Plasmid-based growth hormone-releasing hormone supplementation and its applications

Amir S Khan, Patricia A Brown & Ruxandra Draghia-Akli*

Address
ADViSYS Inc
2700 Research Forest Drive
Suite 180
The Woodlands
TX 77381
USA
Email: ruxandradraghia@advisys.net

***To whom correspondence should be addressed**

Current Opinion in Molecular Therapeutics 2005 **7**(4):306-316
 ISSN 1464-8431

A single dose of a plasmid expressing growth hormone-releasing hormone (GHRH) has been safely used in a number of animal species and applications to physiologically increase growth hormone and insulin-like growth factor-I for over a year. An array of constructs encoding for analogs of, or species-specific, GHRH has been tested to treat anemia and cachexia associated with cancer and its treatment, and renal failure, as well as to increase immune surveillance and animal welfare. The positive results obtained with plasmid-based GHRH in companion and farm animals may be translated to a number of human applications.

Keywords Gene transfer, growth hormone, growth hormone-releasing hormone, insulin-like growth factor-I, plasmid

Introduction

Growth hormone-releasing hormone (GHRH) is a hypothalamic hormone that stimulates the synthesis and secretion of growth hormone (GH) from the anterior pituitary [1]. The administration of recombinant GH (rGH) and GHRH has been studied extensively as a means to enhance or restore growth in humans [2] or to supplement inadequate endogenous levels in GH-deficient patients [3,4]. In a variety of other mammalian species, rGH administration has been used to improve the efficiency of dietary nitrogen utilization, while promoting increases in lean body mass, protein accretion and milk production [5,6]. Nevertheless, the widespread application of rGH or GHRH in the US for animal use has been limited by both economic and feasibility considerations. It is costly and labor intensive, as its short serum half-life necessitates frequent administration [7]. The non-physiological hormonal peaks and troughs that follow rGH injections can result in impaired glucose tolerance and insulin resistance [8]. Moreover, biological responses to exogenous rGH may not be similar to physiological responses to the endogenous isoforms of this protein [9]. While virtually no adverse effects have been reported with GHRH administration, the hormone has an even shorter half-life in serum (6 to 17 min, depending on the assay conditions) than rGH [10•,11]. Efforts were made to increase the half-life of both the GH [12] and GHRH peptide [13,14], but these analog molecules are not widely used therapeutically.

Therefore, the feasibility of employing a plasmid-mediated GHRH approach to ensure physiological levels of GH in both normal and pathological situations has been explored. In this review, the benefits of plasmid transfer technology are examined, the diverse physiological effects of GHRH plasmid administration (Figure 1) on various animal species summarized, and the applications of this technology discussed.

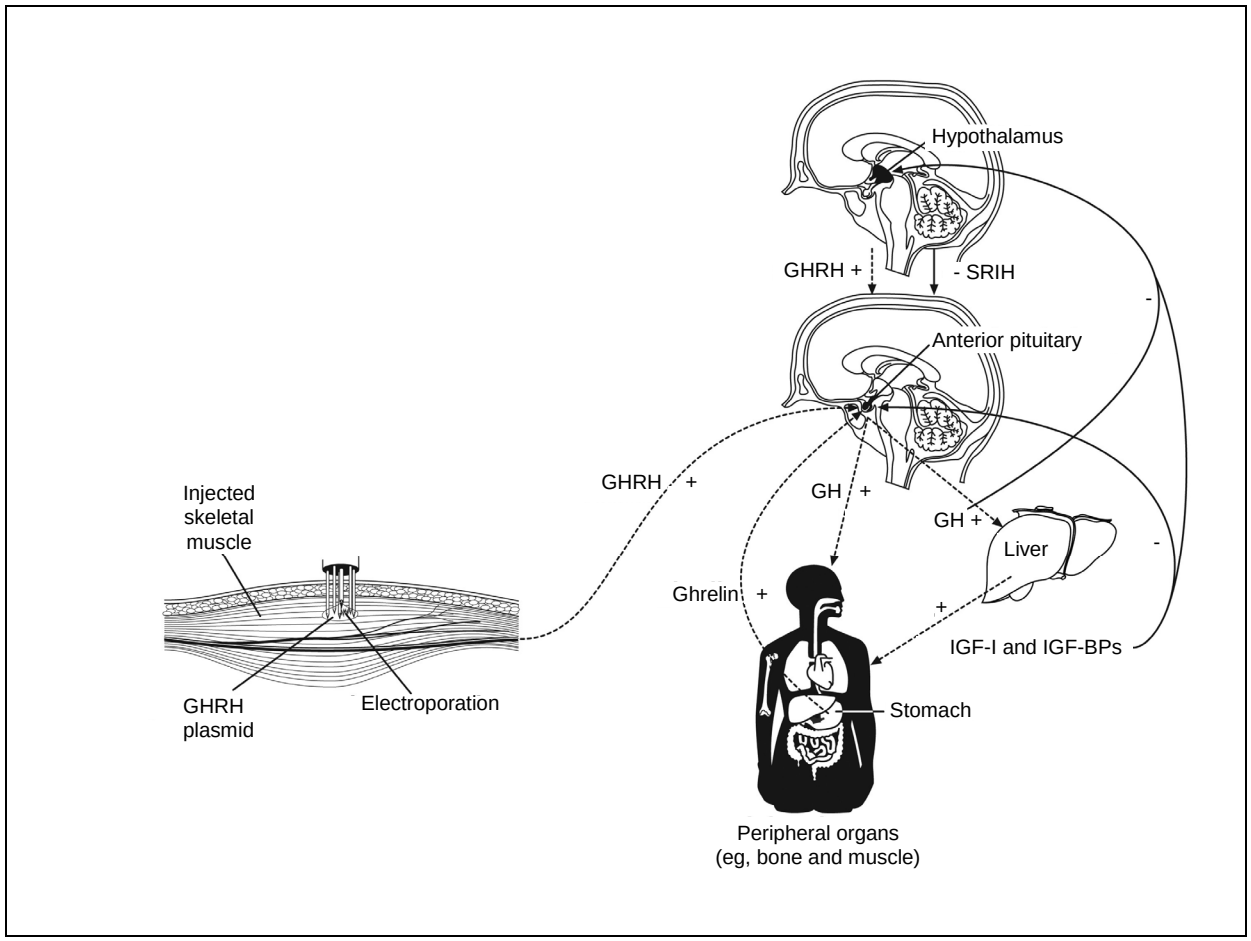
Plasmid delivery technology

Most gene therapy studies have used viral vectors or liposome-based complexes to transfer genes of interest into a subject. Thus, a number of disadvantages have been identified, including severe adverse effects to the vector system itself and costly and complicated manufacturing requirements. In contrast, plasmids are simple to construct and easy to manufacture at relatively low cost, using good manufacturing practice (GMP) techniques that meet regulatory standards. They have a low risk-to-benefit ratio when compared with other delivery methods [15,16], as previously summarized by the Food and Drug Administration's Center for Biologics Evaluation and Research [17••]. Furthermore, plasmids can be efficiently taken up by different cell types and are expressed for various lengths of time, depending on the turnover rate of the cells and their inherent level of nuclease activity [18,19]. One historical concern was that the plasmid DNA would integrate into the recipient host's chromosomes. Numerous animal studies involving plasmid DNA injections have shown that this is not the case. A potential integration event would be extremely infrequent, approximately 3000-fold lower than the spontaneous mutation rate for mammalian genomes [20,21].

Typically, a plasmid contains a backbone with elements necessary for replication and selection (plasmid production), and an expression cassette (Figure 2). The components of the expression cassette are the promoter, signal peptide sequence, transgene, 3'-untranslated region and polyadenylation signal. These are crucial to determine the length and characteristics of expression, including gene silencing [22], and to transgene product localization [23,24]. The promoter directs plasmid expression, and specificity of the promoter typically controls the duration, characteristics and amplitude of expression. For example, a transgene preceded by a cytomegalovirus (CMV) promoter will express in any tissue, whereas a tissue-specific promoter [25] will express only in the target tissue. Several research groups, including ours have been investigating plasmid expression systems that can be regulated [26•,27], and may be more suitable for use in some patients.

