



High mobility group A2 (HMGA2) deficiency in pigs leads to dwarfism, abnormal fetal resource allocation, and cryptorchidism

Jaewook Chung^{a,b,1}, Xia Zhang^{a,b}, Bruce Collins^{a,c}, Renan B. Sper^{a,b,2}, Katherine Gleason^{a,b}, Sean Simpson^{a,b}, Sehwon Koh^{a,b,3}, Jeffrey Sommer^c, William L. Flowers^c, Robert M. Petters^c, and Jorge A. Piedrahita^{a,b,4}

^aComparative Medicine Institute, North Carolina State University, Raleigh, NC 27607; ^bCollege of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607; and ^cDepartment of Animal Science, North Carolina State University, Raleigh, NC 27695

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Expression of *HMGA2* is strongly associated with body size and growth in mice and humans. In mice, inactivation of one or both alleles of *Hmga2* results in body-size reductions of 20% and 60%, respectively. In humans, microdeletions involving the *HMGA2* locus result in short stature, suggesting the function of the *HMGA2* protein is conserved among mammals. To test this hypothesis, we generated *HMGA2*-deficient pigs via gene editing and somatic cell nuclear transfer (SCNT). Examination of growth parameters revealed that *HMGA2*^{-/+} male and female pigs were on average 20% lighter and smaller than *HMGA2*^{+/+} matched controls ($P < 0.05$). *HMGA2*^{-/-} boars showed significant size reduction ranging from 35 to 85% of controls depending on age ($P < 0.05$), and organ weights were also affected ($P < 0.05$). *HMGA2*^{-/+} gilts and boars exhibited normal reproductive development and fertility, while *HMGA2*^{-/-} boars were sterile due to undescended testes (cryptorchidism). Crossbreeding *HMGA2*^{-/+} boars and gilts produced litters lacking the *HMGA2*^{-/-} genotype. However, analysis of day (D) D40 and D78 pregnancies indicated that *HMGA2*^{-/-} fetuses were present at the expected Mendelian ratio, but placental abnormalities were seen in the D78 *HMGA2*^{-/-} concepti. Additionally, *HMGA2*^{-/-} embryos generated by gene editing and SCNT produced multiple pregnancies and viable offspring, indicating that lack of *HMGA2* is not lethal per se. Overall, our results show that the effect of *HMGA2* with respect to growth regulation is highly conserved among mammals and opens up the possibility of regulating body and organ size in a variety of mammalian species including food and companion animals.

HMGA2 | dwarfism | swine | gene editing | organ size

The high mobility group A2 (*HMGA2*) gene encodes a non-histone chromosomal and architectural transcription factor that facilitates 3D changes in chromatin structure (1). *HMGA2* proteins contain a binding domain peptide with three AT-hook motifs that preferentially recognize AT-rich regions in the minor groove of DNA. Of the three AT-hooks, the second motif is the most critical component for *HMGA2* protein function, and the motif is highly conserved among diverse organisms (2), including pigs. Acting as an enhanceosome, chromatin-bound *HMGA2* promotes the recruitment of regulatory protein complexes involved in cell proliferation and differentiation (3). During mouse embryonic development, *Hmga2* expression is detected at a high level throughout the embryo except in the embryonic brain, but it is not detected in normal adult tissues except in the testes (4, 5). In humans, genome-wide association studies showed that variation in *HMGA2* gene affects human height (6), and identification of an intragenic microdeletion in the *HMGA2* gene in short-stature patients further supports a direct role of *HMGA2* in human growth (7). *HMGA2* has also been implicated in dwarfism in rabbits (8), body weight in dogs (9), and beak size in birds (10). Thus, genetic evidence supports a conserved role for *HMGA2* in growth regulation.

Direct evidence for the role of *Hmga2* in growth regulation comes from observations in small or “pygmy” mice with naturally occurring mutations in *Hmga2* (11, 12). *Hmga2* effects on body size were confirmed by inactivation of the mouse *Hmga2* by insertional inactivation or homologous recombination (3, 13). Compared with wild-type controls, adult mice with inactive *Hmga2*, whether due to an induced or a naturally occurring mutation, were 60% smaller, and adult heterozygous mutants were 20% smaller (3, 13). Organ size was also affected (14). In contrast, overexpression of a truncated form of *HMGA2* resulted in gigantism and lipomatosis in mice (15). Despite the documented role in regulation of body size in mice, the definitive function of *HMGA2* in pigs remains elusive; *HMGA2* has been associated with ear size in pigs (16, 17) and higher expression in fetal skeletal muscle of breeds with higher muscularity (18). Previously it has been shown that inactivation of the growth hormone receptor (*GHR*) in pigs results in reduced adult weight and size and increase in obesity. As of yet, however, there are no reports of the effect of *HMGA2* inactivation in pigs. The goal of this project, therefore, was to understand the role of *HMGA2* in body and organ-size regulation in pigs and to determine the usefulness of *HMGA2* modification for

Significance

We show that mutations in *HMGA2* affect fetal resource allocation, testis descent, and the size of pigs and provides a target for gene modification that can be used to modulate size in other mammalian species. This can have implications in agriculture as well as in the development of new strains of companion animals. In addition, most xenograft pig donors have adult organs larger than those of humans. Recently, it has been shown that regulation of organ growth is donor-controlled, not host-controlled, resulting in organ overgrowth and damage after transplantation. We show here that the *HMGA2* gene is a potential target for organ-size regulation in xenotransplantation.

