Am J Physiol Endocrinol Metab 285: E224–E231, 2003.
First published April 1, 2003; 10.1152/ajpendo.00050.2003.

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

Amir S. Khan,[1] Marta L. Fiorotto,[2] Kathleen K. Cummings,[1] Melissa A. Pope,[1] Patricia A. Brown,[1] and Ruxandra Draghia-Akli[1]
[1]*ADViSYS, Inc., The Woodlands 77381; and* [2]*Agricultural Research Service, United States Department of Agriculture, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030*

Submitted 5 February 2003; accepted in final form 29 March 2003

Khan, Amir S., Marta L. Fiorotto, Kathleen K. Cummings, Melissa A. Pope, Patricia A. Brown, and Ruxandra Draghia-Akli. Maternal GHRH plasmid administration changes pituitary cell lineage and improves progeny growth of pigs. *Am J Physiol Endocrinol Metab* 285: E224–E231, 2003. First published April 1, 2003; 10.1152/ajpendo.00050.2003.— Previous studies from our laboratory have demonstrated that administration of a myogenic plasmid that encodes a protease-resistant growth hormone-releasing hormone (HV-GHRH) to pregnant rat dams augmented long-term growth in first-generation progeny. In the present study, gilts were injected intramuscularly at *day 85* of gestation with 0, 0.1, 0.5, 1, or 5 mg of the HV-GHRH-expressing plasmid and were then electroporated. Piglets were weighed and bled periodically from birth to 100 kg. Piglets from gilts treated with 1 and 5 mg of HV-GHRH plasmid were larger at birth and weaning compared with controls. These two groups reached 100 kg 9 days earlier than the other groups. GHRH levels were increased at birth in piglets from treated gilts. IGF-I levels were significantly increased in the 5-mg group beginning at 21 days of age compared with controls. Pituitaries from the 5-mg group contained a significantly increased number of somatotrophs and lactotrophs from birth to 100 kg. This study confirms that enhanced maternal GHRH production results in intergenerational growth augmentation and that the magnitude of the response is dose dependent. The similarity of the response across species suggests that the effect is likely exerted as a fundamental component of gestational and developmental physiology.

growth hormone-releasing hormone; insulin-like growth factor I; electroporation

IN HEALTHY ADULT MAMMALS, growth hormone (GH) is released in a regulated, distinctively pulsatile pattern. This phenomenon is under the control of the hypothalamic hormones, growth hormone-releasing hormone (GHRH) and somatostatin. This pulsatile pattern of GH secretion is fundamental to its biological activity (1) and is required for its physiological effects at the peripheral level (33). Regulated GH secretion is essential for optimal linear growth, for homeostasis of carbohydrate, protein, and fat metabolism, and for promoting a positive nitrogen balance (27). These effects are mediated by both insulin-like growth factor I (IGF-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.

MATERIALS AND METHODS

Plasmid constructs. The plasmid pSPc5–12 contained a 360-bp *Sac*I/*Bam*HI fragment of the SPc5–12 synthetic promoter (23) inserted in the *Sac*I/*Bam*HI 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 *Bam*HI/*Hin*dIII 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

Address for reprint requests and other correspondence: R. Draghia-Akli, ADViSYS, Inc., 2700 Research Forest Drive, Suite 180, The Woodlands, TX 77381 (E-mail: ruxandradraghia@advisys.net).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 plasmid (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 electroporation: 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.