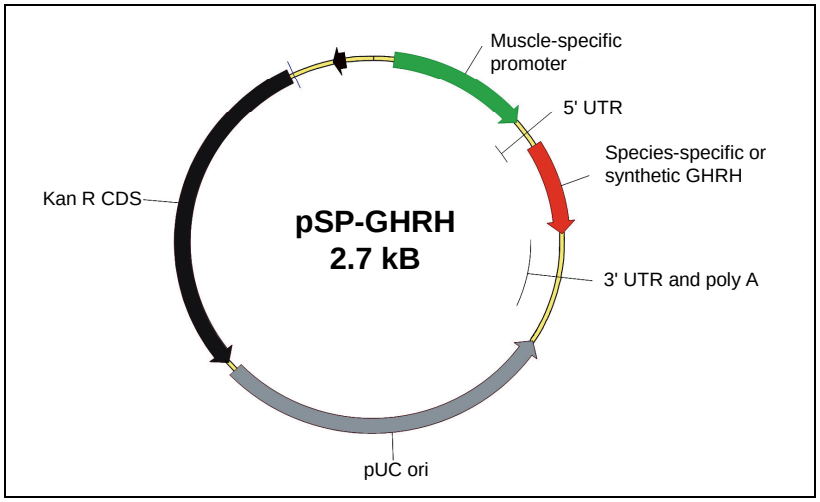
Cis-acting DNA elements within and outside of the plasmid backbone can impact on the length and amplitude of expression [28], thus analysis of the target tissue and tissue-specific transcription factors is imperative when designing plasmids for therapeutic applications [25]. These findings have to be integrated with understanding about the kinetic expression of the transgene, which can be variable depending on many factors, including cellular localization of the protein, physiological activity and regulation [16]. The

Figure 1. The GHRH-GH-insulin-like growth factor-I axis.



An illustration of the GHRH-GH-IGF-I axis and the contribution of plasmid-mediated GHRH supplementation, including target organs and negative feed-back relationships. **GH** growth hormone, **GHRH** growth hormone-releasing hormone, **IGF-BP** insulin-like growth factor-I binding protein, **IGF-I** insulin-like growth factor-I, **SRIH** somatostatin.

Figure 2. Map of a generic 2.7-kb myogenic plasmid expressing a species-specific, or analog, GHRH.



The components of the myogenic plasmid synthetic promoter (**pSP**)-GHRH include, a muscle-specific promoter, preferably a synthetic promoter such as SPc5-12 [25]; a 5' untranslated region (**UTR**); either a species-specific or analog GHRH cDNA sequence, including the species-specific or synthetic signal peptide cDNA sequence; a 3' UTR and polyadenylation (**poly A**) region; a plasmid backbone that includes an optimized origin of replication (**pUC ori**) for increased plasmid replication and yields; and a kanamycin resistance gene (Kan R CDS) for plasmid production purposes.

elements contained within the plasmid described in Figure 2 were optimized for long-term mammalian expression of GHRH or other hormones and small peptides.

Target tissue: Skeletal muscle

Skeletal muscle fibers are especially suitable for plasmid delivery because they are post-mitotic cells and have the capacity to produce and secrete a multitude of proteins into the circulation. Although muscle is not regarded as a secretory tissue, in many cases, gene products enter the systemic circulation. Therefore, it is possible to use this approach to alter levels of endocrine and paracrine factors [29]. A previous limitation of plasmid administration was low transfection efficiency into normal, mature muscle. A variety of techniques have been employed to improve muscle transfection, such as localized muscle damage followed by regeneration, hydrodynamic methods and electroporation. The latter technique increases uptake and, consequently, expression at 100- to 1000-fold versus direct injection. Intramuscular injection of plasmid followed by electroporation has been used successfully in mice [23,30,31], rats [32,33], dogs [34], pigs [35] and cattle [36•] to deliver therapeutic genes that encode for a variety of hormones, cytokines or enzymes. Additionally, the technique has been used successfully to vaccinate ruminants [37•,38].

The efficiency of peptide production and secretion is also influenced by the structure, fiber type and vascularization of the muscle into which the plasmid is administered. To evaluate this efficiency, we used a reporter plasmid that expresses secreted embryonic alkaline phosphatase (SEAP) under the control of a muscle-specific synthetic promoter (pSP). This approach was chosen because SEAP is a reporter that is not present in the plasma of adult mammals, is secreted efficiently into the circulation and specific enzyme activity can be detected as early as 24 h after transfection by a sensitive chemiluminescence-based assay [39]. Large muscles, such as the longissimus dorsi, semitendinosus or sternocranialis, have similar high levels of SEAP expression, while small, flat muscles, such as the masseter, secrete relatively lower quantities [40]. Thus, in all the studies described, we used the larger muscles for the administration of myogenic GHRH-expressing plasmids.

Growth hormone-releasing hormone

GHRH administration increases GH secretion and augments insulin-like growth factor-I (IGF-I) levels proportional to the GHRH dose [41]. Feedback regulation inherent within the GHRH axis ensures the normal production and secretion of hormones (Figure 1). Most importantly, GH secretion is affected at low levels of GHRH (~ 100 pg/ml) in the blood [42,43]. Plasmid-mediated GHRH stimulates the production of GH at physiological levels, as demonstrated in directly treated animals by the lack of short- or long-term systemic adverse effects, such as cardiac hypertrophy, insulin resistance, organomegaly or acromegaly [44-47].

Plasmid-mediated therapy is facilitated by the previous characterization of the cDNAs encoding for GHRH from humans, pigs and other animals [48•,49]. Additionally, the feline and canine GHRH have been cloned [50]. The GHRH gene typically includes five exons [51]: exon 1 includes the 5' non-translated sequence, while exons 2 through 5 encode for the GHRH precursor protein. Pre-pro-hormones are processed post-translationally and two mature protein species are formed: GHRH (1-40) with a carboxyl terminal group and GHRH (1-44) with a carboxyl terminal amide group. The 40-hydroxy and 44-amide isoforms have slightly different half-lives and respond differently to protease degradation [11]. Unlike humans, in most animal species, only the (1-44) isoform has been identified. Part of exon 2, all of exon 3 and part of exon 4 encode for the 31 amino-acid signal peptide (Met to Arg-Arg) and the entire mature peptide (Tyr^{1} to Leu^{44}). As the cellular mechanisms that determine amidation may not be always present in muscle cells, the plasmids used in all our clinical studies encode for a 40-hydroxy form of GHRH. The translation of some species-specific and long half-life analog GHRHs, including their signal peptides, is depicted in Figure 3.

Figure 3. GHRH signal peptide and mature 40-hydroxy forms of mammalian species and consensus amino acid sequences.

Translation of GHRH species:

Human GHRH	(1)	MPLWVFFFVILTLSNSSHCSPPPPLTLRMRRYADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGA ---
Porcine GHRH	(1)	MVLWVFFFVILTLSNSSHCSPPPPLTLRMRRYADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGA ---
Cat GHRH	(1)	MVLWVFFLVILTLDSGSHCSPPS-LPLRMPRYADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGA ---
Dog GHRH	(1)	MVLWVFFLVILTLSSGSHSSPPS-LPIRIPRYADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNREQGA ---
HV-GHRH	(1)	MVLWVFFFVILTLSNSSHCSPPPPLTLRMRRHVDAIFTNSYRKVLAQLSARKLLQDILNRQQGERNQEQGA ---
Bovine GHRH	(1)	MVLWVFFLVTLTLSSGSHGSLPS-QPLRIPRYADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGA
Ovine GHRH	(1)	MVLWVFFLVTLTLSSGSHGSLPS-QPLRIPRYADAIFTNSYRKILGQLSARKLLQDIMNRQQGERNQEQGA
Horse GHRH*	(1)	MVLWVFFFVILTLSNSSHCSPPPPLTLRMRRYADAIFTNNYRKVLGQLSARKILQDIMSR----------
Consensus	(1)	MVLWVFF VILTLSSGSHCSPP LPLRM RYADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGA

GHRH growth hormone releasing hormone, **HV-GHRH** A plasmid expressing GHRH with a long half-life. *The horse GHRH sequence is partial.

Directly treated animals: Therapeutic indications

Anemia and cachexia: Cancer

Cachexia is a frequent complication of late-stage malignancy and other chronic diseases. Its development in cancer patients often precludes further therapy and ultimately contributes to nearly a third of all cancer deaths [52,53]. Patients with cachexia are catabolic, exhibiting an increased breakdown of proteins, decreased protein synthesis, a negative nitrogen balance and increased basal energy expenditure. The release of cytokines such as tumor necrosis factor (TNF)α and interleukins-1 and -6 mediate wasting by suppressing muscle gene products [54,55], further contributing to this condition. Preclinical studies in rodents have suggested that anabolic hormones such as GH, IGF-I, IGF binding protein 3 (IGFBP-3) [56-58] or ghrelin [59] may reverse the catabolic state associated with cachexias of different etiology, but for logistical reasons this strategy has not been tested in large animals with spontaneously occurring tumors.

