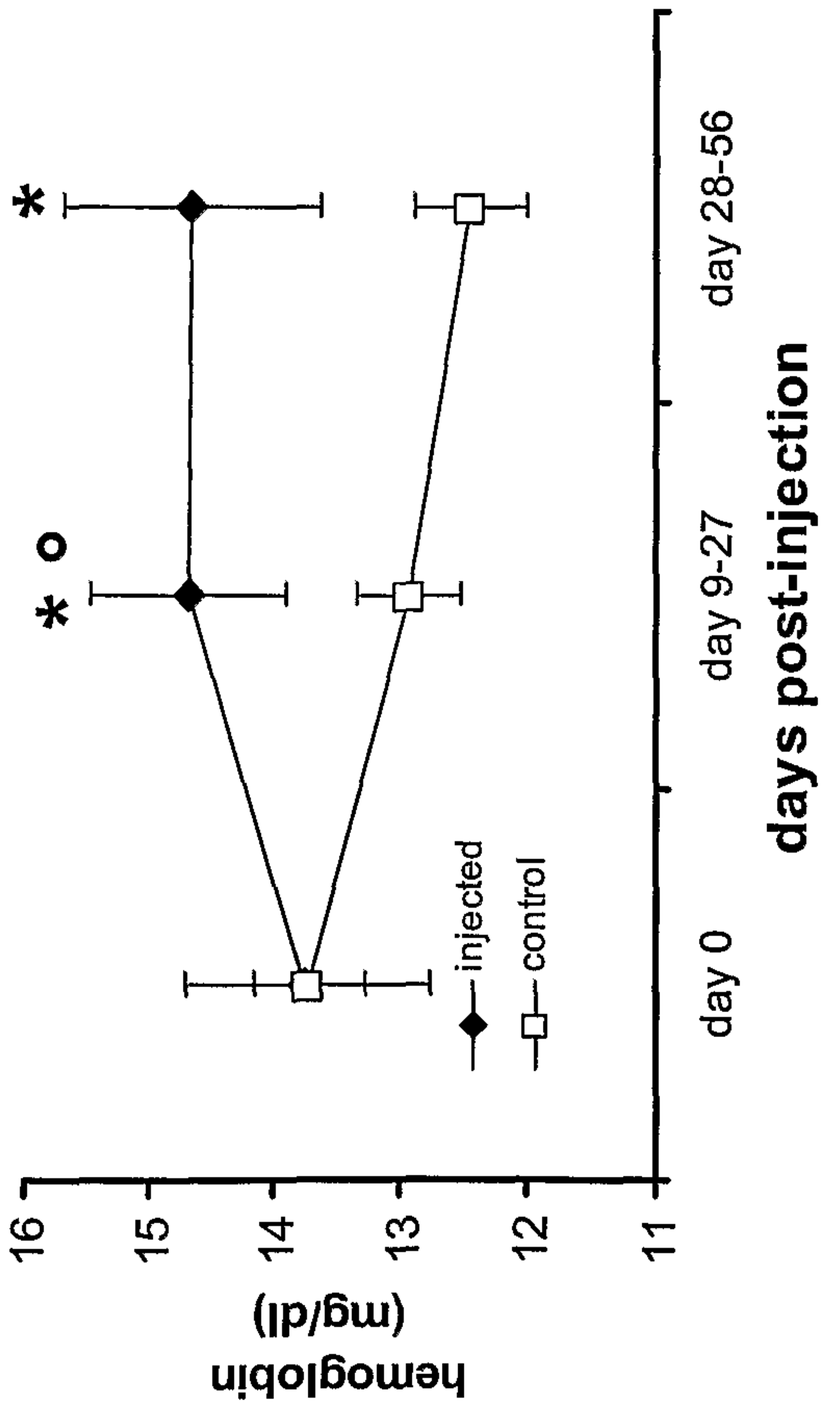

% weight increase d0-d56
5
0
5
0
5.38
7.21
10.63
10.43
control PBS
200 mcrg
600 mcrg
1000 mcrg
Treatment

Figure 5



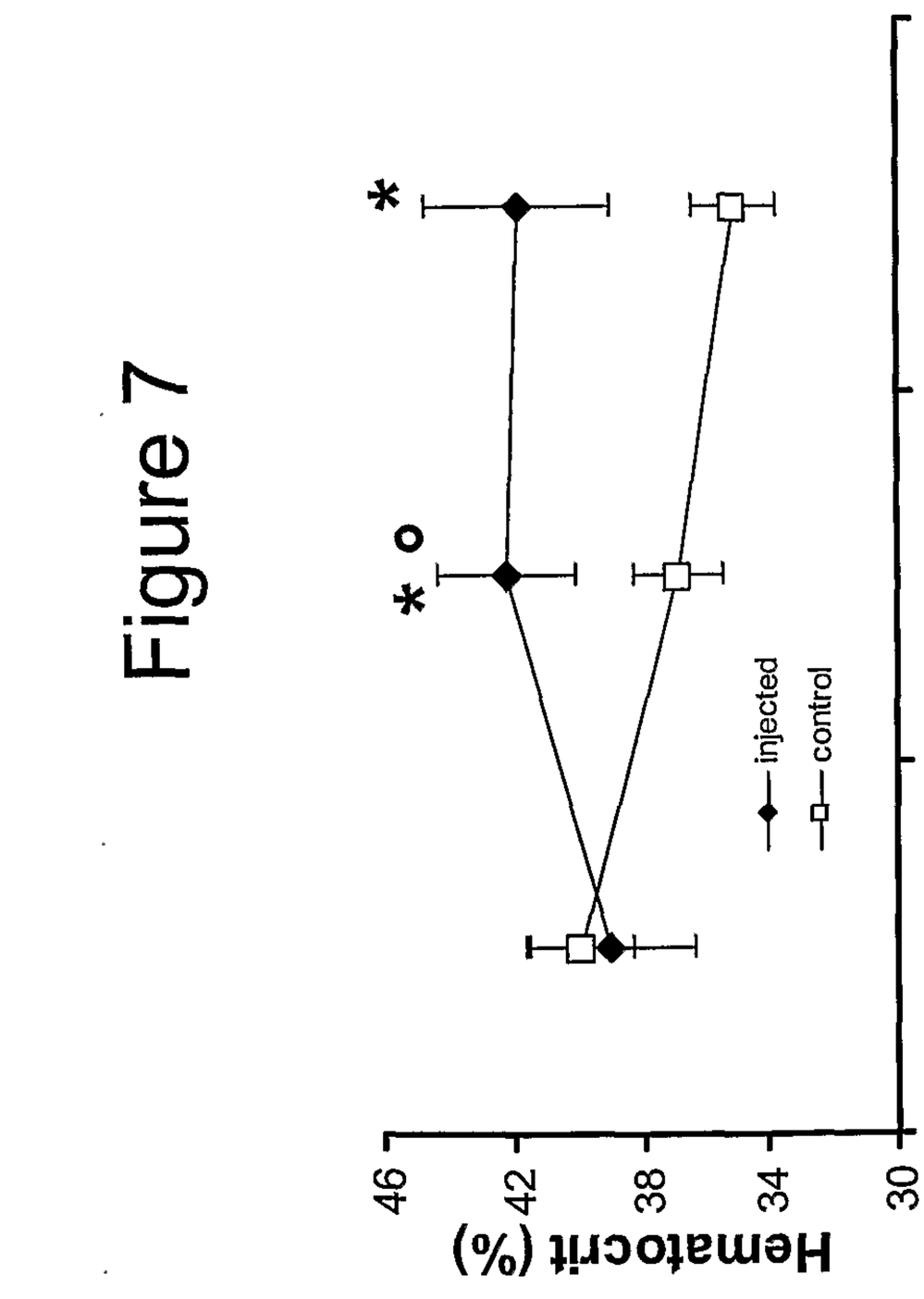
RBC	day 0	day 9-27	day 28-56
injected	5.75 +/- 0.39	6.27 +/- 0.33	6.25 +/- 0.47
control	6.00 +/- 0.24	5.53 +/- 0.19	5.26 +/- 0.19
TTEST pst/pri		0.03	0.06
TTEST pst/ctr		0.03	0.02