Piglet growth and serum end points. Piglets were weighed and numbered immediately at birth, before they were allowed to suckle. The one piglet from each litter with birth weight closest to the average of its respective litter was bled and euthanized, and the pituitary and liver were quickly removed for later analysis. The remaining piglets in the litter stayed with their gilt until 21 days of age. Male piglets were castrated by use of standard procedures before *day 4*, with minimal blood loss. After weaning, the piglets were housed in pens according to treatment group. A small number of piglets were lost in each group due to perinatal or early life mortality or morbidity. At birth, we observed one stillborn in the control group and two stillborns in the 0.1-mg group. One to three piglets in each group (a majority in the 0.5-mg group) were smothered when the gilt lay on them. There were no statistically significant differences in number of piglets lost among groups. The total number of pigs analyzed from birth to the end of the study is summarized in Table 1. After *day 21*, piglets had ad libitum access to water and a 22% protein diet (1.6% lysine). Feed intake was recorded. The pigs were weighed and bled every week for the first month, twice a month for 2 mo, and then once a month. Two groups (1.0 mg and 5.0 mg) were slaughtered at *day 130*, when the groups had reached an approximate average end-of-study weight of 100 kg. The other groups (control, 0.1 mg, and 0.5 mg) were slaughtered at *day 139*, when the groups had reached an approximate average end-of-study weight of 100 kg. A licensed veterinarian examined all internal organs for gross pathologies. At the end of study, pituitaries were removed for analysis, and carcass data were recorded. Whole blood from gilts and piglets was collected in EDTA and submitted for complete blood count (CBC) analysis (Antech Diagnostics, Irvine, CA). Serum was isolated and aliquoted for hormonal and biochemical analyses.

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

Plasmid Dose, mg	Average Litter Size	Total No. of Piglets Studied	Weight, kg		
			Birth	Weaning	130 Days
0	9	12	1.53 ± 0.08	6.83 ± 0.37	93.7 ± 1.54
0.1	12	17	1.55 ± 0.04	6.18 ± 0.23	94.3 ± 1.38
0.5	8	10	1.43 ± 0.07	5.57 ± 0.25	93.2 ± 1.41
1.0	4	5	1.72 ± 0.14	7.84 ± 0.78	96.7 ± 3.39
5.0	7	8	1.73 ± 0.07	7.86 ± 0.20	103.9 ± 1.39

PCR analysis for presence of plasmid. To test for the presence of plasmid, DNA was extracted from the colostrum, placenta, and amniotic fluid of each treated gilt and from the liver of newborn piglets, according to standard procedures (30). PCR was performed to detect the presence of the origin of replication of the injected HV-GHRH plasmid (forward primer 5′-CGAGCGGTATCAGCTCA-3′ and reverse primer 5′-ATCGTCTCGCTCCATACAT-3′) relative to the presence of β-actin as a control (forward primer 5′-ATGTACGTGGCCATCCA-3′and reverse primer 5′-AGGAGGAGGGACCTCTT-3′). The sensitivity of the conventional PCR reaction is routinely one copy of plasmid per microgram of genomic DNA (22). The typical PCR reaction produces a millionfold amplification, and thus we can detect the presence of 0.2 pg or greater amounts of plasmid DNA.

Pituitary immunohistochemistry. Pituitaries were fixed in 4% paraformaldehyde and imbedded in paraffin. Five-micrometer-thick sections were cut, deparaffinized, and washed in PBS. Sections were blocked using a solution of 5% normal goat serum (Jackson Immunoresearch, West Grove, PA), 1% BSA (Sigma, St. Louis, MO), and 0.05% Tween 20 (Sigma) in PBS for 1 h at room temperature. The sections were incubated overnight at 4°C with one of the primary antibodies, monkey anti-porcine GH [AFP10318545, National Hormone and Peptide Program (NHPP)] diluted 1:5,000 or rabbit anti-porcine prolactin [PRL; AFP084255 (NHPP)] diluted 1:30,000. After washes to remove the primary antibodies, the appropriate secondary antibody was applied for 30 min at room temperature, either peroxidase-coupled goat anti-monkey IgG antibody (Research Diagnostics, Flanders, NJ) at a dilution of 1:500 for GH staining or peroxidase-coupled goat anti-rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:500 for PRL staining. Slides were washed in distilled water between each step of the procedure. Peroxidase activity was revealed by use of diaminobenzidine as substrate (Vector Laboratories, Burlingame, CA) for 4 min. Sections were counterstained with hematoxylin (Fisher, Pittsburgh, PA) to visualize cell morphology and nuclei. The sections were visualized on an Olympus BX51 microscope (Leeds Instruments, Irving, TX) with a ×40 objective, and digital images of the sections were captured using an Optronics MagnaFire digital color camera with MagnaFire 2.0 software (Optronics, Goleta, CA). The observer was blinded to the treatment groups and counted a random set of six fields per group, and these fields were averaged within each group. At slaughter, equal numbers of male and female pituitaries were analyzed for each group.