A single injection of GHRH-expressing plasmid into skeletal muscle can ameliorate the anemia and cachexia in animals with spontaneous malignancies or experimentally implanted tumors [35,60]. A plasmid expressing a GHRH analog with a long half-life, HV-GHRH, was used in an initial study in companion animals [35]. Animals received a single injection of 0.1 mg/kg in up to 1 mg of plasmid. All dogs enrolled in this study, including controls, were also on specific chemotherapy and/or radiotherapy. In severely debilitated companion dogs with naturally occurring tumors [60], at a median of 16 days after intramuscular delivery of the plasmid, serum concentrations of IGF-I were increased from 21 to 120% (median = 49%) of the pre-treatment values and were generally sustained or higher on the final evaluation performed at day 56 of the study. Anemia resolved post-treatment, as indicated by significant increases in mean red blood cell count, hematocrit and hemoglobin concentrations. There was also a significant rise in the percentage of circulating lymphocytes. Treated dogs maintained their weights over the 56-day study and did not show any adverse effects from the GHRH plasmid therapy. Interestingly, of the treated dogs, three had detectable baseline TNFα levels that had decreased by 40% at 56 days post-treatment. These observations demonstrate the feasibility of GHRH plasmid therapy to stimulate the GH axis in large animals, leading to anabolic responses and the correction of anemia and other catabolic processes associated with cancer cachexia. Furthermore, beneficial effects on the quality-of-life, increases in weight, activity level, exercise tolerance, appetite and hematological parameters of the treated dogs persisted for more than a year post-treatment [45].

One ongoing concern is that overstimulation of the GHRH-GH-IGF-I pathway with GHRH while treating cancer cachexia would also enhance tumor cell growth [61]. Thus, experiments were undertaken to evaluate the role of GHRH in tumorigenesis using murine- or human-derived experimental tumors in the absence of a specific antitumor therapy. Lewis lung adenocarcinoma cells were implanted into the left flank of male and female immunocompetent mice [62]. Mice were injected intramuscularly 1 day later with 20 μg of a plasmid expressing human GHRH(1-40)OH. Controls received an *Escherichia coli* β-galactosidase-expressing plasmid. A delay in tumor growth and a reduction in the number of metastases compared with control animals were observed, despite a 13% increase in the serum levels of IGF-I relative to controls. Male and female mice exhibited sexual dimorphism with respect to tumor growth and number of metastases using a variety of tumor types. The gender-specific neuroendocrine regulation of the GHRH axis in mice is largely unknown, and studies addressing this issue are ongoing. In humans with protracted critical illness, a gender dissociation within the axis is evident, with men showing greater loss of pulsatility within the GH secretory pattern than women, and concomitantly lower IGF-I levels [63]; as a clinical consequence, females appear to be in part protected against adverse outcomes in these situations. Animals constitutively expressing GHRH exhibited a decline in tumor growth rate (20% for males, $p < 0.03$ and 11% for females, $p < 0.13$). Histopathological analysis revealed that treated animals were less likely to develop lung metastases (11%) and had no metastases in other organs. The number of metastases/lung was reduced by 57% in female mice with GHRH treatment ($p < 0.006$). Furthermore, animals with advanced tumors had improved kidney function and delayed loss of skeletal muscle mass compared with control animals. When tumor size exceeded 8% of body weight, GHRH-treated mice showed normal urea and creatinine levels and kidney weight, while controls displayed signs of renal insufficiency [62]. This study provides evidence that with plasmid-mediated GHRH supplementation in tumor-bearing mice, tumor growth rate is not increased but is actually attenuated.

Cancer patients are often immunocompromised. Thus, we tested the hypothesis that physiological stimulation of the GHRH axis through a plasmid-based GHRH expression system does not stimulate tumor growth in immunodeficient animals [64]. Nude mice were implanted with two different human tumor cell lines, NCI-H358 human bronchioalveolar carcinoma and MDA-MB-468 human breast adenocarcinoma. Tumor growth was not augmented by GHRH plasmid treatment in any group and was decreased in some groups. Male animals implanted with the NCI-H358 tumor cell line and treated with the GHRH-expressing plasmid exhibited a 40% decrease in the size of the tumors ($p < 0.02$), a 48% increase in white blood cells ($p < 0.025$) and a 300% increase in monocyte count ($p < 0.0001$), as well as an increase in the frequency of activated CD3+ and CD4+ cells in the tumors compared with control animals, suggesting that the mechanism responsible for tumor growth reduction involves immune stimulation. No adverse effects were observed in animals that received the GHRH-plasmid treatment. These results provide support for the use of GHRH-expressing plasmids in the treatment of cancer cachexia in companion animals and humans.

Anemia: Renal failure

Previous studies have shown that GH [65,66] and IGF-I improve renal function in patients with renal failure [67]. Erythroid cell number is regulated primarily by erythropoietin (EPO), but is impacted by many factors.

There are data to suggest that IGF-I rather than EPO is the primary mediator of erythropoiesis during catabolic states and in uremic patients [68] and can induce a proportional increase in body mass and oxygen transport capacity [69]. The increase in hemoglobin and hematocrit confirms the *in vivo* erythropoietic-promoting effects of GH observed during GH treatment in GH-deficient children or adults [70,71]. In veterinary applications, the current standard therapy for chronic renal failure (CRF)-associated anemia is EPO of human origin. The effective response to the treatment decreases over time due to development of antibodies in response to the human protein [72]. Canine recombinant EPO has been used experimentally in dogs, resulting in an increase in appetite and energy along with an increase in hematocrit, but results were mitigated by etiology and development of systolic hypertension in half of the treated animals [73]. A recent study using a recombinant adeno-associated virus (AAV) vector encoding for feline EPO administered intramuscularly to healthy cats demonstrated a dose-related increase in hematocrit over 7 weeks [74]. Unfortunately, from a practical point of view, AAV vectors are difficult to produce, include steps performed *ex vivo*, result in low production titers per batch, have a risk of adenoviral contamination, and are extremely expensive to manufacture using GMP [75,76]. In addition, the EPO treatment does not correct the other complications of CRF, such as wasting.

Plasmid-mediated GHRH supplementation maintained renal function in mice and dogs with cancer [60,62] and in cats and dogs with CRF [50]. The primary objective of this study was to evaluate the safety and efficacy of a single intramuscular dose of plasmid DNA that expresses HV-GHRH to dogs (0.35 mg of plasmid; average age = 13.1 years) and cats (0.1 mg of plasmid; average age = 13.2 years) for the management of CRF [50]. Inclusion criteria included blood urea nitrogen (BUN) values of > 25 mg/dl or creatinine values of > 1.6 mg/dl for dogs, and BUN values of > 30mg/dl and creatinine values of > 2mg/dl for cats. These animals had a clinically determined life expectancy of at least 90 days. The secondary objectives of this 75-day study were to assess the effects of the GHRH therapy on blood, serum chemistry and urinalysis values, weight and quality-of-life in these dogs and cats. After a 75-day follow-up of dogs and cats with CRF treated with the GHRH-expressing plasmid, body weight increased 10 to 20%, plasma IGF-I levels were increased in both species ($p < 0.05$) (Figure 4), circulating iron concentrations almost doubled in treated dogs ($p < 0.05$), protein metabolism as measured by increased total protein concentration in serum was significantly improved in treated cats and, most importantly, several hematological parameters, including red blood cells, hematocrit and hemoglobin were significantly improved as early as day 20 in both species. Many of these improvements persisted until day 75 (Tables 1 and 2). Kidney function, as measured by BUN and creatinine, was maintained throughout the study [50].

Osteoarthritis

In 1997, approximately 16% of the US population had some form of arthritis [77]. Osteoarthritis is one of the most common disorders in the elderly population. Osteoarthritis is thus a significant social and economic problem and continued research and improvements in therapy are needed. GHRH-GH-IGF-I axis function undergoes considerable decline in aging mammals. The resulting reduction in GH secretion and IGF-I production is associated with sarcopenia, osteoporosis, arthritis, increased fat deposition and decreased lean body mass [78,79]. The development of these changes can be offset by recombinant GH therapy. The production of hyaluronan and chondroitin sulfate proteoglycans is regulated *in vitro* by GH and IGF-I, and these molecules may be of importance in the therapy of joint pathology [80,81]. For example, gene transfer of IGF-I into rabbit knee joints promoted proteoglycan synthesis without significantly affecting inflammation, or causing cartilage breakdown or adverse effects. As a result, local gene transfer of IGF-I to joints was suggested as a therapeutic strategy to stimulate new matrix synthesis in both rheumatoid arthritis and osteoarthritis [82]. In addition, increased levels of IGFBPs in arthritis may result in the reduced availability of free IGFs that can bind to IGF receptors. The observed changes in the IGF system thus may participate in catabolic processes in rheumatoid arthritis and the development of cachexia and wasting in these patients [83]. A therapy that addresses both the arthritic disease and the wasting would be an important step forward in the well-being and quality-of-life of patients.

Horses with chronic laminitis/arthritis (at least one year in duration) were used in a pilot study [P Brown, D Hood, R Draghia-Akli, unpublished data]. These horses demonstrated a significant improvement in lameness status as detected by both physical and force plate assessment. At 6-months post-treatment, the horses no longer require systemic analgesics and were rated as pasture sound. Radiographic evaluation of the hooves demonstrated resolution of the inflammation and correction of the third phalynx rotation. This proof-of-concept study will be followed by further detailed analysis in a larger population of animals.

