Maternal GHRH plasmid administration changes pituitary cell lineage and improves progeny growth of pigs

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MATERIALS AND METHODS

Plasmid constructs. The plasmid pSP5–12 contained a 360-bp SacI/BamHI fragment of the SPC5–12 synthetic promoter (23) inserted in the SacI/BamHI sites of the pSK-GHRH backbone (9). To obtain pSP-HV-GHRH, the porcine GHRH cDNA modified to extend its long half-life (HV-GHRH) was cloned into the BamHI/HindIII sites of pSP-GHRH, followed by the 3’-untranslated region and poly(A) signal of the human (h)GH gene (8). HV-GHRH is a GHRH I, the downstream effector of GH, and direct effects of the hormone on target tissues.

The administration of recombinant porcine GH and GHRH has been extensively studied as a means of enhancing or restoring growth in a variety of mammalian species (4, 5, 10, 21, 35). These peptide proteins are degraded in the serum by proteases; therefore either they require relatively frequent dosing or the peptide sequence must be modified to increase its half-life. Under many circumstances, this becomes both time-consuming and expensive. Recently, we demonstrated that plasmid delivery followed by electroporation is an efficient strategy for long-term enhancement of GHRH production (9). Our method of myogenic plasmid delivery requires only a single dose, exerts a long-lasting effect on growth, and, because it does not require the use of viral or lipid particles, is advantageous compared with other methods of gene delivery (26).

We also have demonstrated that pregnant rat dams treated with this system give birth to pups that have increased numbers of somatotrophs and lactotrophs, as well as long-term increase in body weight (18). Together, these studies suggest that stable production of the protease-resistant GHRH can produce a number of physiological effects on the injected organism directly, as well as intergenerational effects on the offspring. Our hypothesis was that similar effects could be obtained in the porcine model despite a longer gestation time and different placental structure compared with the rodent model. Furthermore, if successful in the porcine model, this novel technology could be useful in the agricultural industry.
Analog with a His1 and Val2 substituted with the Tyr1 and Ala2, Gly15 substituted with Ala15, and Met27 and Ser28 with Leu27 and Asn28. A control plasmid, pSP-β-gal, contained the Escherichia coli β-galactosidase gene under control of the same muscle-specific promoter. Plasmids were grown in E. coli DH5α, (GIBCO-BRL, Carlsbad, CA). Endotoxin-free plasmids (Qiagen, Chatsworth, CA) preparations were diluted to 5 mg/ml in sterile water and stored at −80°C before use.

Gilt treatment with HV-GHRH plasmid. Gilts (n = 10, 11 mo of age) were divided randomly into five groups of two gilts per group and fertilized by artificial insemination on the same day with semen from the same boar, and parturition was induced 113 days later. Animals were given ad libitum access to food and water. On day 85 of gestation, gilts were anesthetized with Telazol (1 mg/100 kg), and the pSP-HV-GHRH plasmid was injected into the semitendinosus muscle of each gilt in a constant volume of 0.5 ml at doses of 0.1, 0.5, 1, or 5 mg. The control group was injected with 0.5 mg of pSP-β-gal in a 0.5-ml volume. The injection was followed by electroprostration: 2 min after injection, a pentagonal electrode array composed of 2-cm-long, 21-gauge needles, 1 cm apart, was inserted surrounding the injection point, and electric pulses (1 A for 50 ms, with 1 s between pulses) were applied. Animal protocols were approved by the Baylor College of Medicine and Texas A&M University Animal Care and Use Committees and conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

At parturition, samples of placenta and amniotic fluid were collected, and colostrum was expressed before the piglets were allowed to suckle. Milk was also collected 7, 14, and 21 days postpartum.