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¹Present address: Department of Animal & Avian Sciences, University of Maryland, College Park, MD 20742.

²Present address: Department of Surgery, University of San Francisco School of Medicine, San Francisco, CA 94143.

³Present address: Department of Neurobiology, Duke University Medical School, Durham, NC 27710.

⁴To whom correspondence should be addressed. Email: Jorge_Piedrahita@ncsu.edu.

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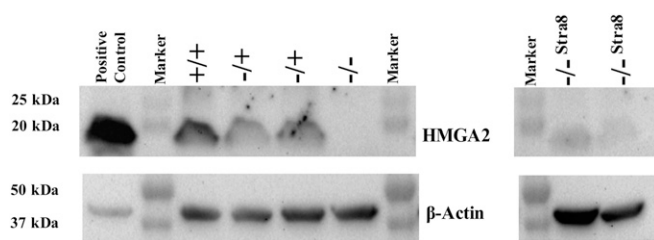


Fig. 1. HMGA2 protein in gene-edited fetal fibroblasts. HMGA2 protein levels for $HMGA2^{+/+}$, $HMGA2^{+/-}$, $HMGA2^{-/-}$, and $HMGA2^{-/-Stra8}$ fetal fibroblasts were detected by Western blot. Proteins were extracted from primary D40 fetal fibroblasts of all three $HMGA2$ genotypes generated from the breeding of $HMGA2^{+/+}$ founders. $HMGA2^{-/-Stra8}$ samples were isolated from primary D43 fetal fibroblasts generated by SCNT. $HMGA2^{+/+}$ had twice as much protein as $HMGA2^{+/-}$ samples, suggesting there was no compensation from the remaining active allele (*SI Appendix, Fig. S2*). There was no protein detected from $HMGA2^{-/-}$ or $HMGA2^{-/-Stra8}$ fibroblasts. β -Actin was used as internal control. HEK293 lysate was used as positive control.

the regulation of size in genetically modified pigs to be used for biomedical research including xenotransplantation. Reduced size will allow for better match between organ receipt and donor (19) as well as facilitate animal housing and management in a biomedical setting.

Results

Generation of HMGA2-Deficient Pigs. To study the effect of disruption of the $HMGA2$ gene on fetal and adult growth $HMGA2^{+/+}$ male and female fetal fibroblasts cell lines were generated as somatic cell nuclear transfer (SCNT) donors. As $HMGA2$ is expressed in fetal fibroblasts, conventional homologous recombination using β -geo and a gene-trap approach was used (20) (*SI Appendix, Fig. S1 and Table S1*). Gene targeting events in single cell-derived colonies were confirmed as described in *SI Appendix*. Targeting rates were 20/34 (58.8%) for male cell lines and 4/28 (14.3%) for female cell lines. These rates are comparable to those reported in sheep somatic cells using gene trap and selection (21). $HMGA2^{+/-}$ female and male pigs generated by SCNT were crossbred and litters analyzed. In addition, we generated $HMGA2^{-/-}$ offspring by gene editing with transcription activator-like effector nucleases (TALENs) the remaining wild-type allele in the $HMGA2^{+/-}$ fetal fibroblasts. We also used TALENs to generate knock-out/knock-in lines that disrupted the endogenous $HMGA2$ locus while also introducing an $HMGA2$ coding sequence under the control of the mouse $Stra8$ promoter (*SI Appendix, Fig. S1*). This results in inactivation of the $HMGA2$ locus but testis-specific expression of an $HMGA2$ cDNA via the $Stra8$ promoter. This was done to potentially reduce previously reported spermatogenesis defects in $Hmga2^{-/-}$ mice (5), without affecting the influence of $HMGA2$ on overall body size. $HMGA2^{-/-Stra8}$ colonies were identified by PCR screening followed by sequencing, and 8/40 (20%) cell lines analyzed had the correct modification. To confirm functional disruption of $HMGA2$ fibroblast cell lines derived from $HMGA2^{+/+}$, $HMGA2^{-/-Stra8}$, $HMGA2^{-/-}$, and control fibroblast lines were analyzed by Western blot (Fig. 1). Both $HMGA2^{-/-}$ and $HMGA2^{-/-Stra8}$ cell lines showed the absence of HMGA2, while $HMGA2^{+/-}$ cells had reduced levels compared with wild-type controls. Densitometry analysis showed that $HMGA2^{+/-}$ cells had approximately half the level of HMGA2 compared with wild-type cells (*SI Appendix, Fig. S2*). Using $HMGA2$ mutant cell lines we generated 8 $HMGA2^{+/+}$ boars, 11 $HMGA2^{-/-Stra8}$ boars, 5 $HMGA2^{-/-}$ boars, and 6 $HMGA2^{+/-}$ gilts. As no statistical differences in any phenotype measured were observed between $HMGA2^{-/-}$ and $HMGA2^{-/-Stra8}$ pigs, data from both lines were combined and referred to as $HMGA2$ -null heretofore.