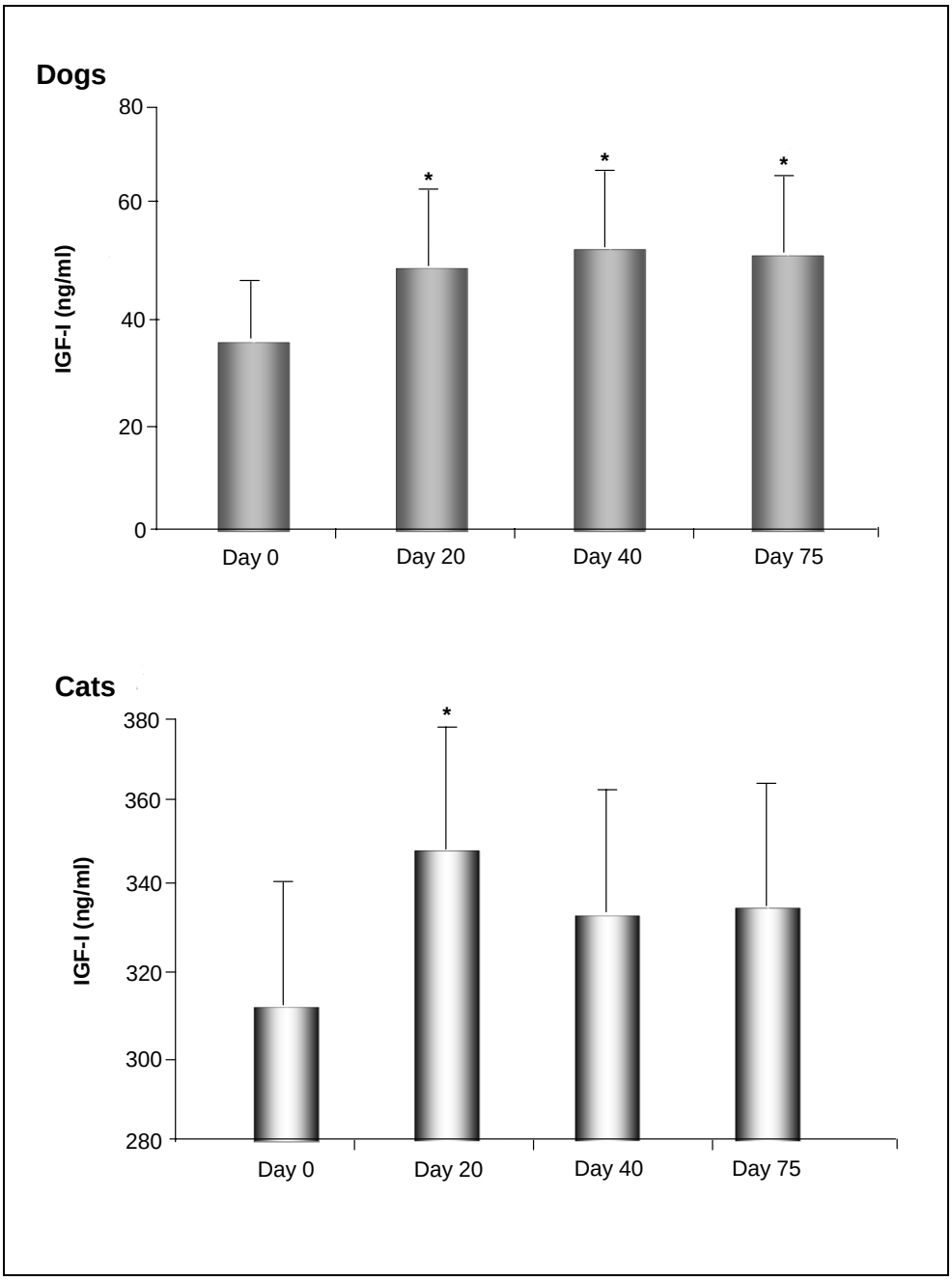
Directly treated animals: Growth, development and welfare

Plasmid-mediated GHRH and immune function

The neuroendocrine and immune systems are inter-related and interdependent [84]. Numerous hormones affect immune function, including those of the GHRH-GH-IGF-I axis. Both GHRH [85,86] and GH have immunomodulatory properties [86-88]. The importance of GHRH in the modulation of immune status under physiological and pathological conditions has been described [85], both through stimulation of the GH-IGF-I axis and directly as an immune modulator [89,90]. However, the mechanisms involved with GHRH mediating those effects or the impact of GHRH treatment on vaccination and pathogen challenge remain elusive.

Plasmid-mediated GHRH supplementation resulted in positive changes in the immune system in pregnant cows [36•]. Compared with controls, in GHRH-treated animals, CD2+αβ T-cells were increased by 14% ($p < 0.004$), CD25+CD4+ cells by 13% ($p < 0.001$) and CD4+CD45R+ cells by 53% ($p < 0.016$). These increases were maintained long-term after treatment and correlated with plasmid expression.

Figure 4. **Serum IGF-I in dogs and cats with chronic renal failure treated with a GHRH-expressing plasmid.**



Measurements were taken at days 0, 20, 40 and 75. Data are expressed as mean ± SEM. *****p < 0.05.

Table 1. Improvements in hematological parameters in dogs with renal failure treated with HV-GHRH plasmid.

Hematological parameter	Day 0	Day 20	Day 40	Days 60 to 75
RBC	6.16 ± 0.3	6.71 ± 0.3 8.9% (p = 0.002)	6.35 ± 0.3 3.1% (p = 0.125)	6.42 ± 0.3 4.2% (p = 0.035)
HCT	27.6 ± 1.1	30.5 ± 1.1 10.5% (p = 0.002)	28.2 ± 1.1 2.2% (p = 0.32)	29.2 ± 1.3 5.8% (p = 0.053)
HGB	9.02 ± 0.4	10.1 ± 0.4 12.0% (p < 0.0001)	9.65 ± 0.3 7.0% (p = 0.024)	9.82 ± 0.5 8.9% (p = 0.003)

Hematological parameters are significantly improved in HV-GHRH plasmid-treated dogs with chronic renal failure. Data are presented as mean ± SEM % change from baseline. **HCT** hematocrit, **HGB** hemoglobin, **RBC** red blood cell count.

Table 2. Improvements in hematological parameters in cats with renal failure treated with HV-GHRH plasmid.

Hematological parameter	Day 0	Day 20	Day 40	Days 60 to 75
RBC	4.84 ± 0.3	5.26 ± 0.3 8.7% (p = 0.05)	5.13 ± 0.3 6.0% (p = 0.18)	5.19 ± 0.3 7.2% (p = 0.30)
HCT	35.0 ± 1.9	37.4 ± 1.8 6.9% (p = 0.19)	36.7 ± 2.1 4.9% (p = 0.40)	37.1 ± 2.2 6.0% (p = 0.50)
HGB	11.5 ± 0.6	12.7 ± 0.6 10.4% (p < 0.01)	12.5 ± 0.7 8.7% (p = 0.03)	12.7 ± 0.8 10.4% (p = 0.05)

Hematological parameters are significantly improved in HV-GHRH plasmid-treated cats with chronic renal failure. Data are presented as mean ± SEM % change from baseline. **HCT** hematocrit, **HGB** hemoglobin, **RBC** red blood cell count.

expression. At 300 days post-GHRH therapy, the frequency of CD45R+/CD45R0-naive lymphocytes was significantly increased. Natural killer lymphocytes were also increased. As a consequence of improved health status, the body condition scores of treated animals improved significantly, and the morbidity and mortality of treated heifers decreased. During the 360-day study, none of the treated heifers died, while 20% of control heifers had to be culled ($p < 0.003$). However, animals in this experiment were not studied after specific vaccination or pathogenic challenges [36•].

A model was developed to test the hypothesis that plasmid-mediated GHRH administration could impact vaccine efficacy and clinical outcome [91]. This study evaluated the effect of a single dose of 0.625 mg of a porcine GHRH-expressing plasmid on the clinical outcomes of pigs vaccinated against and challenged with *Mycoplasma hyopneumoniae* (*M hyo*) and/or porcine reproductive and respiratory syndrome virus (PRRSV), as a model for both human and animal disease. One week after the plasmid injection, pigs were vaccinated against *M hyo* at 2-week intervals, challenged with either *M hyo* and/or PRRSV 2 weeks after the second vaccination, and necropsied at 17 and 36 days after challenge. Clinical parameters associated with *M hyo* challenge were improved with the GHRH treatment. Average daily gain between challenge and necropsy was improved ($p = 0.04$). Respiratory scores for *M hyo*-challenged pigs tended to be lower in GHRH-treated animals than in control animals and coughing scores were significantly improved by the treatment ($p = 0.01$). Macroscopic lung lesions associated with *M hyo* infection were less numerous in the group that received GHRH-expressing plasmid. No differences in the macroscopic pneumonia associated with PRRSV, or in serum antibodies to *M hyo* or PRRSV were observed with GHRH treatment. Nevertheless, IgG in the bronchioalveolar lavage was increased by GHRH treatment in *M hyo*-challenged animals ($p < 0.03$) [91]. The results of this study suggest that GHRH supplementation prior to vaccination may enhance protection against *M hyo*-induced pneumonia, and that a single dose of GHRH-expressing plasmid was sufficient to elicit an improved clinical outcome in this disease challenge model.

