

A new plasmid-mediated approach to supplement somatotropin production in pigs^{1,2}

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ABSTRACT: 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. A parallel and significant effort is now focused on the discovery and development of economically feasible gene delivery technologies. Plasmid-mediated GHRH gene transfer has emerged as an excellent candidate for agricultural applications to optimize production and animal welfare. We have engineered a GHRH-expressing plasmid that is efficiently expressed in skeletal muscle following intramuscular injection enhanced by electroporation. The GHRH is synthesized in the injected muscle, from which it is secreted to circulate and stimulate normal pituitary GH production and release. Young pigs directly injected with as little as 0.1 mg of a GHRH-expressing plasmid had greater ($P <$

0.01) weight gain than controls, and a increase ($P < 0.05$) in fat-free mass. We also have demonstrated that the offspring of gilts injected intramuscularly at d 85 of gestation with a GHRH-expressing plasmid have optimized growth characteristics due to both improved intrauterine weight gain and enhanced maternal lactation performance. Thus, the piglets from treated gilts were larger at birth and weaning compared to controls and reached market weight earlier ($P < 0.001$). Additionally, pituitaries collected from this group contained an increased number of somatotrophs and lactotrophs ($P < 0.001$) at birth and at 100 kg. An additional advantage of administering the GHRH plasmid to the gilt compared with the administration of growth-promoting agents to the individual adult animal is a substantial decrease in offspring morbidity and mortality ($P < 0.01$), which has always represented a major economic loss for the swine industry.

Key Words: Body Composition, Insulin-Like Growth Factor-I, Intrauterine Growth, Pituitary Development, Somatotropin

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Introduction

Growth hormone administration has widespread use in livestock production to improve the efficiency of di-

etary nitrogen utilization while promoting greater protein accretion and milk production (Etherton and Bauman, 1998; Klindt et al., 1998), and in humans to supplement inadequate endogenous levels, as in the case of GH-deficiencies (Henwood et al., 2002; Wit, 2002). The benefits of GH for animal agriculture also extend to the environment, in that the improved feed utilization decreases the quantity of nitrogenous waste produced. Nevertheless, the widespread application of GH in the United States has been curtailed by economic considerations. It is costly, and its short serum half-life necessitates frequent administration (Leung et al., 2002), which is labor intensive. Additionally, adverse effects to the health of the animals, such as mastitis (Pell et al., 1992), hyperthermia due to heat stress (Cole and Hansen, 1993), and the development of ulcers (Smith et al., 1991), have been observed with chronic GH administration.

Alternative approaches have been directed toward the physiological stimulation of endogenous GH secretion via its releasing factor, GHRH, a hypothalamic

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hormone (Buonomo and Baile, 1990). Growth hormone-releasing hormone peptide administration enhances growth performance and milk production, and maintains physiological feedback and regulation of the GH axis (Dubreuil et al., 1990; Farmer et al., 1992). However, the widespread use of a recombinant GHRH treatment is also limited by cost and the frequency of administration that is required to produce biological effects. Therefore, we have explored the feasibility of employing a plasmid-mediated GHRH supplementation approach that is conceptually similar to recombinant DNA vaccines. Plasmid-mediated GHRH treatment of young pigs optimizes growth and produces positive changes in their body composition (Draghia-Akli et al., 2003). In pregnant gilts, the treatment increases the number of somatotrophs and lactotrophs in the pituitary of the offspring (Khan et al., 2003). Moreover, we have encountered no adverse effects resulting from plasmid delivery to enhance GHRH expression.

In the present review, we summarize the diverse physiological effects of GHRH plasmid administration to pigs and discuss the underlying mechanisms that result in the improved growth response in treated animals and the possible applications of the technology.

Recombinant Protein Therapy vs. Plasmid-Mediated Therapy

The administration of recombinant porcine GH and GHRH has been extensively studied as a means to enhance or restore growth in a variety of mammalian species (Blanchard et al., 1991; Etienne et al., 1992; Wester et al., 1998; Kulinski et al., 2002). The native peptides are degraded in the serum by proteases and therefore require relatively frequent dosing in order to produce significant responses. For example, GHRH has a serum half-life of 6 to 17 min, depending on the assay conditions (Frohman et al., 1984, 1986). Three conceptually distinct approaches have been pursued to circumvent the short circulation time. Firstly, the peptides have been manipulated so that they are in a form that is protected from degradation and are released slowly over a longer period. Secondly, recombinant forms of GHRH peptide have been produced in which the AA sequence has been altered to reduce the rate of proteolysis and prolong the half-life. Lastly, a DNA plasmid that contains a GHRH complementary DNA (cDNA) to direct ectopic production of GHRH can be administered. The increase in circulating GHRH then enhances endogenous GH secretion. We have developed the latter solution for use in swine, dogs, and other species.