Fetal Viability of HMGA2-Null Concepti. Mating of $HMGA2^{+/+} \times HMGA2^{+/+}$ founders resulted in three pregnancies to term,

producing a total of 19 piglets: 6 $HMGA2^{+/+}$ and 13 $HMGA2^{-/-}$, no live $HMGA2^{-/-}$, and 8 highly degraded dead fetuses (mummies) that could not be genotyped (*SI Appendix, Fig. S3*). A χ^2 test to validate the goodness of fit for Mendelian ratios genotypes indicated that the live offspring data did not meet the expected ratios ($P < 0.05$). To determine the timing of fetal loss and to identify a cause for the loss, one pregnancy at day (D) D40 and two pregnancies at D78 were examined. At D40 and D78 $HMGA2$ genotyping of fetuses confirmed the presence of all genotypes, including $HMGA2^{-/-}$, at the expected Mendelian frequency. Size differences were evident at fetal stages, with both male and female $HMGA2$ -null fetuses examined at D40 and D78 of gestation being smaller and lighter than $HMGA2^{+/+}$ fetuses (Fig. 2 and *SI Appendix, Fig. S4*). While no obvious defects beyond size reduction were detected in the $HMGA2^{-/-}$ D78 fetuses, abnormalities were observed in the D78 $HMGA2^{-/-}$ placentas at both the macroscopic and microscopic level (Fig. 3). Macroscopically, $HMGA2^{-/-}$ D78 placentas showed extensive degeneration and detached easily from the uterus (Fig. 3). At the histological level, $HMGA2^{-/-}$ placentas had abnormal rugae at the contact area between chorionic membrane and uterine endometrium. The calculated perimeter (micrometers) and area (square micrometers) in contact between fetal membranes and endometrium were significantly reduced between $HMGA2^{+/+}$ and $HMGA2^{-/-}$ placentas by 9% and 32%, respectively ($P < 0.05$). To determine if there was an interaction between the $HMGA2$ deficiency and uterine crowding, unilateral oviduct ligation was performed on two $HMGA2^{+/-}$ F1 gilts. This results in smaller litter sizes and reduced uterine crowding. The resulting pregnancies produced nine live offspring (five and four piglets per pregnancy) and one degraded mummy. Of the nine live piglets, four were $HMGA2^{+/+}$ and five were $HMGA2^{-/-}$. No live $HMGA2^{-/-}$ fetuses survived to term.

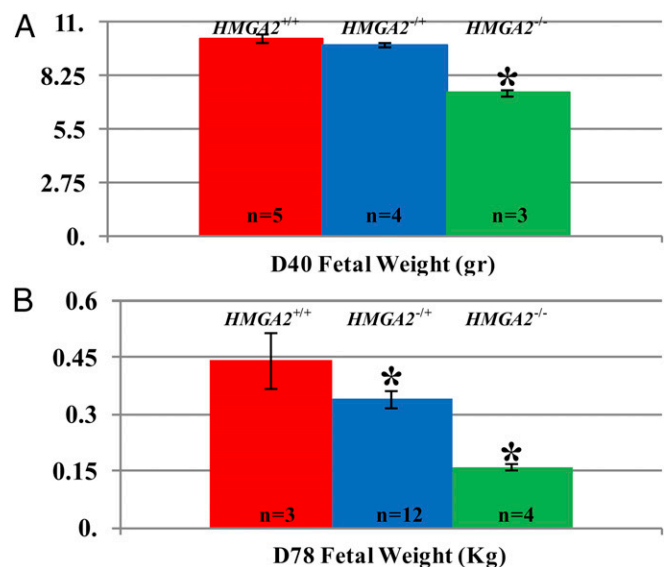


Fig. 2. Fetal weights (D40 and D78) of $HMGA2$ gene-edited pigs. Pregnancies were generated by crossbreeding $HMGA2^{+/+}$ gilts and boars. (A) Fetuses with all expected genotypes were detected at D40 at the expected Mendelian frequency. Weights of $HMGA2^{+/-}$ fetuses were comparable to those of $HMGA2^{+/+}$ fetuses, but the $HMGA2^{-/-}$ fetuses were significantly smaller (30% reduced) compared with the other two genotypes. Two females and one male $HMGA2^{-/-}$ fetuses were collected, all size-restricted. (B) At D78, $HMGA2^{+/-}$ fetuses were 24% smaller and $HMGA2^{-/-}$ were 72% smaller than $HMGA2^{+/+}$ fetuses. Both $HMGA2^{+/-}$ and $HMGA2^{-/-}$ male and female fetuses were affected (nine females:three males for $HMGA2^{+/-}$ and one female:three males for $HMGA2^{-/-}$).