Increases in weight and body composition in the porcine model

Tremendous progress has been made in the identification of the stimulatory molecules that regulate growth, the mechanisms of action and the potential application of these molecules for livestock production [92,93]. A parallel and significant effort is now focused on the discovery and development of economically feasible gene delivery technologies that would enhance prolificacy and reproductive performance, increase feed utilization and growth rate, improve carcass composition, improve milk production and/or composition, and increase disease resistance [94]. Because of its characteristics, plasmid-mediated GHRH gene transfer has emerged as an excellent candidate for agricultural applications to increase animal health and welfare, while improving on the production parameters within physiological parameters (eg, animals that are healthier maintain a good average daily gain in the face of a microbial challenge).

Initial experiments in our laboratory compared weight and body composition changes, as well as hormonal and biochemical profiles *in vivo*, in groups of young pigs treated with plasmids that directed the expression of wild-type (wt) porcine GHRH, a novel serum protease-resistant porcine GHRH, HV-GHRH or β-galactosidase (for controls) [35]. Treated 3-week-old piglets received a single high-dose intramuscular injection of plasmid (up to 10 mg). Serum GHRH levels were elevated 2- to 4-fold, which increased GH secretion and enhanced serum IGF-I levels 3- to 6-fold compared with control pigs. Animals were fed a 24% protein, 1.6% lysine diet at 6% of their body weight. Under these conditions, the average increase in body weight over 65 days in the pSP-HV-GHRH-injected pigs was 37% greater than in controls and 21% greater in pigs treated with the pSP-wt-GHRH. Additionally, as usually encountered with GH treatment, serum urea concentrations were significantly reduced as a result of reduced amino acid catabolism [35].

Subsequently, dose-response and time-course studies were performed [40]. Young pigs, 7 to 10 days-old, were treated with escalating doses of plasmid (0.1 to 3 mg of pSP-HV-GHRH). The group treated with 0.1 mg of plasmid had greater weight gain over 53 days (22.4 ± 0.8 kg versus 19.7 ± 0.03 kg in control pigs, $p < 0.01$). Body composition studies by dual X-ray absorptiometry showed a 22% decrease in fat deposition ($p < 0.05$) and a 10% increase in bone mineral density ($p < 0.004$). A small number of pigs (< 5%) that had been treated with 3 mg of HV-GHRH-expressing plasmid developed neutralizing antibodies and consequently did not show any improvement in their rates of weight gain compared with controls.

Subsequent studies were performed under farm conditions in two separate trials of 150 and 180 young pigs. Groups of 20 animals were treated with 0.05 to 2 mg of pSP-HV-GHRH

or pSP-wt-GHRH, with one group as control. Average daily gain over the 143-day experiment was improved by 5.5 to 9.0% for the groups treated with 0.1, 0.5 or 1 mg of pSP-HV-GHRH (0.85 ± 0.02 kg/day for GHRH plasmid-treated pigs versus 0.78 ± 0.02 kg/day for control pigs, $p < 0.03$). At slaughter, dressing percentage (the weight of the dressed carcass compared with the weight of the live animal) was 4.4% higher with GHRH plasmid treatment ($p < 0.001$). Additionally, 2 months after treatment, the value of the CD4+/CD8+ ratio, a marker of immune function, was assayed and found to be 25% higher in the GHRH plasmid-treated pigs ($p < 0.05$). Increased immune surveillance may explain the improvements in animal health.

Inter-generational effects of plasmid-mediated GHRH supplementation

Results from several studies on rats and pigs have demonstrated that when pregnant animals were treated with a GHRH-expressing plasmid, they gave birth to offspring that had increased numbers of somatotrophs and lactotrophs [46,47]. When studies in large animals were conducted, perinatal morbidity and mortality were also significantly decreased. Together, these studies suggest that stable production of GHRH can produce a number of direct physiological effects on the injected organism, as well as inter-generational effects on the offspring.

Gilts (female pigs that have never given birth) were treated with 0.1 to 5 mg of a GHRH-expressing plasmid at 85 to 90 days of gestation, and piglets were followed from birth to slaughter. The results revealed a dose threshold. Effects were clear for the offspring born to gilts treated with 5 mg of plasmid and included enhanced birth (13%, $p < 0.025$) and slaughter weights (11%, $p < 0.001$), improved feed efficiency (3.5%, $p < 0.026$), higher serum GHRH (20%) and IGF-I concentrations ($p < 0.05$), and increased numbers of pituitary somatotrophs (19% at birth, $p < 0.027$; 50% at slaughter, $p < 0.0012$) and lactotrophs (62.4% at birth, $p < 0.015$; 31% at slaughter, $p < 0.001$) in the progeny [46]. In a subsequent trial conducted on a larger number of gilts ($n = 80$/group), we also recorded a decreased mortality in the offspring of treated gilts (57%) compared with untreated animals, and this effect was maintained for at least the first two consecutive litters following treatment of the sows [46].

To establish the extent to which these effects on the progeny were the result of intra-uterine versus lactational consequences of the GHRH-plasmid treatment, cross-fostering studies were conducted. When the progeny of control gilts ($n = 12$ to 24/treatment group) were cross-fostered at birth to GHRH plasmid-treated gilts, they were 12% heavier at weaning compared with the control piglets nursed on control gilts. The progeny of GHRH plasmid-treated gilts nursed on treated gilts were 28% heavier ($p < 0.055$) at weaning compared with progeny of control gilts that nursed on controls, while their littermates that nursed on control gilts were 22% heavier [95]. These observations indicate that although treated gilts produced more milk, intra-uterine effects must have been responsible for the changes present at birth of their progeny and their subsequent capacity to grow faster post-natally, even when cross-fostered to non-treated control gilts. The higher numbers of somatotrophs and lactotrophs maintained from birth to slaughter indicate that this profile is maintained permanently and thus can sustain prolonged effects on a variety of parameters from growth and feed efficiency to immune function and overall mortality [46,95].

The exact mechanisms responsible for these responses are unknown and may involve both direct and indirect effects of GHRH on the fetus. Moreover, the relative importance of individual mechanisms may be species dependent. Improved fetal and neonatal growth could be due partially to indirect effects of the enhanced maternal GH production on nutrient transport from the mother to the fetus pre-natally [96] and on milk production post-natally [97]. On the other hand, the pituitary changes could be ascribed to direct effects of GHRH on the pituitary [46]. During early development, chronic exposure to GHRH enhances somatotroph proliferation, increases GHRH receptor mRNA expression [98], and stimulates GH gene transcription and secretion. Importantly, the immature pituitary is more responsive to GHRH than the mature gland. The GHRH-induced responses are mediated largely by the transcription factor Pit-1 [99], the expression of which is stimulated by GHRH [100]. However, there is no direct evidence to date for the trans-placental transfer of GHRH. There are data in humans to suggest that GHRH and other comparably small proteins, such as insulin and IGF-I, do not cross the placenta [101]. Nonetheless, based on our previous results, it is our hypothesis that GHRH is one of the variety of proteins that can access fetal circulation and affect pituitary development.

Conclusions

GHRH is a member of the hypothalamic hormone family. Originally recognized simply as factors controlling anterior pituitary secretions [102••], these peptides now comprise their own chapter of physiology and medical endocrinology. It is well-known that GHRH and its receptors are ubiquitous and have profound effects on a large number of organs and functions.

In this review, a novel method of augmenting GH secretion through the single administration of a GHRH-expressing plasmid has been described. This GHRH-expressing plasmid can treat the cachexia and anemia associated with cancer and renal failure, decrease tumor growth, enhance immune function and body composition, increase weight and decrease perinatal morbidity and mortality of the offspring after treatment of a pregnant animal. This research is facilitated by new molecular biology tools that allow the study of short- and long-term effects of GHRH in both physiological and pathological situations in several species of various sizes, from cows and horses, to companion animals such as dogs and cats. The strength of this technology is that beneficial effects of plasmid-mediated GHRH administration have been demonstrated across species. We believe that this technology has the potential to transition from veterinary applications to human applications in the near future.