Plasmid Delivery and Target Organ

Plasmid vectors are simple to construct and are easy to manufacture at relatively low cost, using good manufacturing practice techniques that meet regulatory standards. They have a low risk:benefit ratio compared with viral vectors, as summarized by the FDA Center

for Biologics Evaluation and Research (Frederickson et al., 2003). 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 (Lechardeur et al., 1999; Wilber et al., 2002). The plasmid does not become incorporated into the animal's genomic DNA. Skeletal muscle fibers are especially suitable because of their slow rate of turnover and their capacity to produce and secrete a multitude of nonmuscle proteins. A previous limitation of plasmid administration has been a low efficiency of transfection. A variety of techniques have been used to improve muscle transfection, such as localized muscle damage followed by regeneration, hydrodynamic methods, and electroporation. The latter technique increases uptake, and consequently expression, 100- to 1,000-fold. Intramuscular injection of plasmid followed by electroporation has been used very successfully in mice (Lucas et al., 2001; Vilquin et al., 2001; Lesbordes et al., 2002), 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. Additionally, the technique has been used successfully in ruminants for vaccination purposes (Babiuk et al., 2003; Tollefsen et al., 2003).

A further benefit of the plasmid administration approach is that expression of the encoded protein can be largely restricted to one tissue through the use of tissue-specific promoters. This is an important safety issue because plasmid will not be expressed in other cell types. In the case of plasmids driven by ubiquitous promoters, cells such as antigen presenting cells could express the peptide and mount an immune response against the plasmid construct or transgene product, potentially resulting in the destruction of the surrounding muscle fibers (Davis et al., 1997). In addition to restricting the site of expression, we have taken advantage of the well-understood regulation of muscle gene expression to develop synthetic muscle promoters that result in much higher levels of expression than do endogenous muscle promoters, such as skeletal actin, creatine kinase, or viral promoter sequences, such as the CMV promoter (Li et al., 1999; Hagstrom et al., 2000; Roscilli et al., 2002).

The proteins encoded by the plasmid are secreted from the muscle and are transported via the circulation to their target organs, which, in the case of GHRH, is the anterior pituitary. The efficiency of peptide production and secretion is also influenced by the anatomical structure of the muscle into which the plasmid is administered. To evaluate this efficiency, we have used a reporter plasmid that expresses a secreted embryonic alkaline phosphatase under the control of the same muscle specific synthetic promoter (pSP). This reporter approach was chosen because secreted embryonic alkaline phosphatase, which normally is not present in plasma, is secreted efficiently into the circulation and enzyme activity can be detected as early as 24 h after

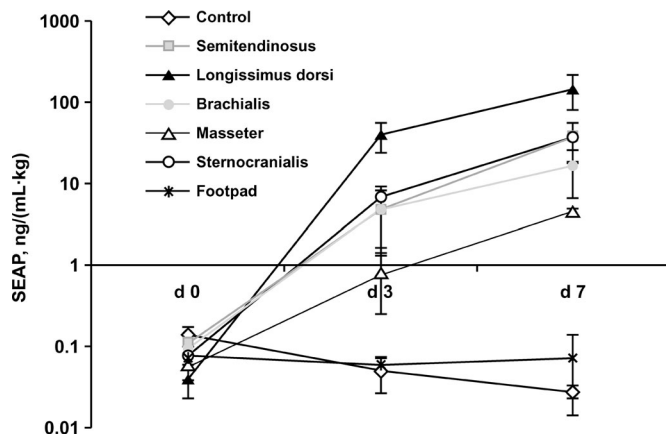


Figure 1. Secreted reporter peptide, secreted embryonic alkaline phosphatase (SEAP), and plasma concentration in pigs at 0 to 7 d postinjection ($n = 6/\text{group}$). Longissimus dorsi, semitendinosus, and sternocranialis muscles gave similar, high levels of expression, whereas small, flat muscles, such as the masseter, produced and secreted relatively lower peptide quantities. The peptide is not expressed in nonmuscle tissues, such as the footpad. Values are means \pm SE.