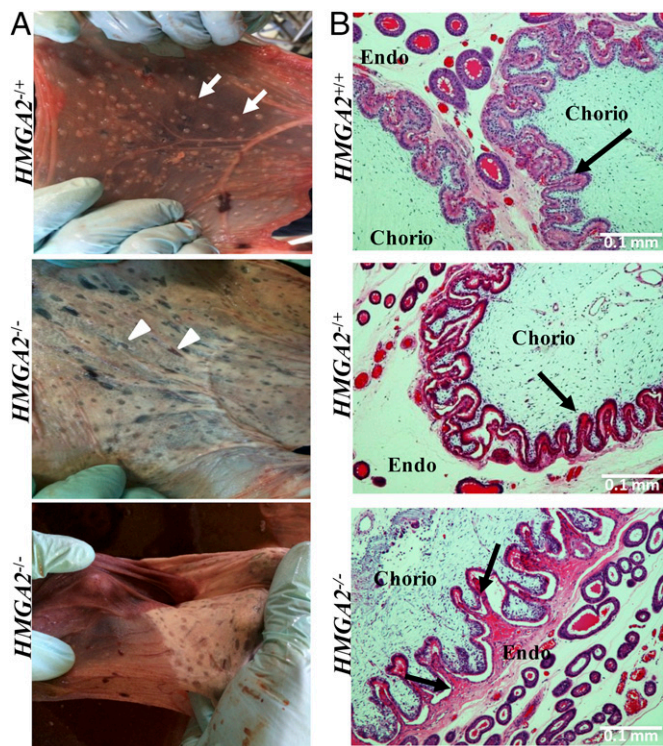


Fig. 3. Placental morphology in *HMGGA2* concepti at D78 of gestation. (A) *HMGGA2*^{+/+} placentas were healthy and well-vascularized, while *HMGGA2*^{-/-} placentas showed lack of absorptive structures (areolae; arrowheads compared with arrows) and vascularization. (B) Histological analysis of placenta and endometrium from *HMGGA2*^{+/+}, *HMGGA2*^{+/-}, and *HMGGA2*^{-/-} fetuses. Villi were severely degenerated in *HMGGA2*^{-/-} placenta compared with other genotypes. Furthermore, poor contact of villi between uterine endometrium (Endo) and chorionic membrane (Chorio) was detected for *HMGGA2*^{+/-} (arrow), suggesting the connection between uterus and placenta was deficient. (Scale bars: 100 μ m.)

Effects of *HMGGA2* Modifications on Growth Parameters. Due to the inability to generate *HMGGA2*^{-/-} offspring by breeding, growth comparisons were carried out using SCNT-generated animals. In boars, for the first 3 mo, length, height, circumference, and body weights of *HMGGA2*^{+/-} SCNT animals were not significantly different from *HMGGA2*^{+/+} SCNT controls. However, from 4 to 6 mo of age, *HMGGA2*^{+/-} SCNT boars were 17% ($P < 0.05$) and 16% ($P < 0.05$) lighter compared with *HMGGA2*^{+/+} SCNT boars and naturally bred boars, respectively (Fig. 4). Body length, height, and circumference measurements showed similar reductions in size (Fig. 4). *HMGGA2*-null SCNT boars showed a dramatic reduction in all measured growth/size parameters from birth (Fig. 4). A representative photograph of all *HMGGA2* genotypes at 6 mo of age is shown in Fig. 5. To determine if the *HMGGA2* deficiency was affecting symmetry, an allometric growth analysis was performed. Each set of measurements (body length, height, circumference, and weight) was log-transformed and plotted to determine the scaling exponent, represented by the slope of the line of best fit. Standardized major axis regression analysis indicated that these slopes were not significantly different from one another (SI Appendix, Fig. S5). This suggests that none of the *HMGGA2* mutations affected the scaling exponent, indicating that the relationship between each measurement changes with growth is similar in both mutant and control pigs. However, significant differences in the y-intercepts of the regression lines of best fit were observed in the length vs. circumference ($P < 0.05$) and circumference vs. weight comparisons ($P < 0.05$). Pairwise comparisons revealed that at the same given length the circumference of the *HMGGA2*^{-/-} pigs was

5% less than the SCNT and wild-type controls ($P < 0.05$), and that the heterozygous circumference measurements were 5% larger than the SCNT and wild-type controls ($P < 0.05$). At the same circumference, the weight for heterozygous mutant was lower than that of the SCNT and wild-type controls by 6% and 7%, respectively ($P < 0.05$), while the weight of the homozygous mutant was greater than that of the controls by 21% and 18%, respectively ($P < 0.05$). Essentially the *HMGGA2*^{-/-} mutants had a greater weight-to-size ratio (higher density/leaner) compared with a wild-type pigs of the same size. These morphological differences apply at birth and are retained throughout the animal's life, as indicated by identical regression slopes. In gilts, disruption of one *HMGGA2* allele resulted in a 35% reduction in growth parameters, compared with control gilts at 30 wk of age (SI Appendix, Fig. S6). This reduction remained significant for *HMGGA2*^{+/-} gilts until the end of the study, at 59 wk of age. In addition to examining allometric whole-body growth parameters,