Acknowledgements

We would like to thank our fellow employees at ADViSYS as well as our collaborators and supporters over the years, Douglas Kern, Marta Fiortto, Louis Smith, Jeffrey Nordstrom and Malcolm Brenner. We would also like to

thank Ryan Person for his technical assistance, Melissa Pope for her excellent laboratory skills and Catherine Tone for her editorial correction of this manuscript.

References

•• of outstanding interest
• of special interest

1. Veldhuis JD, Bowers CY: **Human GH pulsatility: An ensemble property regulated by age and gender.** *J Endocrinol Invest* (2003) **26**(9):799-813.

2. Harris M, Hofman PL, Cutfield WS: **Growth hormone treatment in children: Review of safety and efficacy.** *Paediatr Drugs* (2004) **6**(2):93-106.

3. Henwood MJ, Grimberg A, Moshang T Jr: **Expanded spectrum of recombinant human growth hormone therapy.** *Curr Opin Pediatr* (2002) **14**(4):437-442.

4. Abdul Shakoor SK, Shalet SM: **Effects of GH replacement on metabolism and physical performance in GH deficient adults.** *J Endocrinol Invest* (2003) **26**(9):911-918.

5. Etherton TD, Bauman DE: **Biology of somatotropin in growth and lactation of domestic animals.** *Physiol Rev* (1998) **78**(3):745-761.

6. Klindt J, Yen JT, Buonomo FC, Roberts AJ, Wise T: **Growth, body composition, and endocrine responses to chronic administration of insulin-like growth factor I and (or) porcine growth hormone in pigs.** *J Anim Sci* (1998) **76**(9):2368-2381.

7. Rehfeldt C, Nissen PM, Kuhn G, Vestergaard M, Ender K, Oksbjerg N: **Effects of maternal nutrition and porcine growth hormone (pGH) treatment during gestation on endocrine and metabolic factors in sows, fetuses and pigs, skeletal muscle development, and postnatal growth.** *Domest Anim Endocrinol* (2004) **27**(3):267-285.

8. Angelopoulos TJ, Seip RL, Cole TG: **Effect of short-term recombinant growth hormone administration on plasma lipoproteins in elderly adults.** *Gerontology* (1998) **44**(4):228-231.

9. Satozawa N, Takezawa K, Miwa T, Takahashi S, Hayakawa M, Ooka H: **Differences in the effects of 20 K- and 22 K-hGH on water retention in rats.** *Growth Horm IGF Res* (2000) **10**(4):187-192.

10. Frohman LA, Jansson JO: **Growth hormone-releasing hormone.** *Endocr Rev* (1986) **7**(3):223-253.

• *This paper provides one of the most comprehensive reviews of the then novel field studying GHRH and its early uses as therapy for GH deficiencies.*

11. Frohman LA, Downs TR, Heimer EP, Felix AM: **Dipeptidylpeptidase IV and trypsin-like enzymatic degradation of human growth hormone-releasing hormone in plasma.** *J Clin Invest* (1989) **83**(5):1533-1540.

12. Osborn BL, Sekut L, Corcoran M, Poortman C, Sturm B, Chen G, Mather D, Lin HL, Parry TJ: **Albutropin: A growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys.** *Eur J Pharmacol* (2002) **456**(1-3):149-158.

13. Tang SS, Chen ZL, Liu JJ: **Production and enhanced biological activity of a novel GHRH analog, hGHRH with an *N*-terminal Pro-Pro extension.** *Protein Expr Purif* (2004) **34**(2):296-301.

14. Esposito P, Barbero L, Caccia P, Caliceti P, D'Antonio M, Piquet G, Veronese FM: **PEGylation of growth hormone-releasing hormone (GRF) analogues.** *Adv Drug Deliv Rev* (2003) **55**(10):1279-1291.

15. Quezada A, Larson J, French M, Ponce R, Perrard J, Durland R, Coleman M: **Biodistribution and safety studies of hDel-1 plasmid-based gene therapy in mouse and rabbit models.** *J Pharm Pharmacol* (2004) **56**(2):177-185.

16. Bloquel C, Fabre E, Bureau MF, Scherman D: **Plasmid DNA electrotransfer for intracellular and secreted proteins expression: New methodological developments and applications.** *J Gene Med* (2004) **6**(Suppl 1):S11-S23.

17. Frederickson RM, Carter BJ, Pilaro AM: **Nonclinical toxicology in support of licensure of gene therapies. Arlington, VA, USA, March 13-14, 2003.** *Mol Ther* (2003) **8**(1):8-10.

•• *This paper familiarizes both industrial and academic investigators with the regulatory requirements for non-clinical studies in support of labeling for licensure of gene therapies.*

18. Lechardeur D, Sohn KJ, Haardt M, Joshi PB, Monck M, Graham RW, Beatty B, Squire J, O'Brodovich H, Lukacs GL: **Metabolic instability of plasmid DNA in the cytosol: A potential barrier to gene transfer.** *Gene Ther* (1999) **6**(4):482-497.

19. Wilber A, Lu M, Schneider MC: **Deoxyribonuclease I-like III is an inducible macrophage barrier to liposomal transfection.** *Mol Ther* (2002) **6**(1):35-42.

20. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG 2nd, Harper LB, Schock HB, Zhang H, Faris JE, Way PA *et al*: **Plasmid DNA vaccines: Assay for integration into host genomic DNA.** *Dev Biol* (2000) **104**:33-43.

21. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG 2nd, Harper LB, Beare CM, Bagdon WJ, Nichols WW: **Plasmid DNA vaccines: Investigation of integration into host cellular DNA following intramuscular injection in mice.** *Intervirology* (2000) **43**(4-6):258-272.

22. Chen ZY, He CY, Meuse L, Kay MA: **Silencing of episomal transgene expression by plasmid bacterial DNA elements *in vivo*.** *Gene Ther* (2004) **11**(10):856-864.

23. Molnar MJ, Gilbert R, Lu Y, Liu AB, Guo A, Larochelle N, Orlopp K, Lochmuller H, Petrof BJ, Nalbantoglu J, Karpati G: **Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles.** *Mol Ther* (2004) **10**(3):447-455.

24. Xie QF, Wu CX, Meng QY, Li N: **Ghrelin and truncated ghrelin variant plasmid vectors administration into skeletal muscle augments long-term growth in rats.** *Domest Anim Endocrinol* (2004) **27**(2):155-164.

25. Li X, Eastman EM, Schwartz RJ, Draghia-Akli R: **Synthetic muscle promoters: Activities exceeding naturally occurring regulatory sequences.** *Nat Biotechnol* (1999) **17**(3):241-245.

26. Draghia-Akli R, Li X, Schwartz RJ: **Enhanced growth by ectopic expression of growth hormone releasing hormone using an injectable myogenic vector.** *Nat Biotechnol* (1997) **15**(12):1285-1289.

• *This paper describes the effects of plasmid-based GHRH administration in rodents. Mice treated with a single intramuscular injection of 100 μg of GHRH-expressing plasmid were significantly larger than controls in only 14 days and this increase was maintained to 21 days post-treatment.*

27. Terada Y, Tanaka H, Okado T, Shimamura H, Inoshita S, Kuwahara M, Akiba T, Sasaki S: **Ligand-regulatable erythropoietin production by plasmid injection and *in vivo* electroporation.** *Kidney Int* (2002) **62**(6):1966-1976.

28. Ehrhardt A, Peng PD, Xu H, Meuse L, Kay MA: **Optimization of *cis*-acting elements for gene expression from nonviral vectors *in vivo*.** *Hum Gene Ther* (2003) **14**(3):215-225.

29. Goldspink G: **Skeletal muscle as an artificial endocrine tissue.** *Best Pract Res Clin Endocrinol Metab* (2003) **17**(2):211-222.

30. Long YC, Jaichandran S, Ho LP, Tien SL, Tan SY, Kon OL: **FVIII gene delivery by muscle electroporation corrects murine hemophilia A.** *J Gene Med* (2005) **7**(4):494-505.

31. Jeong JG, Kim JM, Ho SH, Hahn W, Yu SS, Kim S: **Electrotransfer of human IL-1Ra into skeletal muscles reduces the incidence of murine collagen-induced arthritis.** *J Gene Med* (2004) **6**(10):1125-1133.

32. Tanaka S, Shimizu M, Debari K, Furuya R, Kawawa T, Sasaki T: **Acute effects of ovariectomy on wound healing of alveolar bone after maxillary molar extraction in aged rats.** *Anat Rec* (2001) **262**(2):203-212.

33. Xue F, Takahara T, Yata Y, Minemura M, Morioka CY, Takahara S, Yamato E, Dono K, Watanabe A: **Attenuated acute liver injury in mice by naked hepatocyte growth factor gene transfer into skeletal muscle with electroporation.** *Gut* (2002) **50**(4):558-562.

34. Fewell JG, MacLaughlin F, Mehta V, Gondo M, Nicol F, Wilson E, Smith LC: **Gene therapy for the treatment of hemophilia B using PINC-formulated plasmid delivered to muscle with electroporation.** *Mol Ther* (2001) **3**(4):574-583.