Figure 6



Hb	day 0	day 9-27	day 28-56
injected	13.74 +/- 0.97	14.68 +/- 0.78	14.65 +/- 1.03
control	13.71 +/- 0.43	12.94 +/- 0.41	12.44 +/- 0.44
TTEST pst/pri		0.02	0.08
TTEST pst/ctr		0.02	0.02

Figure 7



Ht	day 0	day 9-27	day 28-56
injected	39 +/- 2.62	42.22 +/- 2.17	41.88 +/- 2.86
control	39.92 +/- 1.65	36.88 +/- 1.44	35.12 +/- 1.33
TTEST pst/pri		0.03	0.07
TTEST pst/ctr		0.02	0.01

Figure 8

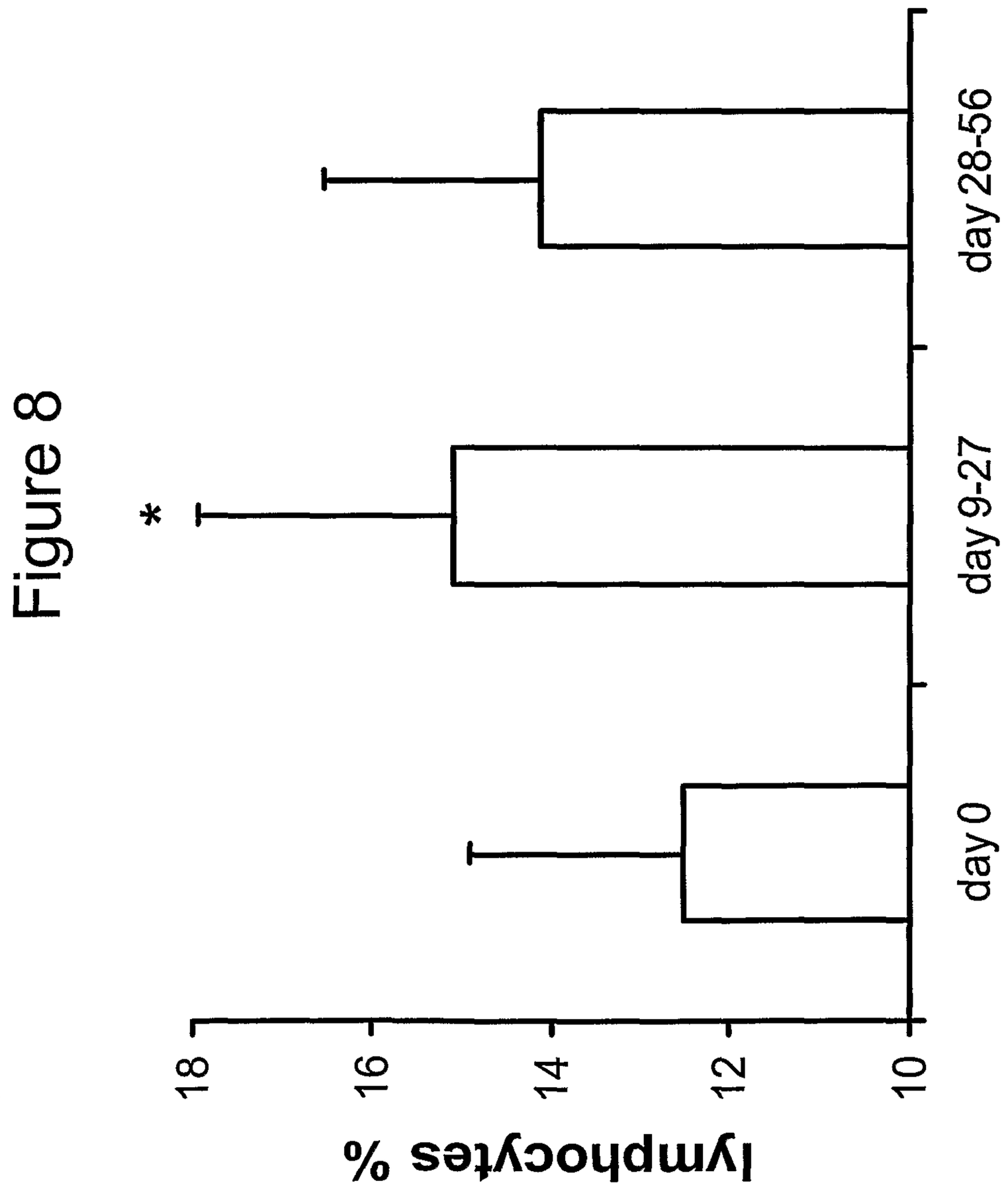


Figure 9