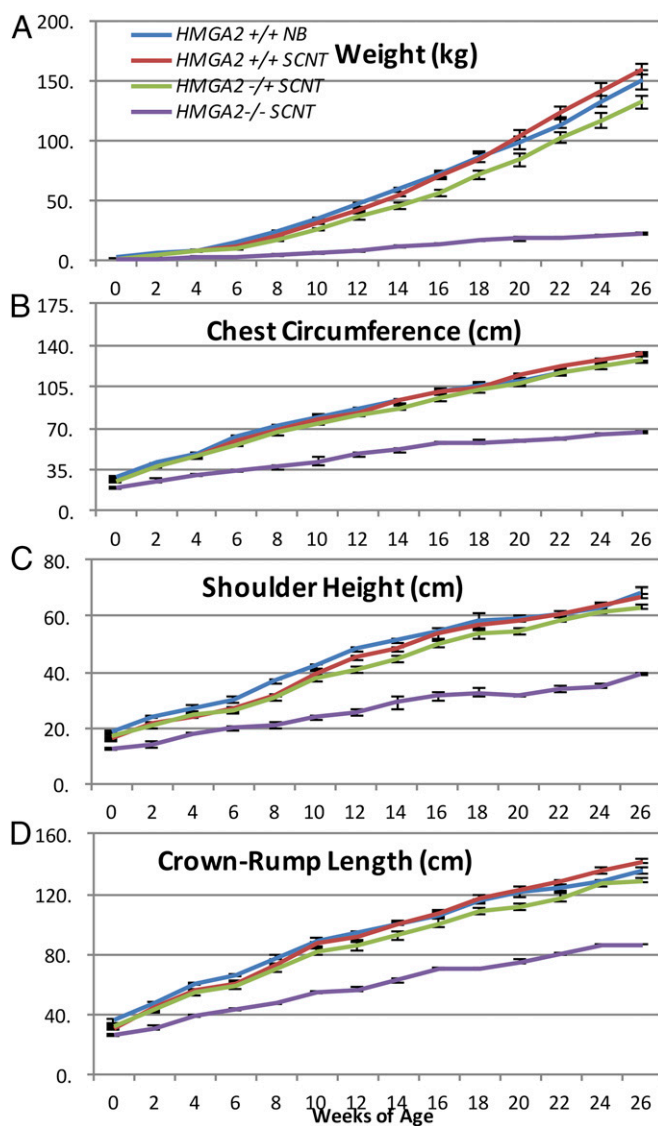


Fig. 4. Growth comparison of *HMGGA2*^{-/-} SCNT, *HMGGA2*^{+/-} SCNT, *HMGGA2*^{+/+} SCNT, and *HMGGA2*^{+/+} naturally bred (NB) boars. Measurements for weight (A), chest circumference (B), shoulder height (C), and crown-rump length (D) were taken every 2 wk over a 26-wk period. *HMGGA2*^{+/+} NB, $n = 5$; *HMGGA2*^{+/+} SCNT, $n = 6$; *HMGGA2*^{+/-}, $n = 6$; *HMGGA2*^{-/-}, $n = 3$ (one *HMGGA2*^{-/-} died at 22 wk so last 4 wk $n = 2$). Error bars indicate SEM.

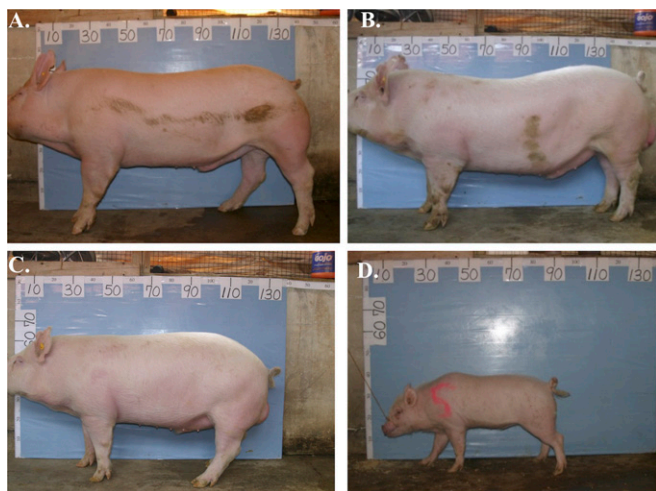


Fig. 5. Visual comparison of representative *HMGA2*^{+/+}, *HMGA2*^{-/-}, and *HMGA2*^{-/-} SCNT boars. At 26 wk of age boars were photographed in front of the same measuring board for to facilitate size comparisons. Board numbering is in centimeters. (A) *HMGA2*^{+/+} NB, (B) *HMGA2*^{+/+} SCNT, (C) *HMGA2*^{-/-} SCNT, (D) *HMGA2*^{-/-} SCNT. Note drastic reduction in size and stocky appearance of the *HMGA2*^{-/-} boar. NB, naturally bred.

we examined whether organ weights were affected. As shown in Table 1, *HMGA2*^{-/+} boars at 9 mo of age had smaller heart and testis weights, while no statistically significant reductions were observed in other organs examined. Compared with *HMGA2*^{+/+} gilts, *HMGA2*^{-/+} gilts at 2.5 y of age also had reduced organ weights with the exception of brain and lung (Table 2). In *HMGA2*^{-/-} pigs, organ sizes at 1 wk of age (Table 3) and 15 wk (SI Appendix, Fig. S7 and Table S2) were affected compared with controls and all organs showed significant reductions. Some organs, however, were proportionally more affected than others (Tables 1–3).

Effect of *HMGA2* on Male and Female Reproductive Characteristics.

Both *HMGA2*^{-/+} boars and *HMGA2*^{-/+} F1 gilts showed normal estrus behavior and reproductive characteristics. Sperm collected from *HMGA2*^{-/+} boars had normal morphology and concentration. LacZ staining assay on embryos generated by in vitro fertilization of *HMGA2*^{-/+} oocytes with *HMGA2*^{-/+} sperm confirmed its ability to fertilize as well as germ-line transmission of the β -*geo* transgene (SI Appendix, Fig. S8). Gross pathological and histological examination of the testes and ovaries from the *HMGA2*^{-/+} boars and gilts revealed they were small but otherwise normal (Fig. 6 and SI Appendix, Figs. S9 and S10). In contrast, all *HMGA2*-null boars had undescended testes (cryptorchidism) at all time points examined, and upon dissection those testes were smaller in size than those of age-matched controls (Tables 1 and 3 and SI Appendix, Fig. S11). Comparison of neonate and 4-mo cryptorchid *HMGA2*^{-/-}-*Stra8* boars to age-matched *HMGA2*^{+/+} testes showed that the internal structure and organization of seminiferous tubules in the *HMGA2*-null testes were morphologically normal in the neonates (Fig. 6). However, the seminiferous tubules in *HMGA2*^{-/-}-*Stra8* undescended testes showed no evidence of spermatogenesis at 4 mo (Fig. 6). Analysis of expression of *HMGA2* in control and *HMGA2*^{-/-}-*Stra8* testis confirmed expression of *HMGA2* from the mouse *Stra8* promoter (Fig. 6C and SI Appendix, Tables S3 and S4).