transfection by a very sensitive chemiluminescence-based assay (Yang et al., 1997). Therefore, it provides a highly sensitive alternative for assessing promoter function and transfection efficiency *in vivo*. Additionally, changes within an animal can be followed longitudinally as this method does not require that animals be euthanized for tissue collection. We demonstrated that large muscles, such as the longissimus dorsi, semitendinosus, or sternocranialis, have similarly high levels of expression (Draghia-Akli et al., 2003), whereas small, flat muscles, such as the masseter, secrete relatively lower peptide quantities (Figure 1). We have demonstrated that GHRH plasmid administration with electroporation is scalable to domestic animals and represents a promising approach to induce long-term production and regulated secretion of proteins in large animals (Draghia-Akli et al., 1999; Draghia-Akli et al., 2002).

Porcine GHRH Analog in Young Pigs

Initial experiments in our laboratory compared weight and body composition changes, as well as hormonal and biochemical profiles *in vivo*, in three groups of piglets treated with plasmids that directed the expression of wild-type (wt) GHRH, a novel serum protease-resistant porcine GHRH, **HV-GHRH** (Draghia-Akli et al., 1999), or β -galactosidase (for controls) under the control of a muscle-specific synthetic promoter, SPc5-12 (Li et al., 1999). Treated 3-wk-old piglets received a single i.m. injection of 10 mg of plasmid followed by electroporation using external electrodes that were applied to the skin surface. Serum GHRH levels

were elevated by two- to fourfold, which increased GH secretion and enhanced serum IGF-I levels by three- to sixfold over control pigs. Animals were fed a 24% protein, 1.6% lysine diet at 6% of their BW. Under these conditions, the average increase in BW over 65 d 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.

Subsequently, the efficacy of a lower plasmid dose on growth and body composition was tested after optimization of the injection/electroporation technique that now uses internal needle electrodes. Dose-response and time-course studies were performed (Draghia-Akli et al., 2003). Young pigs, 7 to 10 d old, were treated with escalating doses of plasmid: 0.1, 1, and 3 mg of pSP-HV-GHRH. The group treated with 0.1 mg plasmid had greater weight gain over 53 d (22.4 ± 0.8 kg vs. controls 19.7 ± 0.03 kg, $P < 0.01$). 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 (less than 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.

The challenge of developing technologies for animal agriculture lies in the transfer of experimental results obtained in laboratory facilities to farm conditions. To develop this application, two separate trials on 150 and 180 young pigs were performed. Groups of 20 animals were treated with 0.05 to 2 mg of pSP-HV-GHRH, whereas one group served as controls. The animals were treated at 7 to 10 d of age and fed a high protein, 1.6% lysine diet *ad libitum* until slaughter. Average daily gain over the 143-d-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/d for GHRH plasmid-treated pigs vs. 0.78 ± 0.02 kg/d for control pigs, $P < 0.03$). At slaughter, dressing percent was 4.4% higher with GHRH plasmid treatment ($P < 0.001$). Additionally, 2 mo 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 ($P < 0.05$). The improved immune surveillance may explain the health and growth benefits.

However, on closer examination of the weight growth curves, we determined that the most rapid rate of weight gain for treated pigs occurred in the first 30 d after treatment: the slope of the linear regression for body weight vs. time of treatment for plasmid HV-GHRH-treated pigs was 0.42 g/(kg·d) vs. 0.34 g/(kg·d) for controls ($P < 0.02$)—an improvement of 22%. Between d 30 and d 143 of treatment, the corresponding values were 0.96 g/(kg·d) vs. 0.89 g/(kg·d), respectively ($P < 0.02$), an improvement of 7%. This response likely

resulted from the fact that the level of ectopic GHRH production remained unchanged, while the volume of GHRH distribution continued to increase as the animal grows. Thus, the effective concentration of GHRH decreased over time. In this case, the strategy to maintain GHRH supplementation at levels that are stimulatory would be to administer a second dose of plasmid.