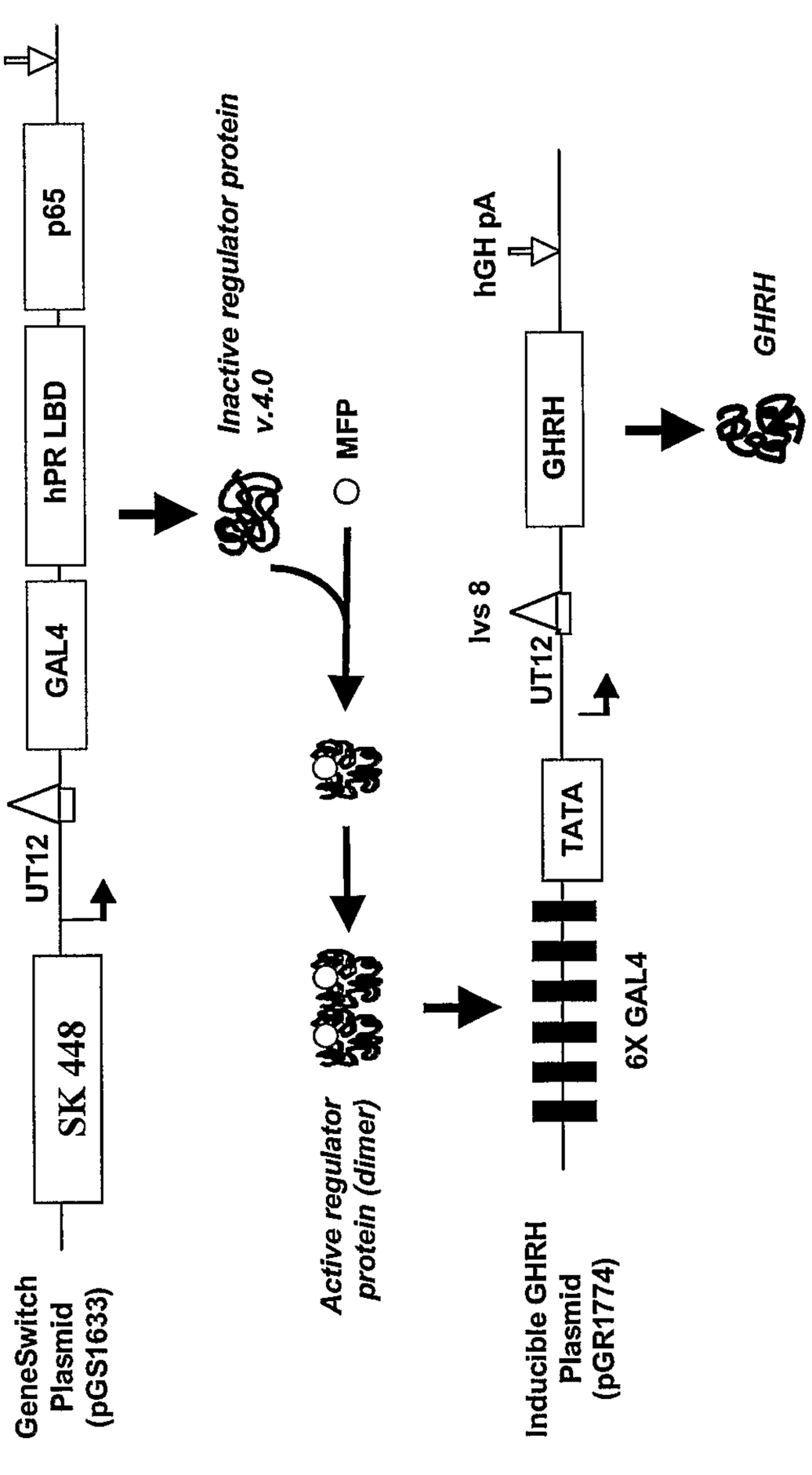
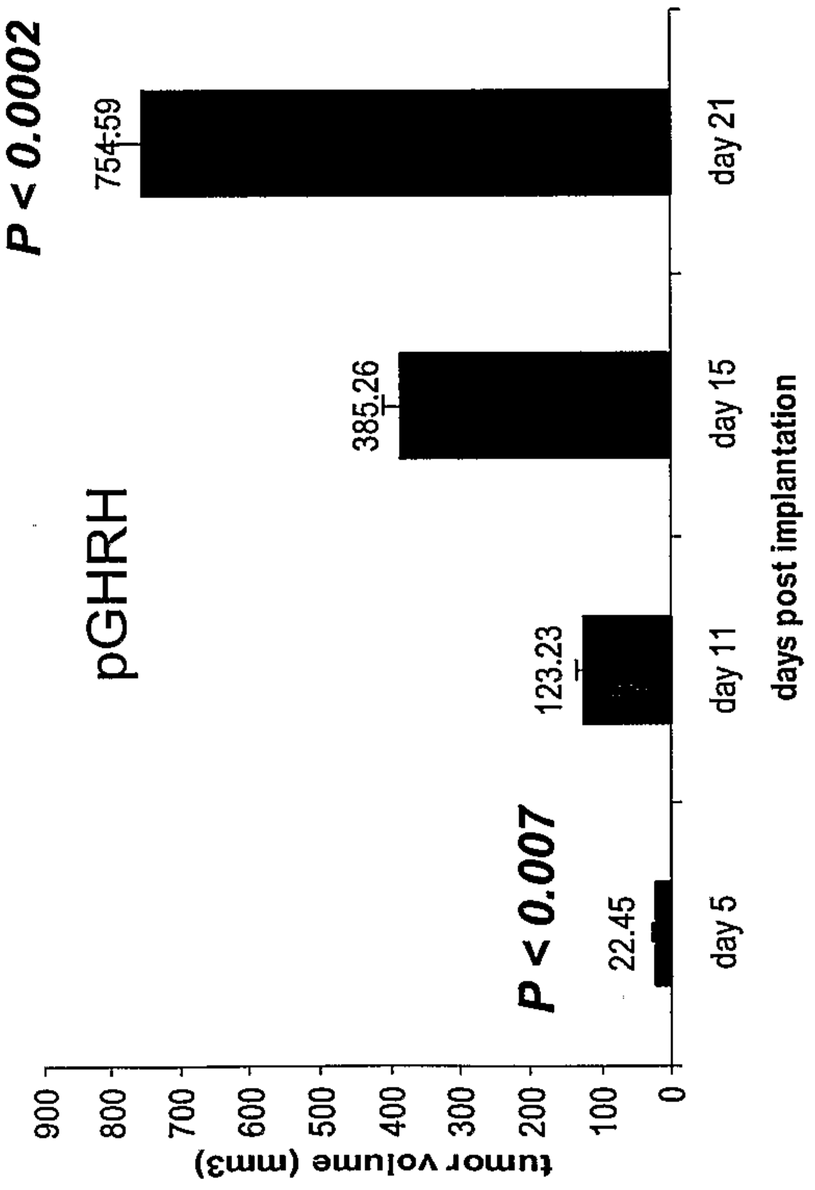

GeneSwitch Plasmid (pGS1633)
lvs 8
UT12
GAL4
hPR LBD
p65
hGH pA
SK 448
Inactive regulator protein v.4.0
MFP
Active regulator protein (dimer)
Inducible GHRH Plasmid (pGR1774)
6X GAL4
TATA
lvs 8
UT12
GHRH
hGH pA
GHRH

Figure 10


pGHRH
P < 0.0002
P < 0.007
tumor volume (mm3)
900
800
700
600
500
400
300
200
100
0
754.59
385.26
123.23
22.45
day 5
day 11
day 15
day 21
days post implantation