35. Draghia-Akli R, Fiorotto ML, Hill LA, Malone PB, Deaver DR, Schwartz RJ: **Myogenic expression of an injectable protease-resistant growth hormone-releasing hormone augments long-term growth in pigs.** *Nat Biotechnol* (1999) **17**(12):1179-1183.

36. Brown PA, Davis WC, Draghia-Akli R: **Immune enhancing effects of growth hormone-releasing hormone delivered by plasmid injection and electroporation.** *Mol Ther* (2004) **10**(4):644-651.
• *The expression of physiologically significant levels of GHRH in dairy cows for almost a year is described in this paper. The results suggest that this method of plasmid delivery followed by electroporation could prove to be efficient for the systemic production of therapeutic proteins.*

37. Babiuk S, Baca-Estrada ME, Foldvari M, Storms M, Rabussay D, Widera G, Babiuk LA: **Electroporation improves the efficacy of DNA vaccines in large animals.** *Vaccine* (2002) **20**(27-28):3399-3408.
• *This paper describes the improvement of DNA vaccine efficacy with electroporation using hepatitis B surface antigen and bovine herpes virus D glycoprotein in pigs.*

38. Tollefsen S, Vordermeier M, Olsen I, Storset AK, Reitan LJ, Clifford D, Lowrie DB, Wiker HG, Huygen K, Hewinson G, Mathiesen I, Tjelle TE: **DNA injection in combination with electroporation: A novel method for vaccination of farmed ruminants.** *Scand J Immunol* (2003) **57**(3):229-238.

39. Yang TT, Sinai P, Kitts PA, Kain SR: **Quantification of gene expression with a secreted alkaline phosphatase reporter system.** *Biotechniques* (1997) **23**(6):1110-1114.

40. Draghia-Akli R, Ellis KM, Hill LA, Malone PB, Fiorotto ML: **High-efficiency growth hormone-releasing hormone plasmid vector administration into skeletal muscle mediated by electroporation in pigs.** *FASEB J* (2003) **17**(3):526-528.

41. Bercu BB, Walker RF: **Growth hormone secretagogues in children with altered growth.** *Acta Paediatr* (1997) **423**:102-106.

42. Corpas E, Harman SM, Pineyro MA, Roberson R, Blackman MR: **Continuous subcutaneous infusions of growth hormone (GH) releasing hormone 1-44 for 14 days increase GH and insulin-like growth factor-I levels in old men.** *J Clin Endocrinol Metab* (1993) **76**(1):134-138.

43. Esch FS, Bohlen P, Ling NC, Brazeau PE, Wehrenberg WB, Thorner MO, Cronin MJ, Guillemin R: **Characterization of a 40 residue peptide from a human pancreatic tumor with growth hormone releasing activity.** *Biochem Biophys Res Commun* (1982) **109**(1):152-158.

44. Draghia-Akli R, Cummings KK, Khan AS, Brown PA, Carpenter RH: **Effects of plasmid-mediated growth hormone releasing hormone supplementation in young, healthy Beagle dogs.** *J Anim Sci* (2003) **81**(9):2301-2310.

45. Tone CM, Cardoza DM, Carpenter RH, Draghia-Akli R: **Long-term effects of plasmid-mediated growth hormone releasing hormone in dogs.** *Cancer Gene Ther* (2004) **11**(5):389-396.

46. Khan AS, Fiorotto ML, Cummings KK, Pope MA, Brown PA, Draghia-Akli R: **Maternal GHRH plasmid administration changes pituitary cell lineage and improves progeny growth of pigs.** *Am J Physiol Endocrinol Metab* (2003) **285**(1):E224-E231.

47. Khan AS, Fiorotto ML, Hill LA, Malone PB, Cummings KK, Parghi D, Schwartz RJ, Smith RG, Draghia-Akli R: **Nonhereditary enhancement of progeny growth.** *Endocrinology* (2002) **143**(9):3561-3567.

48. Mayo KE, Cerelli GM, Lebo RV, Bruce BD, Rosenfeld MG, Evans RM: **Gene encoding human growth hormone-releasing factor precursor: Structure, sequence, and chromosomal assignment.** *Proc Natl Acad Sci USA* (1985) **82**(1):63-67.
• *This paper describes the isolation of the human GHRH gene to chromosome 20.*

49. Mayo KE, Vale W, Rivier J, Rosenfeld MG, Evans RM: **Expression-cloning and sequence of a cDNA encoding human growth hormone-releasing factor.** *Nature* (1983) **306**(5938):86-88.

50. Brown PA, Draghia-Akli R, Sonntag WC: **Improved clinical outcome in cats and dogs in chronic renal failure after plasmid-mediated GHRH treatment.** *Soc Neuroscience* (2004) **34**:Abs 997.10.

51. Frohman LA, Downs TR, Chomczynski P, Frohman MA: **Growth hormone-releasing hormone: Structure, gene expression and molecular heterogeneity.** *Acta Paediatr Scand Suppl* (1990) **367**:81-86.

52. Nelson KA: **The cancer anorexia-cachexia syndrome.** *Semin Oncol* (2000) **27**(1):64-68.

53. Barber MD, Ross JA, Fearon KC: **Cancer cachexia.** *Surg Oncol* (1999) **8**(3):133-141.

54. Acharyya S, Ladner KJ, Nelsen LL, Damrauer J, Reiser PJ, Swoap S, Guttridge DC: **Cancer cachexia is regulated by selective targeting of skeletal muscle gene products.** *J Clin Invest* (2004) **114**(3):370-378.

55. Ramos EJ, Suzuki S, Marks D, Inui A, Asakawa A, Meguid MM: **Cancer anorexia-cachexia syndrome: Cytokines and neuropeptides.** *Curr Opin Clin Nutrition Med Care* (2004) **7**(4):427-434.

56. Kotler DP: **Cachexia.** *Ann Intern Med* (2000) **133**(8):622-634.

57. Colao A, Cuocolo A, Di Somma C, Cerbone G, Della Morte AM, Nicolai E, Lucci R, Salvatore M, Lombardi G: **Impaired cardiac performance in elderly patients with growth hormone deficiency.** *J Clin Endocrinol Metab* (1999) **84**(11):3950-3955.

58. Wang W, Iresjo BM, Karlsson L, Svanberg E: **Provision of rhIGF-I/IGFBP-3 complex attenuated development of cancer cachexia in an experimental tumor model.** *Clin Nutr* (2000) **19**(2):127-132.

59. Li WG, Gavrila D, Liu X, Wang L, Gunnlaugsson S, Stoll LL, McCormick ML, Sigmund CD, Tang C, Weintraub NL: **Ghrelin inhibits proinflammatory responses and nuclear factor-κB activation in human endothelial cells.** *Circulation* (2004) **109**(18):2221-2226.

60. Draghia-Akli R, Hahn KA, King GK, Cummings K, Carpenter RH: **Effects of plasmid-mediated growth hormone-releasing hormone in severely debilitated dogs with cancer.** *Mol Ther* (2002) **6**(6):830-836.

61. Kiaris H, Koutsilieris M, Kalofoutis A, Schally AV: **Growth hormone-releasing hormone and extra-pituitary tumorigenesis: Therapeutic and diagnostic applications of growth hormone-releasing hormone antagonists.** *Expert Opin Investig Drugs* (2003) **12**(8):1385-1394.

62. Khan AS, Anscombe IW, Cummings KK, Pope MA, Smith LC, Draghia-Akli R: **Effects of plasmid-mediated growth hormone-releasing hormone supplementation on LL-2 adenocarcinoma in mice.** *Mol Ther* (2003) **8**(3):459-466.

63. Van den Berghe G, Baxter RC, Weekers F, Wouters P, Bowers CY, Veldhuis JD: **A paradoxical gender dissociation within the growth hormone/insulin-like growth factor I axis during protracted critical illness.** *J Clin Endocrinol Metab* (2000) **85**(1):183-192.

64. Khan AS, Smith LC, Anscombe IW, Cummings KK, Pope MA, Draghia-Akli R: **Growth hormone releasing hormone plasmid supplementation, a potential treatment for cancer cachexia, does not increase tumor growth in nude mice.** *Cancer Gene Ther* (2005) **12**(1):54-60.

65. Ziegler TR, Lazarus JM, Young LS, Hakim R, Wilmore DW: **Effects of recombinant human growth hormone in adults receiving maintenance hemodialysis.** *J Am Soc Nephrol* (1991) **2**(6):1130-1135.

66. Lilien MR, Schroder CH, Levtchenko EN, Koomans HA: **Growth hormone therapy influences endothelial function in children with renal failure.** *Pediatr Nephrol* (2004) **19**(7):785-789.