Radioimmunoassay for GHRH. Porcine GHRH was measured by a heterologous human assay 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 immunora-

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

$$\text{PFFL} = 100 \times \{8.588 - (21.896 \times \text{10th rib fat, in.}) + (3.005 \times \text{loin eye area, in.}^2) + [0.465 \times \text{carcass weight (kg} \times 2.20264)]/[\text{carcass weight (kg} \times 2.20264)]\} \quad (1)$$

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.

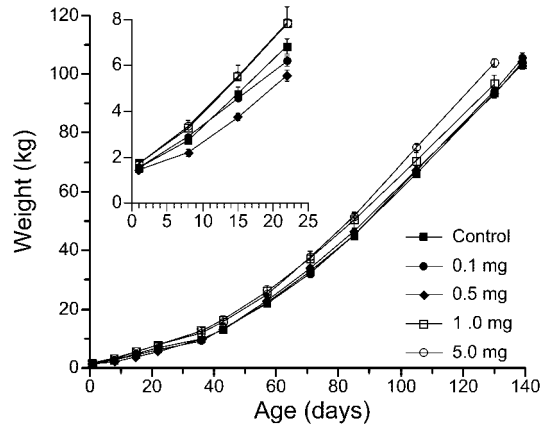

Fig. 1. Growth curves for piglets born from control gilts and gilts treated with 0.1 mg, 0.5 mg, 1 mg, and 5 mg of growth hormone-releasing hormone analog (HV-GHRH) plasmid. Body weights in 1-mg and 5-mg groups are greater than in other groups at birth, and these groups maintain the accelerated growth rate until the end of the study (*day 130*). *Inset*, growth curve from birth to weaning at *day 21*.

To further evaluate the differences in growth patterns, a quadratic equation was fitted to individual animal growth curves (adjusted r^2 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

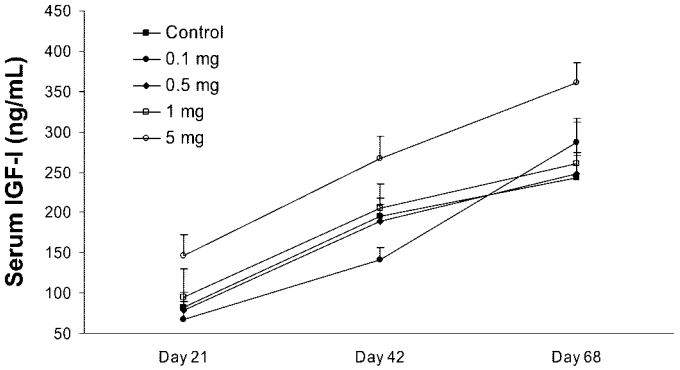

Fig. 2. Serum IGF-I levels in piglets from different treatment groups. Values in 5-mg treatment group are significantly higher than those in controls (*$P < 0.05$), although values from other groups were not significantly different at any time point tested.