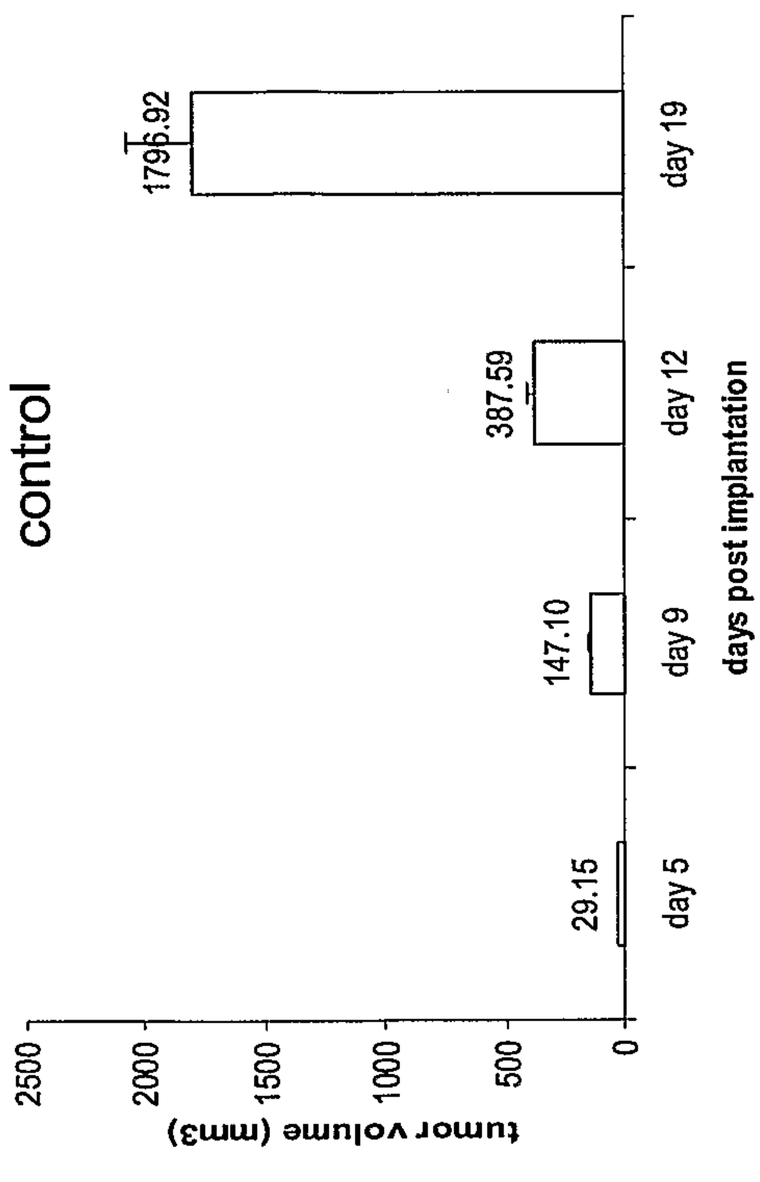

control
tumor volume (mm3)
2500
2000
1500
1000
500
0
1796.92
387.59
147.10
29.15
day 5
day 9
day 12
day 19
days post implantation

Figure 11

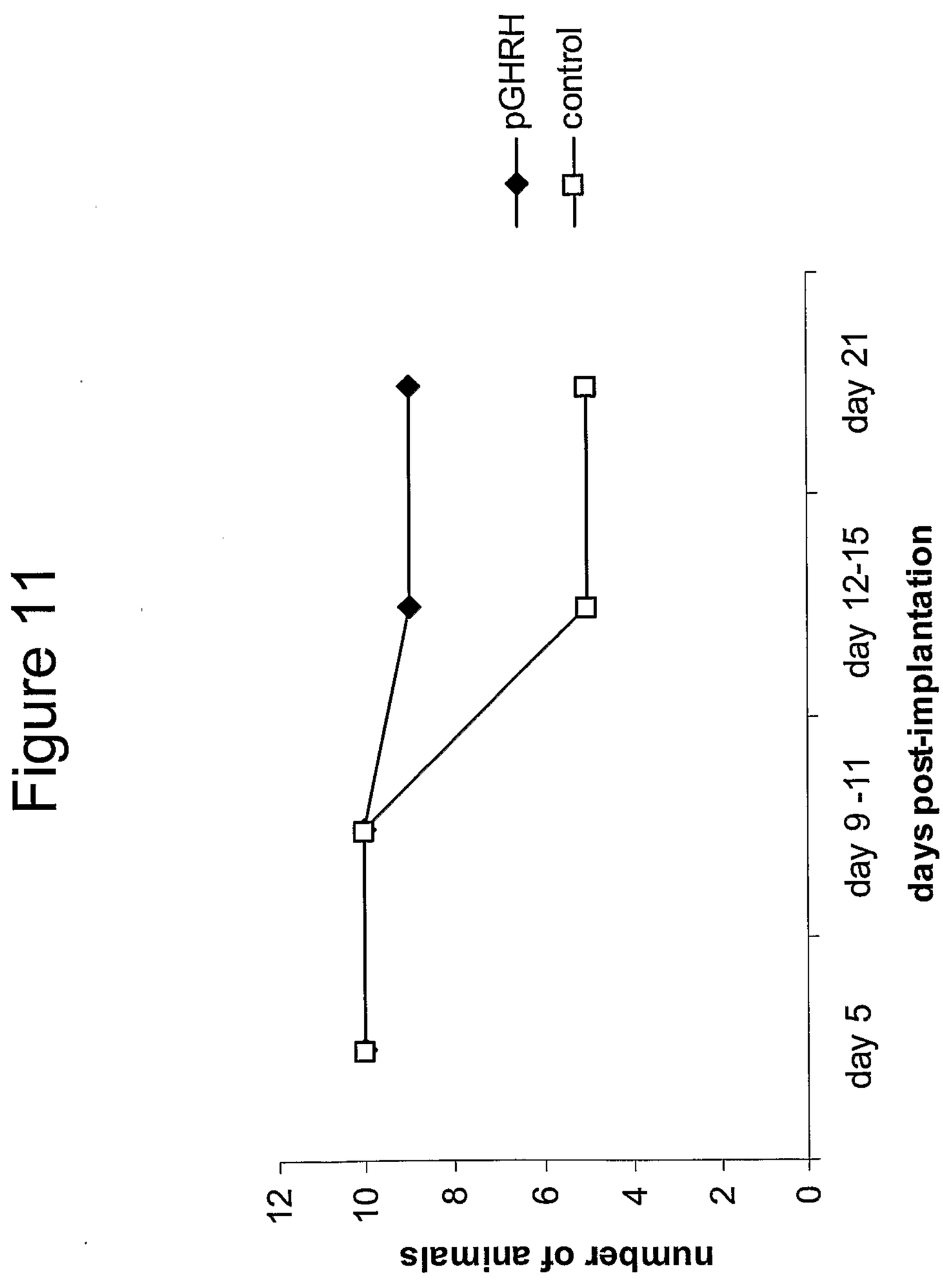

pGHRH
control
day 21
day 12-15
day 9 -11
day 5
days post-implantation
number of animals
0
2
4
6
8
10
12