Table 1. Characteristics of pigs analyzed from birth to end of study

<table>
<thead>
<tr>
<th>Plasmid Dose, mg</th>
<th>Average Litter Size</th>
<th>Total No. of Piglets Studied</th>
<th>Weight, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>9</td>
<td>12</td>
<td>1.53 ± 0.08</td>
</tr>
<tr>
<td>0.1</td>
<td>12</td>
<td>17</td>
<td>1.55 ± 0.04</td>
</tr>
<tr>
<td>0.5</td>
<td>8</td>
<td>10</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
<td>5</td>
<td>1.72 ± 0.14</td>
</tr>
<tr>
<td>5.0</td>
<td>7</td>
<td>8</td>
<td>1.73 ± 0.07</td>
</tr>
</tbody>
</table>

Radioimmunoassay for GHRH. Porcine GHRH was measured in a heterologous antibody sandwich RIA system (Peninsula Laboratories, Belmont, CA). Sensitivity of the assay is 1 pg/tube. All samples were run in the same assay. The intra-assay variability was 3%.

Radioimmunoassay for IGF-I. Serum and milk IGF-I levels were measured using a heterologous human immunoradioimmunoassay...
diometric assay kit (Diagnostic System Labs, Webster, TX). The inter- and intra-assay variabilities were both 4%. Cross-reactivity of human IGF-I antibody for porcine IGF-I is 100%.

Biochemistry and CBC values. After blood was collected from gilts and piglets, plasma was isolated and aliquoted for complete blood chemical and biochemical analyses (Antech Diagnostics). Results were averaged, and corresponding values from treated animals were compared with those from controls. Serum biochemical end points included alanine aminotransferase, total bilirubin, alkaline phosphatase, total protein, albumin, globulin, cholesterol, blood urea nitrogen, creatinine, phosphorus, calcium, glucose, sodium, potassium, chloride, and creatine phosphokinase.

Meat quality at slaughter. Once the pigs in a treatment group attained an approximate average weight of 100 kg, they were slaughtered, and meat quality, drip loss, Hunter color score, pH, and yield data were collected following standard protocols and procedures at the Texas A&M University, Rosenthal Meat Science Center (15, 34). Percentage of fat-free lean (PFFL) was calculated according to the following formula

\[
\text{PFFL} = 100 \times (8.588 - (21.896 \times 10 \text{th rib fat, in.})
+ (3.005 \times \text{loin eye area, in.}^2) + (0.465 \times \text{carcass weight})
/(\text{carcass weight})
\]

\[
(\text{kg} \times 2.20264)/[(\text{carcass weight}) \times (\text{kg} \times 2.20264))]
\]

Statistical analyses. There were no differences in growth, compositional, or biochemical parameters among the progeny of the gilts that received the two lowest doses, i.e., 0.1 and 0.5 mg, and control piglets. In this case, the variability between groups in the mean values of the various assessed parameters was not greater than the variability within each group. The data from these groups of animals, therefore, were combined for comparison with the 1- and 5-mg groups. Specific P values were obtained using Student’s t-test or ANOVA (P < 0.05 was set as the level of statistical significance). Values are reported as means ± SE.

RESULTS

Body weight. The average birth weights of piglets born to gilts that had received the 5-mg dose of plasmid were significantly (P < 0.025) heavier than birth weights of piglets born to control gilts or to the 0.1- and 0.5-mg groups (Table 1). Piglets born to the 1-mg gilts were larger at birth, but the difference was not statistically significant because of small litter sizes. Litter size varied from 4 to 15 piglets per gilt, with no obvious relationship of litter size to treatment group. However, the small litters with four piglets each were both born to the gilts administered the 1-mg dose. The trend in the postnatal weight gain of the piglets reflected the dose of plasmid administered to the gilt. There were no significant differences across groups between the numbers of male and female piglets born. There was no significant difference in weight gain of male piglets immediately postcastration compared with female littermates. Average daily weight gain was not different between birth and 130 days of age among the control, 0.1-, and 0.5-mg groups (710 ± 6 g/day) and was significantly greater (P < 0.001) in the 1- and 5-mg groups (753 ± 15 and 786 ± 10 g/day, respectively); the difference between the 1- and 5-mg groups was not significant (Fig. 1). Given that birth weight is a strong predictor of postnatal weight gain in pigs, we determined by analysis of covariance whether the effect of maternal treatment on average daily gain of the progeny was still present once differences in birth weight were adjusted. Both birth weight (P < 0.02) and plasmid dose (P < 0.001) remained as significant independent predictors of average daily gain. The differences observed at birth were maintained until the end of the study. At 130 days of age, the progeny of the 5-mg gilts weighed an average of 10 kg more than the progeny of controls (P < 0.001). Offspring of the control and 0.1-, and 0.5-mg gilts required an additional 9 days to attain the same weight as the 5-mg group.