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 ($P <$

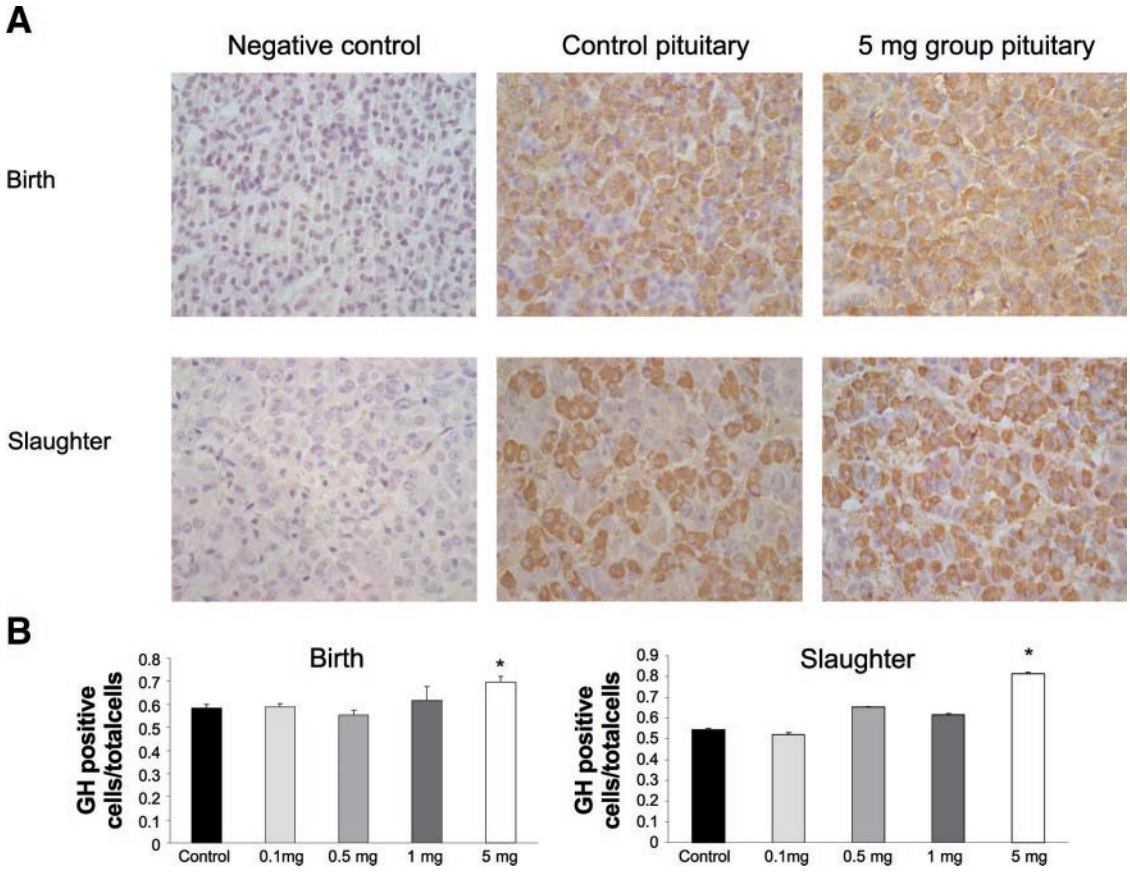

Fig. 3. Immunohistochemical analysis for growth hormone (GH)-secreting cells from piglet pituitaries at birth and at slaughter. *A*: negative control slides are stained without use of primary antibody. Representative images are shown from control group and 5-mg treatment group. *B*: at birth, the proportion of GH-positive cells was significantly increased in 5-mg group compared with the control group (*$P < 0.027$). At slaughter, the proportion of GH-positive cells in 5-mg group was significantly increased over that in controls (*$P < 0.001$).