Figure 12

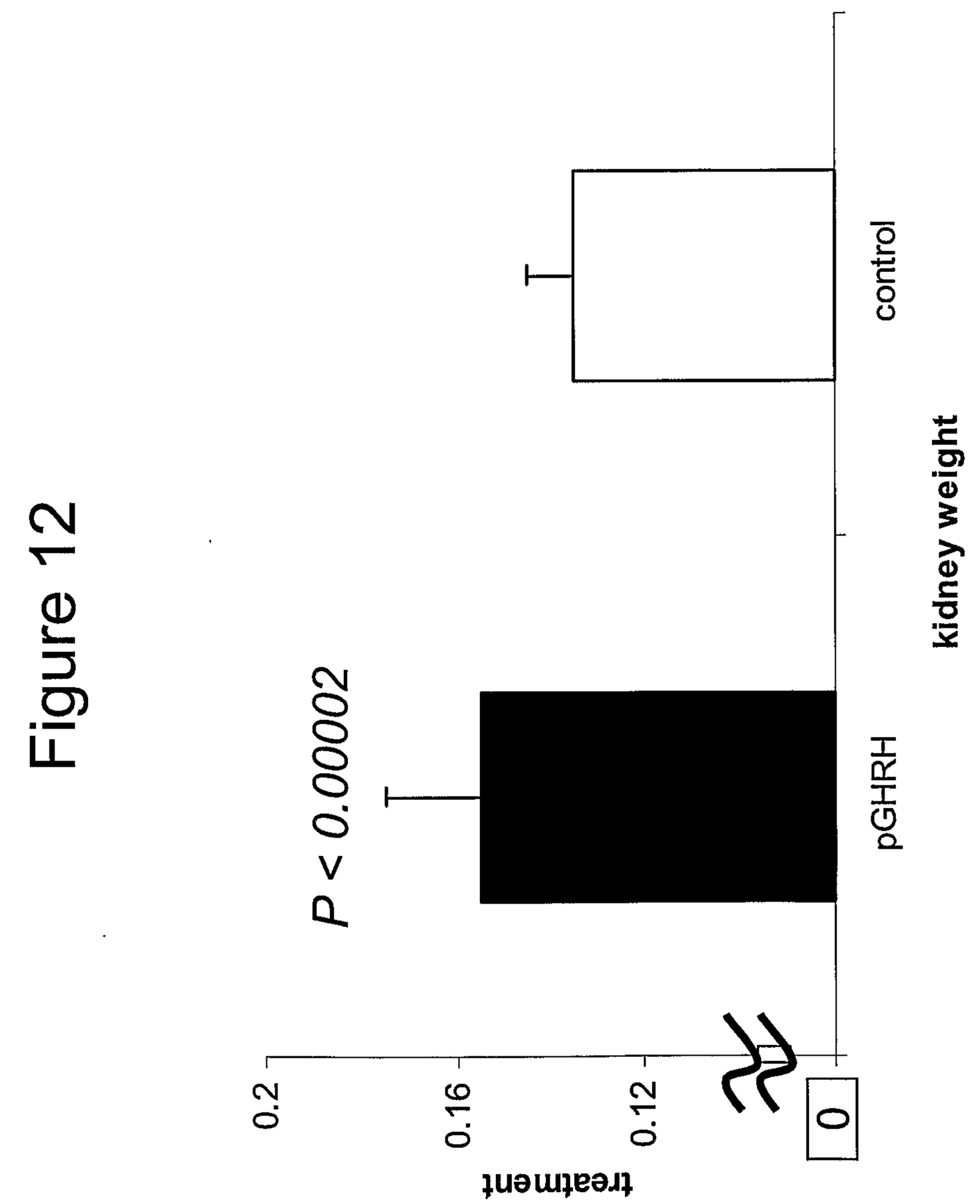
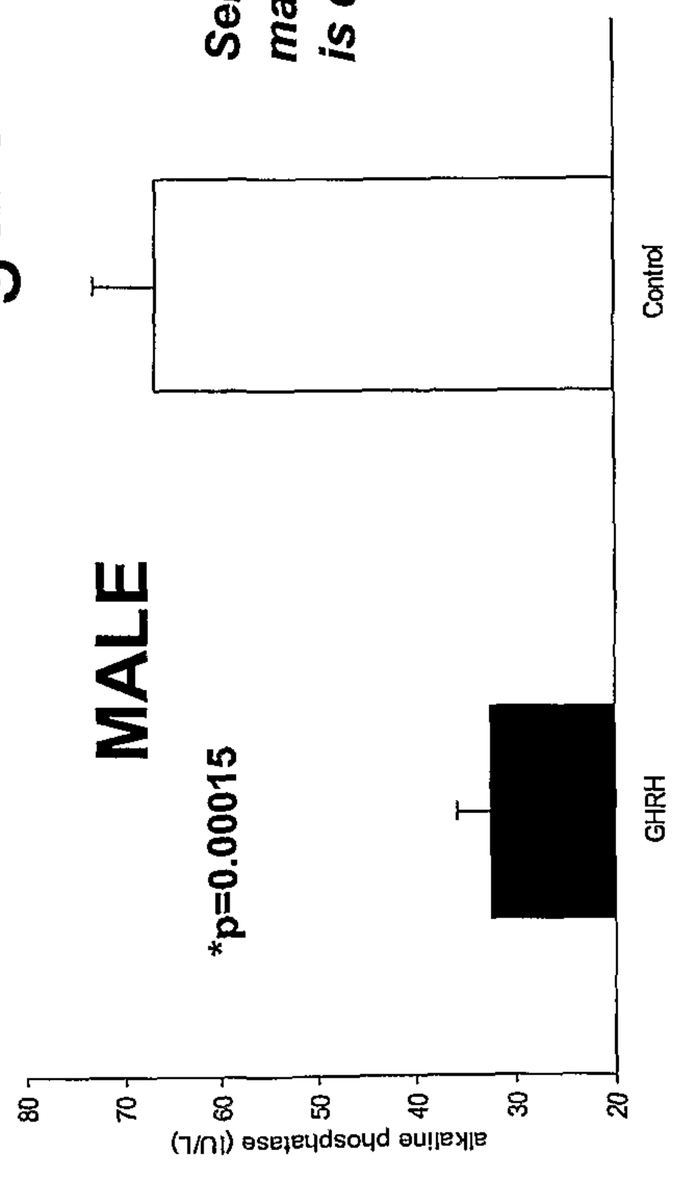


Figure 13

Serum alkaline phosphatase, a marker of neoplastic metastasis is decreased in treated animals.



Treatment with plasmid-mediated GHRH reduces metastasis in male and femaleC57/Bl6 mice with LL tumors

Numerous control animals had lung metastases.