67. Vijayan A, Franklin SC, Behrend T, Hammerman MR, Miller SB: **Insulin-like growth factor I improves renal function in patients with end-stage chronic renal failure.** *Am J Physiol* (1999) **276**(4 Pt 2):R929-R934.

68. Urena P, Bonnardeaux A, Eckardt KU, Kurtz A, Drueke TB: **Insulin-like growth factor I: A modulator of erythropoiesis in uraemic patients?** *Nephrol Dial Transplant* (1992) **7**(1):40-44.

69. Kurtz A, Matter R, Eckardt KU, Zapf J: **Erythropoiesis, serum erythropoietin, and serum IGF-I in rats during accelerated growth.** *Acta Endocrinol* (1990) **122**(3):323-328.

70. Valerio G, Di Maio S, Salerno M, Argenziano A, Badolato R, Tenore A: **Assessment of red blood cell indices in growth-hormone-treated children.** *Horm Res* (1997) **47**(2):62-66.

71. Christ ER, Cummings MH, Westwood NB, Sawyer BM, Pearson TC, Sonksen PH, Russell-Jones DL: **The importance of growth hormone in the regulation of erythropoiesis, red cell mass, and plasma volume in adults with growth hormone deficiency.** *J Clin Endocrinol Metab* (1997) **82**(9):2985-2990.

72. Giampaoli S, Facciabene A, Mennuni C: **A protocol to guide development of a sensitive ELISA for canine erythropoietin.** *Vet Clin Pathol* (2003) **32**(4):199-201.

73. Randolph JE, Scarlett J, Stokol T, MacLeod JN: **Clinical efficacy and safety of recombinant canine erythropoietin in dogs with anemia of chronic renal failure and dogs with recombinant human erythropoietin-induced red cell aplasia.** *J Vet Intern Med* (2004) **18**(1):81-91.

74. Beall CJ, Phipps AJ, Mathes LE, Stromberg P, Johnson PR: **Transfer of the feline erythropoietin gene to cats using a recombinant adeno-associated virus vector.** *Gene Ther* (2000) **7**(6):534-539.

75. Liu YL, Wagner K, Robinson N, Sabatino D, Margaritis P, Xiao W, Herzog RW: **Optimized production of high-titer recombinant adeno-associated virus in roller bottles.** *Biotechniques* (2003) **34**(1):184-189.

76. Qiao C, Li J, Skold A, Zhang X, Xiao X: **Feasibility of generating adeno-associated virus packaging cell lines containing inducible adenovirus helper genes.** *J Virol* (2002) **76**(4):1904-1913.

77. Reginster JY: **The prevalence and burden of arthritis.** *Rheumatology* (2002) **41**(Suppl 1):3-6.

78. Caroni P, Schneider C: **Signaling by insulin-like growth factors in paralyzed skeletal muscle: Rapid induction of IGF1 expression in muscle fibers and prevention of interstitial cell proliferation by IGF-BP5 and IGF-BP4.** *J Neurosci* (1994) **14**(5 Pt 2):3378-3388.

79. Veldhuis JD, Iranmanesh A, Weltman A: **Elements in the pathophysiology of diminished growth hormone (GH) secretion in aging humans.** *Endocrine* (1997) **7**(1):41-48.

80. Pavasant P, Shizari T, Underhill CB: **Hyaluronan synthesis by epiphysial chondrocytes is regulated by growth hormone, insulin-like growth factor-1, parathyroid hormone and transforming growth factor-β1.** *Matrix Biol* (1996) **15**(6):423-432.

81. Erikstrup C, Pedersen LM, Heickendorff L, Ledet T, Rasmussen LM: **Production of hyaluronan and chondroitin sulphate proteoglycans from human arterial smooth muscle - the effect of glucose, insulin, IGF-I or growth hormone.** *Eur J Endocrinol* (2001) **145**(2):193-198.

82. Mi Z, Ghivizzani SC, Lechman ER, Jaffurs D, Glorioso JC, Evans CH, Robbins PD: **Adenovirus-mediated gene transfer of insulin-like growth factor 1 stimulates proteoglycan synthesis in rabbit joints.** *Arthritis Rheum* (2000) **43**(11):2563-2570.

83. Neidel J: **Changes in systemic levels of insulin-like growth factors and their binding proteins in patients with rheumatoid arthritis.** *Clin Exp Rheumatol* (2001) **19**(1):81-84.

84. Steinman L: **Elaborate interactions between the immune and nervous systems.** *Nat Immunol* (2004) **5**(6):575-581.

85. Marshall L, Perras B, Fehm HL, Born J: **Changes in immune cell counts and interleukin (IL)-1β production in humans after a somnogenically active growth hormone-releasing hormone (GHRH) administration.** *Brain Behav Immun* (2001) **15**(3):227-234.

86. Siejka A, Lawnicka H, Komorowski J, Stepien T, Krupinski R, Stepien H: **Effect of growth hormone-releasing hormone (GHRH) and GHRH antagonist (MZ-4-71) on interferon-γ secretion from human peripheral blood mononuclear cells *in vitro*.** *Neuropeptides* (2004) **38**(1):35-39.

87. Bozzola M, De Benedetti F, De Amici M, Jouret B, Travaglino P, Pagani S, Conte F, Tauber M: **Stimulating effect of growth hormone on cytokine release in children.** *Eur J Endocrinol* (2003) **149**(5):397-401.

88. Koo GC, Huang C, Camacho R, Trainor C, Blake JT, Sirotina-Meisher A, Schleim KD, Wu TJ, Cheng K, Nargund R, McKissick G: **Immune enhancing effect of a growth hormone secretagogue.** *J Immunol* (2001) **166**(6):4195-4201.

89. Khorram O, Garthwaite M, Golos T: **The influence of aging and sex hormones on expression of growth hormone-releasing hormone in the human immune system.** *J Clin Endocrinol Metab* (2001) **86**(7):3157-3161.

90. Dialynas E, Brown-Borg H, Bartke A: **Immune function in transgenic mice overexpressing growth hormone (GH) releasing hormone, GH or GH antagonist.** *Proc Soc Exp Biol Med* (1999) **221**(3):178-183.

91. Thacker EL, Holtkamp DJ, Khan AS, Brown PA, Draghia-Akli R: **Plasmid-mediated GHRH efficacy in reducing disease associated with *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus infection.** *J Anim Sci* (2005): in press.

92. Tuggle CK, Trenkle A: **Control of growth hormone synthesis.** *Domest Anim Endocrinol* (1996) **13**(1):1-33.

93. Sillence MN: **Technologies for the control of fat and lean deposition in livestock.** *Vet J* (2004) **167**(3):242-257.

94. Wheeler MB, Walters EM: **Transgenic technology and applications in swine.** *Theriogenology* (2001) **56**(8):1345-1369.

95. Draghia-Akli R, Fiorotto ML: **A new plasmid-mediated approach to supplement somatotropin production in pigs.** *J Anim Sci* (2004) **82**(E-Suppl):E264-E269.

96. Jenkinson CM, Min SH, Mackenzie DD, McCutcheon SN, Breier BH, Gluckman PD: **Placental development and fetal growth in growth hormone-treated ewes.** *Growth Horm IGF Res* (1999) **9**(1):11-17.

97. Allan GJ, Tonner E, Barber MC, Travers MT, Shand JH, Vernon RG, Kelly PA, Binart N, Flint DJ: **Growth hormone, acting in part through the insulin-like growth factor axis, rescues developmental, but not metabolic, activity in the mammary gland of mice expressing a single allele of the prolactin receptor.** *Endocrinology* (2002) **143**(11):4310-4319.

98. Lasko CM, Korytko AI, Wehrenberg WB, Cuttler L: **Differential GH-releasing hormone regulation of GHRH receptor mRNA expression in the rat pituitary.** *Am J Physiol Endocrinol Metab* (2001) **280**(4):E626-E631.

99. Asa SL, Ezzat S: **Molecular determinants of pituitary cytodifferentiation.** *Pituitary* (1999) **1**(3-4):159-168.

100. Gonzalez-Parra S, Chowen JA, Garcia-Segura LM, Argente J: ***In vivo* and *in vitro* regulation of pituitary transcription factor-1 (Pit-1) by changes in the hormone environment.** *Neuroendocrinology* (1996) **63**(1):3-15.

101. de Zegher F, Vanderschueren-Lodeweyckx M, Spitz B, Faijerson Y, Blomberg F, Beckers A, Hennen G, Frankenne F: **Perinatal growth hormone (GH) physiology: Effect of GH-releasing factor on maternal and fetal secretion of pituitary and placental GH.** *J Clin Endocrinol Metab* (1990) **71**(2):520-522.

102. Guillemin R: **Hypothalamic hormones a.k.a. hypothalamic releasing factors.** *J Endocrinol* (2005) **184**(1):11-28.

•• *This is a well written, comprehensive review on hypothalamic hormones, which includes the relevant scientific history of each hormone from a personal point of view.*