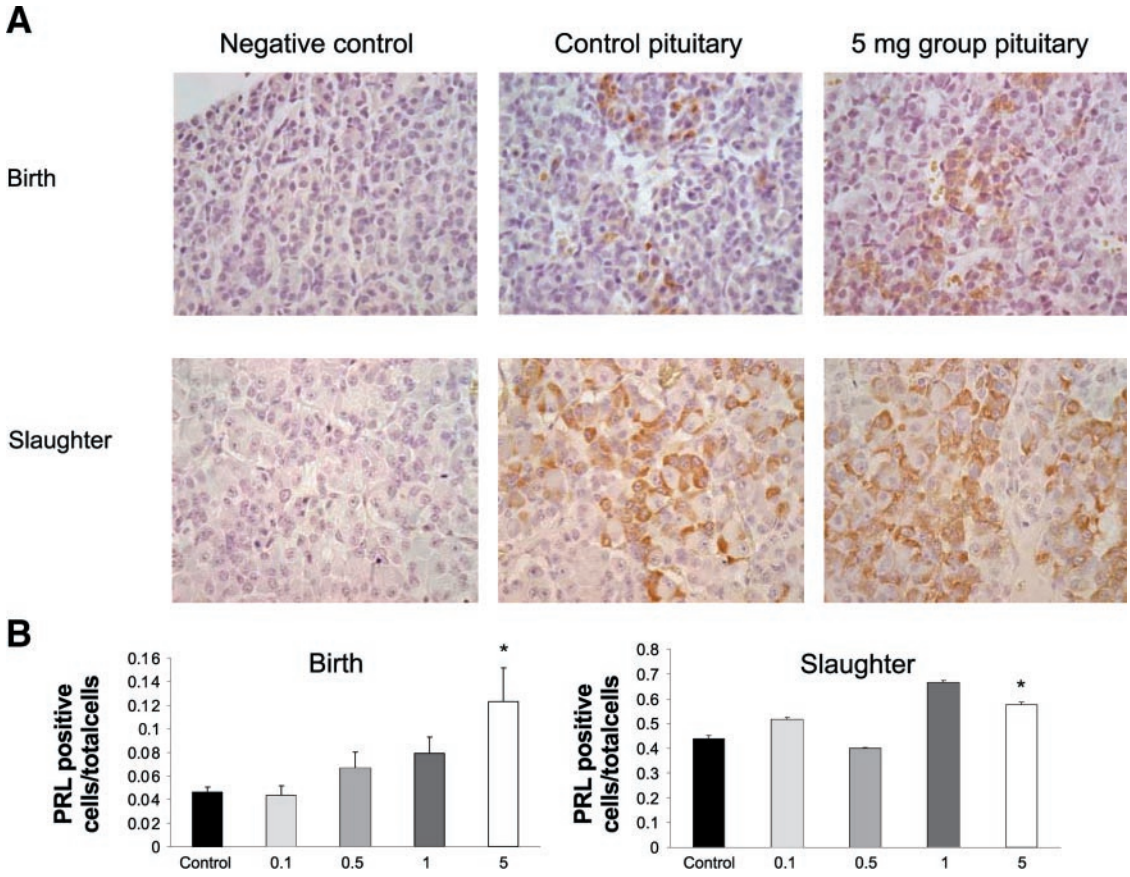

Fig. 4. Immunohistochemical analysis for prolactin (PRL)-secreting cells from piglet pituitaries at birth and at slaughter. *A*: negative control slides are stained without using primary antibody. Representative images are shown from control group and 5-mg group. *B*: at birth, the proportion of PRL-positive 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 ($^*P < 3.31 \times 10^{-6}$).

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 (PFFL, $P < 0.003$). 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 for rats (18) and demonstrates that an intergenerational, nonhereditary biological effect in a large mammal model is possible with the use of this technology.

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 offspring 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 $\sim 1.25 \times 10^{14}$ 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 required to produce a biologically significant amount of hormone. Moreover, because the HV-GHRH plasmid is muscle specific (23), it would be effective only if expressed in myogenic tissue. Thus, had any plasmid leaked out of the injection site and been transferred to the offspring in amounts that are biologically significant, 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 molecule 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 maternal metabolism and physiology that resulted from their higher levels of GHRH production.

The ability of GHRH to traverse the placental barrier 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 possibility cannot be dismissed. Previous studies have pointed to pituitary transcription factor-1 (Pit-1) as a stimulator of somatotroph development (2), and Pit-1 expression 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, differentiation 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 occurring 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 maturation. 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 downstream responses to GH were minimally affected when secretion of GH was impaired. For example, decapitated 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 protein twice a day from 102 days of gestation to parturition (10), there was no effect on birth weight of the piglets. The treatment, however, resulted in significantly less perinatal mortality among piglets, attributable 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) contrast with those of our study, in which differences in birth weight were maintained permanently. These differences 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 metabolism and uteroplacental transport capacity, thereby promoting greater nutrient availability to the fetus and, consequently, more rapid growth. Increased nutrient 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 differentiation 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

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.

We acknowledge Dr. Robert Carpenter for continuous help and encouragement, Karen and Raymond Rosenbaum for their excellent animal husbandry, Dr. Derald Holtkamp for analysis of serum biochemistry values, Dr. Ryan Person of Texas A&M University Rosenthal Meat Science Center for the meat quality data, and Deena Parghi for excellent technical skills and help with the project. We also thank Dr. A. F. Parlow of The National Hormone and Peptide Program for the monkey anti-porcine growth hormone (AFP10318545) and rabbit anti-porcine prolactin (AFP084255) antibodies used in these experiments, and Catherine Tone and Dr. Louis C. Smith for careful editing and correction of the manuscript. We thank Dr. Doug Kern and staff of ADViSYS for their continued support.