Figure 14

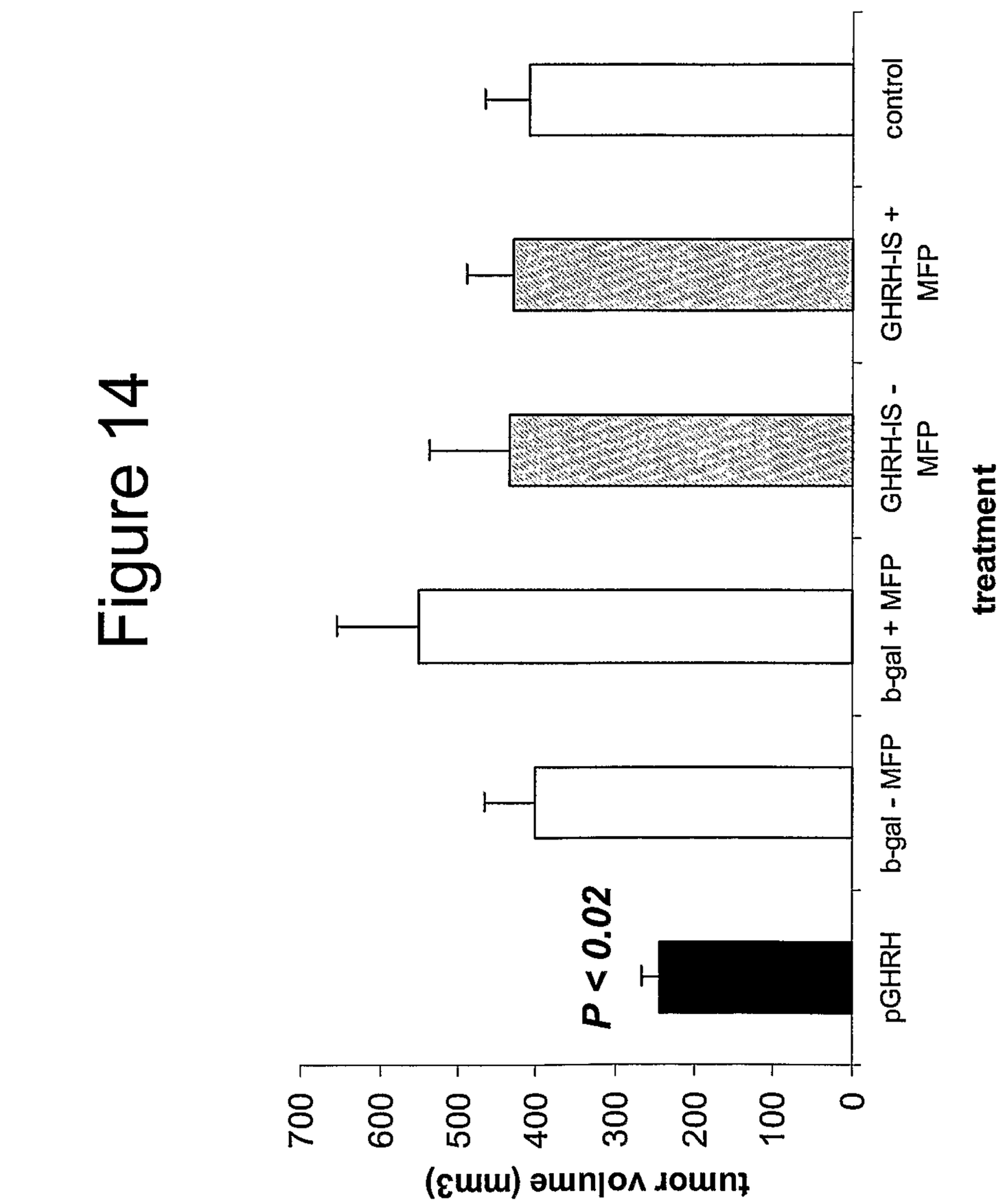

tumor volume (mm3)
0
10
20
30
40
50
60
70
P < 0.02
pGHRH
b-gal - MFP
b-gal + MFP
GHRH-IS - MFP
GHRH-IS + MFP
control
treatment

Figure 15 shows the protein metabolism at 56 days post-injection.

Group I (controls)												
4 Dogs	AST (SGOT)	ALT (SGPT)	T. bilirubin	Alk Phos	GGT	**Tot. Protein**	Albumin	Globulin	A/G Rati	Cholesterol	BUN	Creatinine
Average Value Pre-Injection	19.75	27.75	0.10	43.25	7.00	6.25	3.43	2.83	1.28	164.25	11.75	0.73
Average Value Post-Injection	20.25	29.25	0.10	37.25	2.50	6.23	3.58	2.65	1.40	190.00	14.50	0.68
Average Δ Value	0.50	1.50	0.00	-6.00	-4.50	-0.03	0.15	-0.18	0.13	25.75	2.75	-0.05
% of Δ / average value	2.53	5.41	0.00	-13.87	-64.29	**-0.40**	4.38	-6.19	9.80	15.68	23.40	-6.90
Group II (200 mcg)												
8 Dogs	AST (SGOT)	ALT (SGPT)	T. bilirubin	Alk Phos	GGT	**Tot. Protein**	Albumin	Globulin	A/G Rati	Cholesterol	BUN	Creatinine
Average Value Pre-Injection	25.50	31.75	0.10	32.88	5.75	6.21	3.38	2.84	1.26	161.75	9.88	0.63
Average Value Post-Injection	23.63	31.38	0.11	38.38	4.38	6.44	3.61	2.83	1.39	157.25	11.63	0.70
Average Δ Value	-1.88	-0.38	0.01	5.50	-1.38	0.23	0.24	-0.01	0.13	-4.50	1.75	0.08
% of Δ / average value	-7.35	-1.18	12.50	16.73	-23.91	**3.62**	7.04	-0.44	9.90	-2.78	17.72	12.00
Group III (600 mcg)												
8 Dogs	AST (SGOT)	ALT (SGPT)	T. bilirubin	Alk Phos	GGT	**Tot. Protein**	Albumin	Globulin	A/G Rati	Cholesterol	BUN	Creatinine
Average Value Pre-Injection	24.14	29.43	0.10	33.00	4.75	6.15	3.43	2.73	1.29	172.63	11.88	0.70
Average Value Post-Injection	25.63	29.88	0.10	42.75	4.25	6.31	3.31	2.96	1.28	176.50	13.88	0.69
Average Δ Value	1.48	0.45	0.00	9.75	-0.50	0.16	-0.11	0.24	-0.01	3.88	2.00	-0.01
% of Δ / average value	6.14	1.52	0.00	29.55	-10.53	**2.64**	-3.28	8.72	-0.97	2.24	16.84	-1.79
Group IV (1000 mcg)												
8 Dogs	AST (SGOT)	ALT (SGPT)	T. bilirubin	Alk Phos	GGT	Tot. Protein	Albumin	Globulin	A/G Rati	Cholesterol	BUN	Creatinine
AVERAGE	24.63	29.38	0.10	41.25	6.25	5.98	3.40	2.58	1.33	149.25	11.38	0.69
Average Value Post-Injection	24.75	32.25	0.14	45.88	3.88	5.88	3.58	2.30	1.58	158.25	13.13	0.73
Average Δ Value	0.13	2.88	0.04	4.63	-2.38	-0.10	0.18	-0.28	0.25	9.00	1.75	0.04
% of Δ / average value	0.51	9.79	37.50	11.21	-38.00	-1.67	5.15	-10.68	18.87	6.03	15.38	5.45