Intergenerational Effects of Plasmid-Mediated GHRH Supplementation

Results from several of our recent studies on rats and pigs have demonstrated that when pregnant animals are treated with the HV-GHRH plasmid, they gave birth to offspring that had increased numbers of somatotrophs and lactotrophs, as well as a long-term increase in BW (Khan et al., 2002, 2003). Together, these studies suggest that stable production of GHRH can produce a number of physiological effects on the injected organism directly, as well as intergenerational effects on the offspring. We treated gilts with 0.1 to 5 mg of pSP-HV-GHRH plasmid at 85 to 90 d of gestation, and piglets were followed from birth until slaughter. The results revealed a dose threshold in the responses. The effects were clear for the offspring born to gilts treated with 5 mg of plasmid. These effects included enhanced birth weights, slaughter weights, improved feed efficiency, higher serum GHRH and IGF-I concentrations, and increased numbers of pituitary somatotrophs and lactotrophs in the progeny. In a subsequent trial performed on a larger number of gilts ($n = 80/\text{group}$), we also recorded a decreased mortality in the offspring of treated gilts of 57% compared with untreated animals, and this effect has been maintained for at least the first two consecutive litters following treatment of sows.

To establish the extent to which these effects on the progeny are the result of intrauterine vs. lactational consequences of the GHRH-plasmid treatment, cross-fostering studies were conducted (Figure 2). When progeny of control gilts ($n = 12$ to $24/\text{treatment group}$) 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 than progeny of control gilts that nursed on controls, whereas their littermates that nursed on control gilts were 22% heavier. These observations indicate that, although treated gilts produced more milk, intrauterine effects must have been responsible for the higher birth weight of their progeny and their subsequent capacity to grow faster postnatally, even when cross fostered to untreated control gilts.

Presently, we cannot discern the extent to which improved nutrient availability to the fetus vs. improved nutrient utilization by the fetus or the enhanced immune function and health status contributed to the higher intrauterine rate of weight gain. Although there is no evidence that GHRH, GH, or IGF-I can cross the

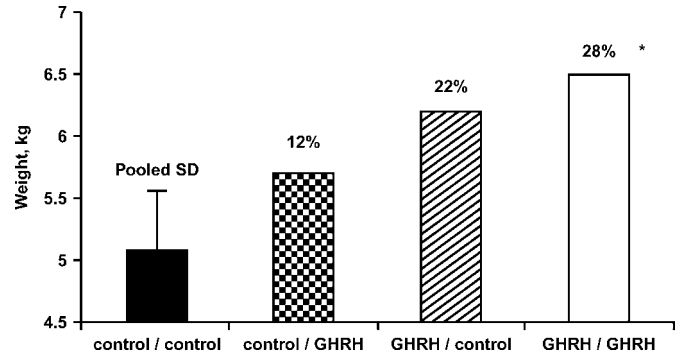


Figure 2. Weaning weights of piglets from cross-fostering studies after plasmid GHRH treatment of pregnant sows. Compared with control piglets suckled on control sows (control/control), an increase in piglet weaning weights was demonstrated in piglets born to control sows that nursed on treated sows (control/GHRH) and piglets born to treated sows nursed on either control (GHRH/control) or treated sows (GHRH/GHRH). Values are means ($n = 17$ to 31 animals/group); pooled SD was 0.764 kg; * $P < 0.055$ for piglets born to treated sows nursed on treated sows vs. control piglets nursed on control sows.

placenta, this possibility cannot be definitely excluded, as there is ample evidence that maternal proteins and cells are present in newborn. Nonetheless, taking the results of these studies together, we hypothesize that maternally derived GHRH can program the fetal pituitary at a critical stage of development to produce the observed changes in pituitary cell composition. The higher abundance of somatotrophs and lactotrophs present at birth and slaughter indicates 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 (Khan et al., 2003).

Implications

Plasmid-mediated growth hormone-releasing hormone treatment conserves the normal physiological regulation of growth hormone secretion, and modulates immune function in treated animals with no apparent adverse effects on treated animals or their offspring. Because of the central role of the growth hormone-releasing hormone–growth hormone–insulin-like growth hormone I axis in the regulation and coordination of anabolic processes of growth and reproduction, the consequences of plasmid-mediated growth hormone-releasing hormone supplementation are far-reaching. During pregnancy, maternal changes affect intrauterine and postnatal development, and promote increased perinatal survival of piglets. Concurrently, direct growth hormone-releasing hormone action induces changes in pituitary cell lineage of the offspring, which can then directly enhance their postnatal growth and

welfare. The treatment decreases morbidity and mortality in directly treated animals and their offspring, and thus may be of economic importance to the producer and may gainfully contribute to the general welfare of production animals.

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