Discussion

While the role of *Hmga2* on mouse body and organ size and fertility has been examined (3, 5, 13), its role in prenatal and postnatal growth, organ size, and reproductive traits in different

Table 1. Body and organ weight comparison of *HMGA2*^{-/+} vs. *HMGA2*^{+/+} boars at 9 mo of age

Body and organs	<i>HMGA2</i> ^{-/+} (n = 5)	<i>HMGA2</i> ^{+/+} (n = 6)	Change in weight, * %
Body [†]	165.2 ± 37.3 kg	221.73 ± 3.6 kg	74.5
Adrenal glands [†]	5.8 ± 0.2 g	6.8 ± 0.7 g	84.9
Brain	86.8 ± 3.2 g	98.5 ± 2.9 g	88.1
Heart [†]	504.4 ± 27.5 g	638.5 ± 15.6 g	79.0
Kidneys	671.6 ± 23.2 g	656.3 ± 30.2 g	102.3
Livers	3,056.6 ± 67.2 g	3,145 ± 193.4 g	97.2
Lungs	830.2 ± 85.6 g	926.8 ± 49.0 g	89.6
Testes [†]	1,081.8 ± 65.5 g	1,282.2 ± 56.9 g	84.4

*Change in weight is the ratio of body or organ weights of *HMGA2*^{-/+} over *HMGA2*^{+/+}.

[†]Significant at *P* < 0.05.

species is still elusive. In pigs, the effect of *HMGA2* deficiency on pig body and organ size, reproduction, and fetal viability has never been reported. Our results show that adult *HMGA2*^{-/+} pigs are ~80% size of wild-type controls, which is in agreement with reports of *Hmga2*^{-/+} mice. *HMGA2*-null pigs were severely growth-restricted at all stages examined, with animals at 3 mo of age being one-fifth the size of controls (Fig. 4); this is a more severe phenotype than the reported 50–60% size reduction in mice (3, 13) and is more in line with the *Hmga1/Hmga2*-null “superpygmy” mice (22). The differences in growth reduction observed between the *HMGA2*^{-/+} lines and the *HMGA2*^{+/+} lines and the Western blot analysis (Fig. 1 and SI Appendix, Fig. S4) support *HMGA2* haploinsufficiency, as reported in the mouse. Moreover, compared with *HMGA2*^{+/+} pigs, allometric analysis showed that the body-size reduction in both *HMGA2*^{-/+} and *HMGA2*-null pigs was proportional for length, weight, and height ratios. When the relationship of circumference to weight and length was examined, however, differences were apparent. This was most striking in the *HMGA2* nulls, which were heavier than controls of similar size, suggesting a leaner phenotype. This is consistent with the reported lean phenotype of *Hmga2*-null mice (23). Organ sizes were also affected in the *HMGA2*-deficient animals (Tables 1–3). As reported previously in mice (14), some organs were proportionally more affected than others, with brain being the least affected. It should be noted that SCNT can cause epigenetic effects that affect growth/weight in mice (24). In pigs, we previously demonstrated that these weight differences are small and nonsignificant in adult SCNT pigs (25).

Table 2. Body and organ weight comparison of *HMGA2*^{-/+} vs. *HMGA2*^{+/+} nonparous gilts at 2.5 y of age

Body and organs	<i>HMGA2</i> ^{-/+} (n = 4)	<i>HMGA2</i> ^{+/+} (n = 2)	Change in weight, * %
Body	253.3 ± 7.5 kg	275.5 ± 15.5 kg	91.9
Adrenal glands [†]	8.0 ± 0.8 g	11.0 ± 1.0 g	72.7
Brain	116.0 ± 0.9 g	118.5 ± 4.5 g	98.3
Heart [†]	497.3 ± 9.0 g	853.0 ± 10.0 g	58.3
Kidneys [†]	381.8 ± 18.5 g	704.5 ± 82.5 g	54.2
Livers [†]	2,372.5 ± 59.3 g	3,224.5 ± 208.5 g	73.6
Lungs	1,238.8 ± 136.2 g	1,161.0 ± 59.0 g	106.7
Uterus [†]	647.0 ± 67.6 g	1,201.0 ± 25.0 g	53.8
Ovaries [†]	29.7 ± 7.8 g [‡]	97.5 ± 61.5 g	30.4

*Change in weight is the ratio of body or organ weights of *HMGA2*^{-/+} over *HMGA2*^{+/+}.

[†]Significant at *P* < 0.05.

[‡]*n* = 3 due to cystic ovary.

Table 3. Body and organ weight comparison of *HMGA2*^{-/-} vs. *HMGA2*^{+/+} boars at 4 d of age

Body and organs	<i>HMGA2</i> ^{-/-} (n = 10)	<i>HMGA2</i> ^{+/+} (n = 2)	Change in weight, %
Body [†]	0.65 ± 0.03 kg	1.44 ± 0.06 kg	45.1
Adrenal glands [†]	0.09 ± 0.01 g	0.36 ± 0.01 g	25.0
Brain [†]	23.30 ± 0.38 g	34.47 ± 0.13 g	67.6
Heart [†]	4.89 ± 0.20 g	14.07 ± 0.27 g	34.8
Kidneys [†]	6.35 ± 0.39 g	14.41 ± 0.76 g	44.0
Livers [†]	26.24 ± 1.49 g	72.76 ± 5.36 g	36.1
Lungs [†]	16.22 ± 1.48 g	30.74 ± 3.45 g	52.8
Testes [†]	0.39 ± 0.03 g	1.40 ± 0.61 g	27.9

*Change in weight is the ratio of body or organ weights of *HMGA2*^{-/-} over *HMGA2*^{+/+}.