To further evaluate the differences in growth patterns, a quadratic equation was fitted to individual animal growth curves (adjusted r² values ranged from 99.2 to 99.6%), and the coefficients were compared. Only the values of the coefficient that described the linear component were greater (P < 0.012) in the 1- and 5-mg groups (0.161 ± 0.049 and 0.143 ± 0.011 g/day) compared with the 0-, 0.1-, and 0.5-mg groups (0.094 ± 0.009 g/day). The coefficients for the quadratic term were not different among control and treated groups, and the latter did not differ among themselves. Unlike average daily gain, however, the effect of treatment on the coefficient was dependent on birth weight (dose × birth weight, P < 0.03), and the effect of treatment was secondary (P < 0.06) once differences in birth weight were taken into account.

Feed efficiency was similar between groups from the 0.1, 0.5, and 1 mg-treated gilts and controls, and was improved by 3.5% in the 5 mg-treated group of gilts (P < 0.026).

PCR analysis for presence of plasmid. We were unable to detect the presence of plasmid in any of the samples analyzed from the injected gilts or in the livers of the newborn piglets.

Serum GHRH and IGF-I levels. Serum GHRH levels were measured in piglets at birth and weaning. At birth, piglets from gilts treated with a 5-mg plasmid
had 20% higher GHRH levels than control piglets (47 ± 3.1 ng/ml in the 5 mg-treated group vs. 39 ± 5.1 ng/ml in controls, *P = 0.13). In piglets from treated gilts, GHRH concentrations increased on average by 7.2% from birth to weaning, whereas in control piglets there was a 36% increase. This result is consistent with the advanced GH axis maturation and increased IGF-I levels (normal feedback mechanism) in piglets from treated gilts. Serum IGF-I concentrations from the 5-mg group were significantly higher from 21 (weaning) to 68 days of age (*P < 0.05). Values among other groups, including the progeny of the 1.0 mg-treated gilts, were not significantly different at any time point (Fig. 2). IGF-I concentrations after 68 days of age were not obtained because of assay failure. Colostrum and milk IGF-I concentrations were not different among groups.

Biochemistry and CBC values. No differences were found among the groups in either CBC or serum biochemistry panels, and these were within the normal range of values for swine. Basal plasma glucose concentrations were normal in the treated and control gilts and treated piglets and controls at all time points tested.

Pituitary immunohistochemistry. Representative digital images of pituitary sections are presented in Figs. 3 and 4. Significant increases in the numbers of immunopositive somatotrophs and lactotrophs were evident in the 5-mg group vs. the control group: at birth (n = 2 pituitaries/group), there were 19% more somatotrophs (*P = 0.027) and 62.4% more lactotrophs (*P = 0.015), and at slaughter (n = 4–6 pituitaries/group), there were 50% more somatotrophs (*P < 0.001).
0.0012) and 31% more lactotrophs (P < 0.001). Equal numbers of pituitaries from male and female piglets were analyzed for all groups. The relative proportions in the progeny from gilts treated with smaller doses of plasmid, including the 1-mg dose, were similar to those observed for control animals.

**Necropsy and meat quality at slaughter.** Necropsy data showed no pathology or organomegaly associated with the treatment (heart, lung, liver, spleen, brain, adrenals, stomach, kidney, pancreas, intestine). Meat quality analysis showed that dressing percentage, carcass length, muscle score, loin-eye area, and average back-fat thickness were not statistically different among the groups. All animals had similar marbling, firmness, meat pH, and color scores. Pigs in the 1-mg group were leaner than controls (PFPL, P < 0.003). Animals in the 5-mg group had less drip loss than controls (P < 0.003), indicative of a higher water-holding capacity, and drip loss correlated with lean mass, which affects appearance and meat quality positively.