Figure 16 shows the blood values in dogs at 56 days post-injection

	Group 1 (control) 4 dogs				Group 2 (200 mcg) 8 dogs				Group 3 (600 mcg) 8 dogs				Group 4 (1000 mcg) 8 dogs		
Blood Values	Ave Pre-Inj	Ave Post-Inj	Ave Δ	%Δ Ave	Ave Pre-Inj	Ave Post-Inj	Ave Δ	%Δ Ave	Ave Pre-Inj	Ave Post-Inj	Ave Δ	%Δ Ave	Ave Pre-Inj	Ave Post-Inj	Ave Δ
WBC	10.88	9.78	-1.10	***-10.1***	10.56	9.90	-0.66	***-6.3***	10.50	13.38	2.88	***27.4***	10.63	11.25	0.63
RBC	6.63	7.60	0.98	***14.7***	6.50	7.56	1.06	***16.3***	6.81	7.30	0.49	***7.2***	7.03	7.39	0.36
HGB	14.90	16.65	1.75	***11.7***	14.20	17.04	2.84	***20.0***	15.10	16.08	0.98	***6.5***	15.63	16.91	1.29
PCV	47.50	53.50	6.00	***12.6***	45.38	52.88	7.50	***16.5***	48.25	51.00	2.75	***5.7***	50.13	52.25	2.13
MCV	72.25	70.75	-1.50	***-2.1***	69.75	70.13	0.38	***0.5***	70.63	69.75	-0.88	***-1.2***	71.13	70.75	-0.38
MCH	22.53	22.03	-0.50	***-2.2***	21.91	22.56	0.65	***3.0***	22.13	21.90	-0.23	***-1.0***	22.23	22.91	0.69
MCHC	31.25	31.25	0.00	***0.0***	31.75	32.38	0.63	***2.0***	31.63	31.38	-0.25	***-0.8***	31.25	32.38	1.13
N (%)	69.25	60.75	-8.50	***-12.3***	66.75	62.88	-3.88	***-5.8***	61.63	65.63	4.00	***6.5***	58.00	62.25	4.25
Lym (%)	20.75	27.50	6.75	***32.5***	24.38	28.00	3.63	***14.9***	24.25	24.63	0.38	***1.5***	33.25	27.00	-6.25
Mono (%)	4.75	4.75	0.00	***0.0***	4.75	3.75	-1.00	***-21.1***	5.50	4.63	-0.88	***-15.9***	3.75	3.50	-0.25
Eos (%)	5.25	7.00	1.75	***33.3***	4.13	5.38	1.25	***30.3***	5.25	5.00	-0.25	***-4.8***	4.88	7.25	2.38
Bas (%)	0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00		0.13	0.00	-0.13
D.BR	0.00	0.00	0.00		0.00	0.01	0.01		0.00	0.01	0.01		0.03	0.03	0.00
LDH	25.75	22.50	-3.25	***-12.6***	47.75	26.50	-21.25	***-44.5***	34.88	24.50	-10.38	***-29.7***	28.00	31.50	3.50
rothrombin Time	7.50	7.70	0.20	***2.7***	8.36	8.34	-0.03	***-0.3***	9.13	8.20	-0.93	***-10.1***	7.93	8.34	0.41
Qnt Plat	118.25	259.75	141.50	***119.7***	140.50	263.50	123.00	***87.5***	239.25	291.25	52.00	***21.7***	271.25	283.88	12.63
Glucose	123.00	110.75	-12.25	***-10.0***	114.25	108.88	-5.38	***-4.7***	119.50	112.13	-7.38	***-6.2***	113.50	109.63	-3.88
Amylase	894.50	819.25	-75.25	***-8.4***	885.50	887.50	2.00	***0.2***	806.50	874.38	67.88	***8.4***	836.63	898.75	62.13
Lipase	218.50	213.00	-5.50	***-2.5***	198.00	224.88	26.88	***13.6***	315.50	262.38	-53.13	***-16.8***	217.00	223.13	6.13
Na	146.75	145.75	-1.00	***-0.7***	146.00	145.00	-1.00	***-0.7***	146.25	145.00	-1.25	***-0.9***	146.00	145.88	-0.13
K	4.33	4.25	-0.08	***-1.7***	4.11	4.09	-0.02	***-0.6***	4.48	4.25	-0.23	***-5.0***	4.38	4.26	-0.11
Na/K Ratio	34.25	34.75	0.50	***1.5***	35.63	35.50	-0.13	***-0.4***	32.75	34.25	1.50	***4.6***	33.50	34.25	0.75
Cl-	110.75	108.00	-2.75	***-2.5***	111.13	107.25	-3.88	***-3.5***	111.25	108.13	-3.13	***-2.8***	110.50	109.00	-1.50
CPK	91.50	125.75	34.25	***37.4***	178.13	119.75	-58.38	***-32.8***	180.50	143.25	-37.25	***-20.6***	128.88	147.75	18.88
TGR	34.25	41.25	7.00	***20.4***	33.38	40.00	6.63	***19.9***	31.50	38.63	7.13	***22.6***	34.75	42.00	7.25
Osmolality, Calc.	304.50	302.75	-1.75	***-0.6***	301.88	300.13	-1.75	***-0.6***	303.25	301.38	-1.88	***-0.6***	302.38	302.63	0.25
Corr. Ca	10.00	10.15	0.15	***1.5***	10.03	10.70	0.67	***6.6***	10.00	10.38	0.38	***3.8***	9.93	10.25	0.32
Mg	1.48	1.48	0.00	***0.0***	1.56	1.50	-0.06	***-4.0***	1.50	1.46	-0.04	***-2.5***	1.51	1.41	-0.10

WO 03/049700 PCT/US02/39509

CA 02469310 2004-06-03

Figure 17 shows the bone metabolism in dogs at 56 days post-injection.