M. L. Fiorotto's work was funded in part by the US Department of Agriculture, Agricultural Research Service, under Cooperative Agreement number 58-6250-6001. The contents of this publication do not necessarily reflect the views or policies of the US Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

REFERENCES

1. **Argente J, Pozo J, and Chowen JA.** The growth hormone axis: control and effects. *Horm Res* 45, *Suppl* 1: 9–11, 1996.

2. **Asa SL and Ezzat S.** Molecular determinants of pituitary cytodifferentiation. *Pituitary* 1: 159–168, 1999.
3. **Asa SL, Kovacs K, Stefaneanu L, Horvath E, Billestrup N, Gonzalez-Manchon C, and Vale W.** Pituitary adenomas in mice transgenic for growth hormone-releasing hormone. *Endocrinology* 131: 2083–2089, 1992.
4. **Blanchard MM, Goodyer CG, Charrier J, Kann G, Garcia-Villar R, Bousquet-Melou A, Toutain PL, and Barenton B.** GRF treatment of late pregnant ewes alters maternal and fetal somatotropic axis activity. *Am J Physiol Endocrinol Metab* 260: E575–E580, 1991.
5. **Chung CS, Etherton TD, and Wiggins JP.** Stimulation of swine growth by porcine growth hormone. *J Anim Sci* 60: 118–130, 1985.
6. **Dacheux F.** Functional differentiation of the anterior pituitary cells in the fetal pig. An ultrastructural immunocytochemical study. *Cell Tissue Res* 235: 623–633, 1984.
7. **De Zegher F, Vanderschueren-Lodeweyckx M, Spitz B, Faijerson Y, Blomberg F, Beckers A, Hennen G, and 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* 71: 520–522, 1990.
8. **Draghia-Akli R, Fiorotto ML, Hill LA, Malone PB, Deaver DR, and Schwartz RJ.** Myogenic expression of an injectable protease-resistant growth hormone-releasing hormone augments long-term growth in pigs. *Nat Biotechnol* 17: 1179–1183, 1999.
9. **Draghia-Akli R, Li XG, and Schwartz RJ.** Enhanced growth by ectopic expression of growth hormone releasing hormone using an injectable myogenic vector. *Nat Biotechnol* 15: 1285–1289, 1997.
10. **Etienne M, Bonneau M, Kann G, and Deletang F.** Effects of administration of growth hormone-releasing factor to sows during late gestation on growth hormone secretion, reproductive traits, and performance of progeny from birth to 100 kilograms live weight. *J Anim Sci* 70: 2212–2220, 1992.
11. **Farmer C, Randall G, and Brazeau P.** In vivo growth hormone (GH) response to human GH-releasing factor (GRF) or somatostatin (SRIF) in foetal pigs. *J Dev Physiol* 17: 93–97, 1992.
12. **Farmer C, Robert S, and Matte JJ.** Lactation performance of sows fed a bulky diet during gestation and receiving growth hormone-releasing factor during lactation. *J Anim Sci* 74: 1298–1306, 1996.
13. **Gatford KL, Owens JA, Campbell RG, Boyce JM, Grant PA, De Blasio MJ, and Owens PC.** Treatment of underfed pigs with GH throughout the second quarter of pregnancy increases fetal growth. *J Endocrinol* 166: 227–234, 2000.
14. **Gonzalez-Parra S, Chowen JA, Garcia-Segura LM, and Argente J.** In vivo and in vitro regulation of pituitary transcription factor-1 (Pit-1) by changes in the hormone environment. *Neuroendocrinology* 63: 3–15, 1996.
15. **Goodson KJ, Miller RK, and Savell JW.** Carcass traits, muscle characteristics, and palatability attributes of lambs. *Meat Sci* 58: 381–387, 2001.
16. **Granz S, Ellendorff F, Grossmann R, Kato Y, Muhlbauer E, and Elsaesser F.** Ontogeny of growth hormone and LH beta-, FSH beta- and alpha-subunit mRNA levels in the porcine fetal and neonatal anterior pituitary. *J Neuroendocrinol* 9: 439–449, 1997.
17. **Harkins M, Boyd RD, and Bauman DE.** Effect of recombinant porcine somatotropin on lactational performance and metabolite patterns in sows and growth of nursing pigs. *J Anim Sci* 67: 1997–2008, 1989.