**DISCUSSION**

The results from this study substantiate our previous observation in rats (18) that an augmentation in GHRH production in the pregnant dams can induce permanent changes in pituitary cell lineage of the progeny and is associated with an enhancement in the rate of weight gain in both fetal and postnatal life. The effects were clear for the animals treated with 5 mg of plasmid, as indicated by the enhanced weight gain, improved feed efficacy, serum GHRH and IGF-I concentrations, and increased number of pituitary somatotrophs and lactotrophs in the progeny. The progeny of the gilts injected with 1 mg of plasmid also were larger than controls at birth, weaning, and day 130. However, because the litters born to gilts injected with 1 mg contained only four piglets each, it is not possible to attribute the differences conclusively to the treatment of the gilt with GHRH plasmid. Such differences in birth weight and subsequent growth could have resulted from the reduced intrauterine crowding and competition for nutrients that would promote enhanced fetal growth. Moreover, the absence of pituitary changes and the presence of control levels of IGF-I further support the suggestion that the 1.0-mg dose, like the 0.5- and 0.1-mg doses, did not result in sufficient GHRH production in the mother to elicit a change in pituitary development in the fetus. Thus this study in pigs confirms that the phenomenon is not species-specific.

![Fig. 4. Immunohistochemical analysis for prolactin (PRL)-secreting cells from piglet pituitaries at birth and at slaughter. A: negative control slides are stained without using primary antibody. Representative images are shown from control group and 5-mg group. B: at birth, the proportion of PRL-positive cells was significantly increased in 5-mg group over that in control group (*P < 0.015). At slaughter, the proportion of PRL-positive cells was also significantly increased over that of controls (*P < 3.31 × 10⁻⁶).](http://ajpendo.physiology.org/)

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**Fig. 4.** Immunohistochemical analysis for prolactin (PRL)-secreting cells from piglet pituitaries at birth and at slaughter. A: negative control slides are stained without using primary antibody. Representative images are shown from control group and 5-mg group. B: at birth, the proportion of PRL-positive cells was significantly increased in 5-mg group over that in control group (*P < 0.015). At slaughter, the proportion of PRL-positive cells was also significantly increased over that of controls (*P < 3.31 × 10⁻⁶).
specific for rats (18) and demonstrates that an inter-
generational, nonhereditary biological effect in a large
mammal model is possible with the use of this tech-
nology.

There are a number of possible explanations for the
responses we observed in the progeny of the 5-mg gilts.
We considered the highly unlikely possibility that the
HV-GHRH plasmid itself was transferred to the off-
spring either transplacentally in the prenatal period
and/or via the ingestion of colostrum in the first few
postnatal days. However, by PCR analysis, we were
unable to demonstrate the presence of plasmid in the
placenta, amniotic fluid, and colostrum from the gilt or
in the livers of the newborn offspring. Treatment with
5 mg of plasmid delivers ~1.25 × 10^14 copies of DNA to
the injection site (24). Our limit of detection is such
that we could detect the presence of a minimum of
5,000 molecules, or 0.2 pg, of plasmid. However, a
substantially greater amount of plasmid would be re-
quired to produce a biologically significant amount of
hormone. Moreover, because the HV-GHRH plasmid is
muscle specific (23), it would be effective only if ex-
pressed in myogenic tissue. Thus, had any plasmid
leaked out of the injection site and been transferred to
the offspring in amounts that are biologically signifi-
cant, we would have been able to detect it under the
parameters of the assay. Thus we conclude that the
changes in pituitary cell programming that affect
growth performance of the progeny were attributable
to the increased expression of the GHRH peptide mol-
ecule in the gilts.

The effects of the peptide on the progeny may have
occurred either subsequently to the transplacental
transfer of GHRH and direct action of the peptide on
the fetus itself and/or secondarily to changes in mater-
nal metabolism and physiology that resulted from their
higher levels of GHRH production.