[†]Significant at *P* < 0.05.

To control for any possible donor cell line/SCNT interaction effects, however, SCNT controls were generated (Fig. 4).

Overall, these data support *HMGA2*'s being a potential target for reducing the size of organs for xenotransplantation into humans, if needed. This can be implemented by the use of CRISPR-Cas9 zygotic injection techniques, bypassing the need for breeding. Recent results indicate that this gene editing approach results in up to 100% of the offspring having both alleles modified (26, 27). Thus, it should be possible to conserve the complex and well-studied genetics of existing pig lines being developed for xenotransplantation and obtain reduced organ sizes by this straightforward procedure. This would bypass the need for breeding and avoid the reproductive issues we observed.

In addition to the drastic body-size reduction phenotype, we observed an unexpected loss of viability of *HMGA2*^{-/-} fetuses in at-term pregnancies of *HMGA2*^{-/+} gilts bred by *HMGA2*^{-/+} boars. We observed mummified piglets at expected frequencies of the *HMGA2*^{-/-} genotype in all litters from this type of mating. While we were not able to obtain DNA from the mummified fetuses for genotyping due to the high degradation of the samples it is likely that they represented the *HMGA2* nulls as we were able to detect *HMGA2*^{-/-} fetuses at D40 and D78 of gestation, with fetuses appearing healthy at both gestational times, but with placentas affected at D78 (Fig. 3). This suggests that gestational stage around D78 is a critical turning point for *HMGA2*^{-/-} fetuses, with the affected placentas eventually leading to fetal loss and mummification (*SI Appendix*, Fig. S3). It has been shown that uterine capacity begins to critically affect litter size after D30 of gestation in pigs when a competition by littermates limits uterine space and resources (28, 29). Although *HMGA2*^{-/+} uteri were smaller than those in *HMGA2*^{+/+} gilts (Table 2 and *SI Appendix*, Fig. S6), reducing uterine crowding by unilateral tubal ligation did not rescue the *HMGA2*-null fetuses, suggesting that the reduced uterine size per se is not the reason. Additionally, while *HMGA2*^{-/-} placental degeneration and midpregnancy fetal loss was seen in pregnancies generated by breeding the *HMGA2* heterozygotes, it was not seen in pregnancies carrying only *HMGA2*^{-/-} fetuses produced by TALEN-induced modification and SCNT. The main differences between the two types of pregnancies are the presence of other genotypes in the uterus in the heterozygote mating, and the fact that the uterus in the heterozygote mating was of an *HMGA2*^{-/+} genotype, while the *HMGA2*^{-/-} only pregnancies were in a wild-type uterus. The evident placental degeneration at D78 in the heterozygote mating supports the notion that *HMGA2*^{-/-} fetuses had difficulties surviving in the presence of other genotypes. The exact mechanism of action of this intriguing loss remains to be determined.

On the male side, *HMGA2*-null boars showed similar but more severe reproductive issues compared with those seen in *Hmga2*-null mice. MacArthur (30) described that fertility of spontaneous homozygous pygmy mice ranged from normal to sterile. However, upon separation of pygmies into two groups based on their size, a small line and a large line, testes in more than half of small-line mice did not descend into the scrotum and most of them had priapism at 6 wk of age (12, 31). In addition, some transgenic *Hmga2*-null mice with normally descended testes were infertile because of the lack of production of mature sperm