Group I (controls)				
4 Dogs	Phosphorus	Calcium	Ca/PO4 Ratio	
Average Value Pre-Injection	4.25	9.83	2.33	
Average Value Post-Injection	4.53	10.38	2.29	
Average Δ Value	0.28	0.55	-0.03	
% of Δ / average value	6.47	5.60	-1.38	
Group II (200 mcg)				
8 Dogs	Phosphorus	Calcium	**Ca/PO4 Ratio**	
Average Value Pre-Injection	4.25	9.83	2.39	
Average Value Post-Injection	4.14	10.40	2.51	
Average Δ Value	-0.11	0.58	0.13	
% of Δ / average value	-2.65	5.85	**5.28**	
Group III (600 mcg)				
8 Dogs	Phosphorus	Calcium	**Ca/PO4 Ratio**	
Average Value Pre-Injection	4.44	10.05	2.29	
Average Value Post-Injection	3.99	10.20	2.56	
Average Δ Value	-0.45	0.15	0.27	
% of Δ / average value	-10.14	1.49	**11.82**	
Group IV (1000 mcg)				
8 Dogs	Phosphorus	Calcium	**Ca/PO4 Ratio**	
AVERAGE	4.83	9.91	2.11	
Average Value Post-Injection	3.74	10.16	2.72	
Average Δ Value	-1.09	0.25	0.61	
% of Δ / average value	-22.54	2.52	**28.71**	

Figure 18 shows a diagnosis specific therapy and survival for dogs

Group	Treatment	Drug		Dose	# Dogs	Cancer Type	# Dogs	Days Survived
Controls	Radiation		GC1	80 Rads (26 visits)	1	Carcinoma	1	254
	Chemotherapy	Vincristine	GC2	0.5-0.65 (mg/M^2)	1	MCT	1	182
		Adriamycin	GC3	30 (mg/M^2)	1	Sarcoma	1	178
		Carboplatin	GC4	90 (mg/M^2)	1	Melanoma	1	133*
		CCNU	GC5	65 (mg/M$_2$)	1	Lymphoma	1	180
		Vin/Cyt	GC6/GC7/GC8/GC9	0.4-0.7 / 200-300 (mg/M^2)	6	Lymphoma	4	104/104/104/189*
			GC10			Sarcoma	1	167
			GC11			Leukemia	1	275
		Vin/Adr	GC12	0.5-0.6 / 30 (mg/M^2)	1	Sarcoma	1	49*
			GC13	0.5-0.6 / 30 (mg/M^2)	1	Lymphoma	1	130
		Cyt/Adr	GC14	? / 30 (mg/M^2)	1	Sarcoma	1	171
		Vin/Cyt/Adr	GC15	0.5-0.6 / 200 / 30 (mg/M^2)	2	Lymphoma	1	180
			GC16			Sarcoma	1	147*
	Rad./ Chemo.	Vincristine/ CCNU	GC17	0.5-0.6 / 60 (mg/M^2) 80 Rads (18 visits)	1	MCT	1	178*
		Adriamycin	GC18	15-30 (mg/M^2) 80 Rads (19 visits)	1	Sarcoma	1	106
		Carboplatin	GC19	90 (mg/M^2) 80 Rads (6 visits)	1	Melanoma	1	258
Experimental	Radiation		GE1/GE2	80 Rads (6-21 visits)	6	Sarcoma	2	167 - 167
			GE3			MCT	1	251
			GE4			Adenoma	1	251
			GE5/GE6			Solid Tumor	2	174 -- 124*
	Chemotherapy	Cytoxin	GE7	350 (mg/M^2)	1	None "Lung Nodules"		72*
		Vin/Cyt/Adr/Car	GE8	0.75 / 300 / 15-30 / 200 (mg/M^2)	1	Sarcoma	1	167
	Rad./ Chemo.	Vin/Cyt/Adr	GE9	0.6-0.7 / 200-300 / 30 (mg/M^2) 80 Rads (2-3 visits)	1	Lymphoma	1	67*
		Vin/Cyt	GE10	0.6 / 200 (mg/M^2) 80 Rads (3 visits)	1	Adenoma	1	83
	Surgical Removal		GE11		1	Solid Tumor	1	97

Figure 19 shows the blood values for dogs with cancer.

Experimental - injected dogs	WBC/HPF	RBC/HPF	HGB	PCV (%)	MCV	MCH	MCHC	Neutrophils	Lymphs (%	Monos (%)	EOS (%)	Basophils
Average Value Pre-Injection	7.97	5.64	13.29	38.29	67.86	23.50	34.86	78.00	13.00	7.43	1.67	1.00
Average Value Post-Injection	7.04	**6.59**	**15.11**	**43.88**	66.75	23.01	34.50	77.13	15.38	5.63	2.60	1.00
Average Δ Value	-1.89	1.03	2.06	6.29	-0.86	-0.41	-0.29	-1.71	3.71	-1.86	0.67	0.00
% of Δ / average value	*-23.66*	*18.23*	*15.48*	*16.42*	-1.26	-1.76	-0.82	-2.20	28.57	-25.00	40.00	0.00
TTEST	**0.04**	**0.01**	**0.02**	**0.02**	0.77	0.27	0.05	0.21	0.35	0.29	0.98	
Controls - non-injected dogs	WBC/HPF	RBC/HPF	HGB	PCV (%)	MCV	MCH	MCHC	Neutrophils	Lymphs (%	Monos (%)	EOS (%)	Basophils
Average Value	12.72	5.72	13.26	38.00	66.40	23.36	35.22	71.91	18.80	6.91	2.76	0.32
Average Δ Value	-1.40	-0.46	-0.91	-1.92	-0.08	0.17	0.44	-3.70	3.43	0.58	0.16	0.04
% of Δ / average value	-11.02	*-8.10*	*-6.82*	*-5.06*	-0.12	0.74	1.24	-5.15	18.24	8.33	5.92	10.89

CA 02469310 2004-06-03

Figure 20 shows the blood values for old healthy dogs

Gulf Coast-Non-cancer/Experimental									
4 Dogs	RBC	HGB	WBC	Lymphocy	T. Protein	Albumin	Globulin	A/G Ratio	Cholesterol
Average Value Pre-Injection	6.53	15.90	8.55	18.50	6.30	3.50	2.80	1.28	247.00
Average Value Post-Injection	6.73	16.33	8.80	14.50	6.50	3.40	3.10	1.10	264.75
Δ Value	0.20	0.42	0.25	-4.00	0.20	-0.10	0.30	-0.18	17.75
% of Δ / average value	**0.03**	**0.03**	**0.03**	**-0.28**	**0.03**	**-0.03**	**0.10**	**-0.16**	**0.07**

Gulf Coast-Non-cancer/Experimental							
4 Dogs	BUN	Creatinine	BUN/Creat	Phosphor	Calcium	Ca/PO4 R	Glucose
Average Value Pre-Inje	13.25	0.73	19.50	4.20	10.65	2.54	110.00
Average Value Post-Inj	12.00	0.83	14.50	3.73	10.78	2.89	104.25
Δ Value	-1.25	0.10	-5.00	-0.48	0.13	0.13	-5.75
% of Δ / average value	**-0.10**	**0.12**	**-0.34**	**-0.13**	**0.01**	**0.04**	**-0.06**