The ability of GHRH to traverse the placental bar-
rrier in the pig has not been demonstrated previously.
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 (7). However, it is well
known that a variety of proteins, and even maternal
cells, can access the fetal circulation; thus the possibil-
ity cannot be dismissed. Previous studies have pointed
to pituitary transcription factor-1 (Pit-1) as a stimula-
tor of somatotroph development (2), and Pit-1 expres-
sion is stimulated by GHRH (14). Maternally derived
GHRH that affects the fetus at a critical stage of
pituitary development could account for the observed
changes in pituitary cell composition. In the pig, dif-
ferentiation of somatotrophs and expression of GH
begin at ~45 days of fetal life. Cells increase rapidly in
number and activity, reaching a maximum at ~95 days
(6, 16). Lactotrophs are the last cells in the pituitary
to differentiate, with the most significant change occur-
ing between day 75 of fetal life and birth (6, 20, 25).
The fetal pituitary responsiveness to GHRH develops
with fetal age. Studies showed that GHRH stimulates
GH release as early as 88 days of gestation and is
maximal at the end of gestation (11). Thus the timing
of the plasmid administration is entirely consistent
with the possibility of a direct effect of GHRH on fetal
pituitary development. Also, the increased circulating
GHRH levels at birth in the piglets from treated gilts
suggest that GHRH can cross the placenta and account
for the changes in pituitary development. The increase
in GHRH levels in controls between birth and weaning
suggests that, at birth, the GHRH/GH/IGF-I axis of
these animals is in a less advanced stage of matura-
tion. Changes in pituitary cell composition are likely to
be of consequence, primarily for the postnatal growth
of the offspring, because there is little evidence to
suggest a role for GH in growth regulation in early life.
In addition to a very low abundance of biologically
active GH receptors, especially in the liver of fetal and
neonatal pigs, it has been observed that the down-
stream responses to GH were minimally affected when
secretion of GH was impaired. For example, decapi-
tated fetuses can maintain relatively normal growth
rates in utero (32), and when GH secretion was ablated
in newborn piglets by the administration of a GHRH
antagonist (36), no significant response in plasma
IGF-I and IGF-binding protein-3 concentrations was
demonstrated despite significantly lower secretion of
GH.

In a previous study, in which GHRH peptide was
administered to pregnant gilts as the recombinant pro-
tein twice a day from 102 days of gestation to parturi-
tion (10), there was no effect on birth weight of the
piglets. The treatment, however, resulted in signifi-
cantly less perinatal mortality among piglets, attribut-
able to a greater maturity at birth as a result of the
prolongation of gestation by 1 day. Although the pigs
were heavier by 2 wk of age, the weight advantage was
not maintained to slaughter. These results (10) con-
trast with those of our study, in which differences in
birth weight were maintained permanently. These dif-
f erences in responses are most probably attributable
to the effective length of exposure to higher GHRH levels
in utero and/or the stage of pregnancy during which
the gilts were treated, as well as the GHRH dose to
which the fetuses were exposed.

The pregnant gilt is highly sensitive to the metabolic
effects of porcine GH and, when treated for various
periods between 25 and 94 days of age, fetal growth is
enhanced (13, 29, 31). GH modulates maternal metab-
olism and uteroplacental transport capacity, thereby
promoting greater nutrient availability to the fetus
and, consequently, more rapid growth. Increased nu-
trient availability is suggested by the higher adipose
tissue content of newborn piglets (10, 29) and increased
fetal liver size (31) that result from GH administration
to gilts. Thus increased maternal GHRH production
during the latter part of pregnancy influenced growth
and maturation of the offspring both acutely, probably
through enhanced substrate transfer from mother to
fetus, and chronically by permanently altering the dif-
f erentiation of pituitary cells toward a greater number
of somatotrophs and lactotrophs. This dual action of
the single GHRH plasmid injection is unique among
the numerous strategies that have been attempted to

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date to enhance the reproductive efficiency in farm animals.