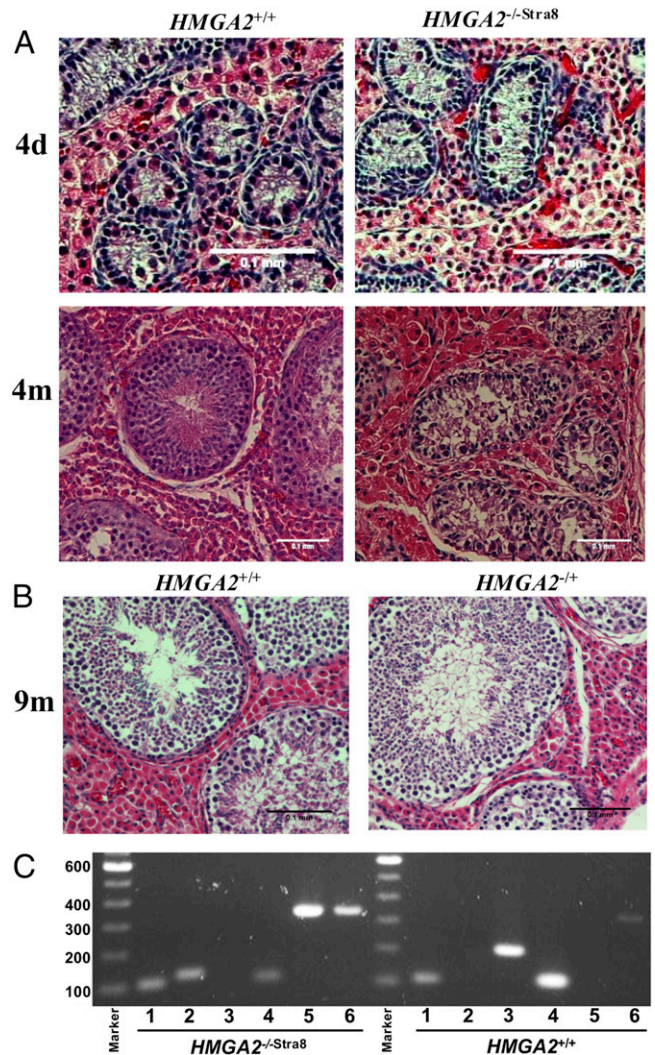


Fig. 6. Testis histology and *HMGA2* expression in control and age-matched *HMGA2*^{-/-Stra8} testis. (A) Testis from control and *HMGA2*^{-/-Stra8} pigs at post-natal D4 and 4 mo of age. At D4, cell structure and arrangement of seminiferous tubules was the same as in age-matched *HMGA2*^{+/+} testis. However, at 4 mo, none of seminiferous tubules in the cryptorchid *HMGA2*^{-/-Stra8} testis showed germ-cell differentiation, while a few tubules in *HMGA2*^{+/+} testis showed evidence of spermatogenesis. (Scale bars: 100 μm.) (B) Testes from an *HMGA2*^{-/-} boar at 9 mo of age compared with the testis of an age-matched *HMGA2*^{+/+} boar, both showing evidence of spermatogenesis. (C) RT-PCR analysis of 4-mo *HMGA2*^{-/-Stra8} and age-matched *HMGA2*^{+/+} testis. Lanes: 1, GAPDH; 2, transgenic β-geo; 3, endogenous *HMGA2* partial CDS (detects only endogenous *HMGA2*); 4, pig *STRA8* CDS; 5, transgenic *HMGA2* (detects only *mStra8*-driven *HMGA2*); and 6, *HMGA2* full-length CDS (detects both endogenous and transgenic *HMGA2*). Results support inactivation of the endogenous *HMGA2* and expression of the transgenic *HMGA2* driven by the *mStra8* promoter in the *HMGA2*^{-/-Stra8} testis.

caused by impaired spermatogenesis (5, 32). Thus, in mice, *Hmga2*-null males can either be fertile or sterile due to either undescended testes or abnormal spermatogenesis. In pigs, the severity of the phenotype was higher, with all *HMGA2*-null boars having small, undescended testes (*SI Appendix*, Figs. S9 and S11). As the testes are sensitive to temperature, exposure of testes to intraabdominal temperatures before adolescence affects spermatogenesis and leads to impaired fertility (33). As shown in Fig. 6, no evidence of spermatogenesis was detected in the *HMGA2*-null pigs despite morphologically and histologically normal structures of testes. Whether normal spermatogenesis can be rescued by surgical correction of the undescended testis (orchiopexy) (34) remains to be determined. Expression of *HMGA2* in the testis only via mStra8-*cHMGA2* was used in an attempt to prevent the previously reported issues with impaired meiosis of normally descended *Hmga2*^{-/-} mouse testes. While this approach did result in expression of *HMGA2* (Fig. 6), it did not rescue cryptorchidism, which is most likely due to a gubernaculum muscle abnormality. If breeding lines of *HMGA2*^{-/-} are required this can be achieved by surgical correction of the cryptorchidic tests (orchiopexy) or by embryo complementation using embryos that can rescue the testis phenotype. This approach has been utilized to rescue nonbreeding or lethal pig lines by others (35). From a practical standpoint, gene editing through zygote injection would bypass the need to carry SCNT, allow its application to species where SCNT is not practical, bypass the reproductive issues reported above, and avoid the need to establish independent *HMGA2*^{-/-} lines. As many transgenic pig lines being generated today, in particular those being used for xenotransplantation, carry multiple transgenic modifications (36), size modification by gene editing using zygotic injection would be a more practical approach than developing and maintaining independent *HMGA2*^{-/-} lines.

In summary, we have been able to demonstrate that inactivation of the *HMGA2* locus in pigs results in a complex phenotype with body-size reduction of over 80%, fetal competition, and cryptorchidism. The magnitude of the size reduction is similar to that seen in the superpygmy mice that lack both *Hmga1* and *Hmga2* (22). Overall, the direct evidence from pigs and mice, and the genetic associations identified between *HMGA2* and growth in dogs and humans, supports that the role of *HMGA2* in body-size regulation in mammals is highly conserved. This opens up the possibility of regulating size in multiple mammalian species and the size reduction of organs to be used in xenotransplantation, thus allowing a better match between the organ size of donor and recipient and avoiding some of the issues that have been recently identified due to organ overgrowth after transplantation (19).

Materials and Methods

HMGA2 mutant pigs were generated by conventional homologous recombination or TALEN-induced gene editing of fetal fibroblasts (FF). Correctly modified FF were used for SCNT to generate animals used in this study. Growth parameters of all genotypes (*HMGA2*^{+/+}, *HMGA2*^{+/-}, and *HMGA2*^{-/-}) were analyzed for up to 59 wk. Effects of *HMGA2* mutation on reproductive parameters of males and females were examined by breeding, analysis of timed pregnancies (D40 and D78), and histological analysis of gonads. See *SI Appendix* for detailed information. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (37) and approved by the Institutional Animal Care and Use Committee of North Carolina State University.

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