18. **Khan AS, Fiorotto ML, Hill LA, Malone PB, Cummings KK, Parghi D, Schwartz RJ, Smith RG, and Draghia-Akli R.** Nonhereditary enhancement of progeny growth. *Endocrinology* 143: 3561–3567, 2002.
19. **Kineman RD, Teixeira LT, Amargo GV, Coschigano KT, Kopchick JJ, and Frohman LA.** The effect of GHRH on somatotrope hyperplasia and tumor formation in the presence and absence of GH signaling. *Endocrinology* 142: 3764–3773, 2001.
20. **Klindt J and Stone RT.** Porcine growth hormone and prolactin: concentrations in the fetus and secretory patterns in the growing pig. *Growth* 48: 1–15, 1984.
21. **Kulinski KM, Thompson DL Jr, Capshaw EL, French DD, and Oliver JL.** Daily treatment of growing foals with equine somatotropin: pathologic and endocrinologic assessments at necropsy and residual effects in live animals. *J Anim Sci* 80: 392–400, 2002.
22. **Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, Harper LB, Schock HB, Zhang H, Faris JE, Way PA, Beare CM, Bagdon WJ, and Nichols WW.** Plasmid DNA vaccines: assay for integration into host genomic DNA. *Dev Biol (Basel)* 104: 33–43, 2000.
23. **Li X, Eastman EM, Schwartz RJ, and Draghia-Akli R.** Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nat Biotechnol* 17: 241–245, 1999.
24. **Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, Griffiths TG, Niu Z, Denisova L, Follmer TT, Pacchione SJ, Wang Z, Beare CM, Bagdon WJ, and Nichols WW.** Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirology* 43: 273–281, 2000.
25. **Meijer JC, Trudeau VL, Colenbrander B, Poot P, Erkens JH, and Van de Wiel DF.** Prolactin in the developing pig. *Biol Reprod* 39: 264–269, 1988.
26. **Muramatsu T, Arakawa S, Fukazawa K, Fujiwara Y, Yoshida T, Sasaki R, Masuda S, and Park HM.** In vivo gene electroporation in skeletal muscle with special reference to the duration of gene expression. *Int J Mol Med* 7: 37–42, 2001.
27. **Murray RD and Shalet SM.** Growth hormone: current and future therapeutic applications. *Expert Opin Pharmacother* 1: 975–990, 2000.
28. **Noblet J and Etienne M.** Estimation of sow milk nutrient output. *J Anim Sci* 67: 3352–3359, 1989.
29. **Rehfeldt C, Fiedler I, Weikard R, Kanitz E, and Ender K.** It is possible to increase skeletal muscle fibre number in utero. *Biosci Rep* 13: 213–220, 1993.
30. **Sambrook J, Fritsch EF, and Maniatis T.** *Molecular Cloning—A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
31. **Sterle JA, Cantley TC, Lamberson WR, Lucy MC, Gerrard DE, Matteri RL, and Day BN.** Effects of recombinant porcine somatotropin on placental size, fetal growth, and IGF-I and IGF-II concentrations in pigs. *J Anim Sci* 73: 2980–2985, 1995.
32. **Stryker JL and Dziuk PJ.** Effects of fetal decapitation on fetal development, parturition and lactation in pigs. *J Anim Sci* 40: 282–287, 1975.
33. **Veldhuis JD.** Neuroendocrine control of pulsatile growth hormone release in the human: relationship with gender. *Growth Horm IGF Res* 8, *Suppl* B: 49–59, 1998.
34. **Wahrmund-Wyle JL, Harris KB, and Savell JW.** Beef retail cut composition. 2. Proximate analysis. *J Food Compos Anal* 13: 243–251, 2000.
35. **Wester TJ, Davis TA, Fiorotto ML, and Burrin DG.** Exogenous growth hormone stimulates somatotropic axis function and growth in neonatal pigs. *Am J Physiol Endocrinol Metab* 274: E29–E37, 1998.
36. **Wester TJ, Fiorotto ML, Klindt J, and Burrin DG.** Feeding colostrum increases circulating insulin-like growth factor I in newborn pigs independent of endogenous growth hormone secretion. *J Anim Sci* 76: 3003–3009, 1998.