The statistical analysis of the individual animal weight gain curves suggests that the accelerated rate of postnatal weight gain was only partly attributable to the differences in birth weight. Several other mechanisms in conjunction with pituitary lineage change likely are implicated in this response. GHRH or GH concentrations in the maternal colostrum may be increased in response to GHRH treatment. Although these are proteins, and normally would be digested in the intestinal tract, the piglet intestine is extremely permeable to large macromolecules in the first day of life, and there is substantial absorption of intact proteins. Because gut permeability decreases rapidly and the half-life of these proteins is short, it is unlikely that the biological effects of the ingested growth factors would be long-lived. Nonetheless, it is recognized that even short-lived alterations in hormonal signals operating at critical stages of development can permanently reprogram certain aspects of brain function. However, the alterations in cell lineage were evident even before the piglets had access to colostrum, which therefore excludes the hypothesis that exposure to high levels of colostrum-derived GHRH contributed to the observed pituitary changes.

An alternative possibility is that the GHRH treatment of the gilt promotes greater milk production and/or increases the nutrient density of the milk. The efficiency of nutrient utilization for growth in the suckling pig is extremely high, and thus growth rate of the piglet is highly correlated with milk nutrient yield (28). Several studies have observed that GH or GHRH treatment during both pregnancy and lactation increases the fat contents of colostrum and milk (12). Evidence in the literature with regard to the effects of GHRH on milk volume in the gilt is equivocal. Although administration of exogenous GH to lactating gilts increased milk volume with parallel changes in piglet growth (17), GHRH administration to gilts on conventional diets did not alter milk volume unless the dietary management of the gilts to improve feed intake was altered concurrently (12).

Our results contrast with the responses observed in transgenic mice that overexpress GHRH in all tissues. In these mice, pituitary weight at birth is about twice as large as in wild-type mice, and the difference is maintained until tumors develop 10–24 mo later (3). Additionally, 70% of the glands of the transgenic animals contain grossly visible adenomas that stain positively for GH, whereas only some show scattered prolactin staining (19). Within our paradigm, animals were first treated in the last third of the gestation period. Thus the pituitaries of the affected animals were exposed to high levels of hormone in utero (and possibly for a day postnatally) for only a limited time, during a critical period of pituitary development. Evidently, this afforded a change in pituitary cell lineage in the offspring, with increased populations of somatotrophs and lactotrophs and without an increase in gland weight or other pathological changes. The increased numbers of somatotrophs and lactotrophs in pigs from treated gilts were present at birth and persisted until the animals were slaughtered. This provides evidence that these alterations in the pituitary are a permanent, but nonpathological, effect of the treatment regimen.

Currently, the daily or weekly injection of somatotropin (GH) is one of the most effective approaches for improving growth performance and meat quality in swine. However, the absence of an economically feasible mode of delivery has precluded its use in this country. The approach we describe here, which employs a plasmid that encodes for a long-lasting GHRH, obviates many of these drawbacks. Animals in our studies experienced an improvement in growth without the frequent individual treatment required by other growth-promoting agents. A one-time treatment of pregnant gilts with an adequate dose of the HV-GHRH plasmid exhibited effects on the progeny that were similar to those observed when animals are injected repeatedly with somatotropin. Administration of GHRH to the gilt, moreover, has an additional advantage over the direct administration of growth-promoting agents to the adult individual animal. By improving fetal growth, GHRH treatment of the gilts could diminish the incidence of neonatal deaths, which has always represented a major economic loss for the swine industry.

Because of the central role of the GHRH/GH/IGF-I axis in the regulation and coordination of anabolic processes of growth and reproduction, the consequences of plasmid-mediated GHRH supplementation to pregnant animals are far-reaching. During pregnancy, maternal changes impact intrauterine and postnatal development and promote increased perinatal survival of piglets. Concurrently, direct GHRH action induces changes in pituitary cell lineage of the offspring, which can then directly enhance their growth and welfare once the postnatal growth comes under the control of GH and IGF-I.

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