ABSTRACT

CHUNG, JAEWOOK. Disruption of the HMGA2 Gene in Swine Reduces Growth. (Under the direction of Dr. Jorge A. Piedrahita.)

The high mobility group AT-hook 2 (HMGA2) has been shown to be a crucial gene for cell growth, proliferation, and apoptosis. HMGA2 is also a strong biological candidate for growth as mutations in this gene alter body size in mice and humans. Compared to wild-type controls, adult mice lacking HMGA2 are 60% smaller, and adult heterozygous mutants are 20% smaller. In humans, a microdeletion in HMGA2 locus resulted in short stature, with no dismorphologies, and normal puberty. In order to determine the effect of HMGA2 on fetal and adult growth in pigs, a transgenic pig line deficient in HMGA2 expression was generated by gene trap approach, using conventional gene targeting with homologous recombination or TALEN mediate homology direct repair (HDR), in fetal fibroblasts (FF).

Somatic cell nuclear transfer (SCNT) was used to generate 8 HMGA2/-+ boars, 11 HMGA2/-+ Stra8 boars, and 6 HMGA2/-+ gilts. Weight data revealed that HMGA2/-+ SCNT boars were 17% and 16% lighter in weight over HMGA2/+ SCNT littermate and naturally bred boars, respectively, since the time point of 14 weeks old. However, trending for 30 weeks was not significant (nonparametric trend test). A HMGA2/-+Stra8 SCNT boar showed severe growth reduction during the early postnatal growth. At the age of 14 weeks, the weight of HMGA2/-+Stra8 SCNT boar was 76.5% lighter than HMGA2+/+ naturally bred boar, the length was 34.7% shorter, the height was 38.9% shorter, and the body cavity was 38.9% smaller. Growth of HMGA2/-+ SCNT gilts showed similar pattern to HMGA2/-+ boars. Weights of HMGA2/-+ SCNT gilts were 20-35% lighter during mid postnatal stages and 25-
30% in late stages compared to *HMGA2*+/+ naturally bred gilts. Nonparametric trend test confirmed that the growth reduction was significant (59 weeks).

Four of six *HMGA2*-/+ SCNT gilts did not show any estrus behavior. Analysis of follicular development visualization and histological analysis on ovaries showed normal folliculogenesis, despite abnormally low progesterone concentrations. Interestingly, *HMGA2*-/+ F1 gilts generated by breeding of *HMGA2*-/+ SCNT founders showed normal estrus at the expected pubertal range for normal gilts. In contrast, *HMGA2*-/+ SCNT boars had normal puberty and sexual behaviors up to the present (2 years and 4 months). Normally organized seminiferous tubules were identified by histological analysis on testis. While *HMGA2*-/+ SCNT boars were normal, all HMGA2 null boars (generated by SCNT or by breeding) had undescended testes (Cryptorchidism) with lack of gubernaculum bulbs, showing no evidence of spermatogenesis until 4 months of age.

Mating of *HMGA2*-/+ SCNT founders resulted in 3 pregnancies to term without HMGA2 null piglets. To determine the timing of fetal loss and to identify a cause for the loss, pregnancies at D40 and D78 were examined, resulting in confirmation of the presence of all four different genotypes, including *HMGA2*-/-.. While no defects were detected in D40 and D78 fetuses per se, placental abnormalities were seen only in *HMGA2*-/- conceptuses from D78 litters at both macroscopic and microscopic level. To determine whether lack of HMGA2 or intrauterine resource depletion was responsible for lethality of HMGA2 null pigs after D78, SCNT was performed to generate a pregnancy carrying only the *HMGA2* genotype. Successful generation of 10 live HMGA2 null clones indicates that HMGA2 deficiency does not cause fetal lethality.
Disruption of the HMGA2 Gene in Swine Reduces Growth.

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BIOGRAPHY

Jaewook Chung was born on January 2, 1980, in Seoul, South Korea. He grew up with his four family members, his father, Chunsoo Chung, his mother, Hye-suk Son, and his brother, Jin-wook Chung. He fulfilled his military service as a ROK marine for two years and two months before he came to study in the United States. He received his Bachelor’s degree in Molecular and Cellular Biology with minors in Chemistry and Computer Science at Utah State University in 2008. Being interested in the biological researches, he joined in Plant Physiology lab, Evolutionary Biology lab, and Tissue Engineering lab as an undergraduate student researcher. To have more research experiences, he worked in Bioinformatics lab for about a year after his undergraduate career. His work for PhD degree was done at North Carolina State University under the direction of Dr. Jorge A. Piedrahita, studying on the transgenic research.
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CHAPTER 1

Literature review
1.1 Somatic Cell Nuclear Transfer (SCNT)

SCNT is a promising technology utilized not only to produce cloned animals, but also to study the mechanisms of reprogramming and to generate genetically modified animals. SCNT has been widely used in many different species, including frogs [Briggs 1952 and Gurdon 1958], rabbits [Chesne 2002, Li 2006, Yang 2007, and Du 2009], sheep [Wilmut 1997, and Schnieke 1997], goats [Baguisi 1999], cows [Cibelli 1998 and Kato 1998], mice [Wakayama 1998 and Wakayama 2001], rat [Zhou 2003] etc. The first successful cloning of pigs was reported in 2000 [Polejaeva 2000 and Betthauser 2000].

Relevant to this thesis, SCNT is a key component of developing genetically modified pigs. In many mammalian species, including pigs, the lack of germ line competent pluripotent stem cell, SCNT is an essential technique to generate genetically modified animals for biomedical research and xenotransplantation. In addition, SCNT, in combination with prescreened genetically modified donor cells, has many advantages when compared to two conventional methods; pronuclear DNA microinjection [Gordon 1981 and Costantini 1981] and lentiviral gene transfer [Jaenisch 1974], Some of these advantages include that the sex of cloned animal can be predetermined by choosing the donor cells, and using cell culturing system, large number of transgenic donor cells can be available for SCNT, eventually giving rise to numerous cloned transgenic animals. In addition, 100% of transgenic animals in a litter can be produced by the proper use of SCNT, and every cell of a transgenic animal carry the transgene without mosaicism.

Despite its success in other animal models, pig SCNT has challenging issues. First, the efficiency of cloning is very low, ranging from 1 to 5 % as defined by number of live
offspring per embryo transferred [Colman 2000 and Vajta 2007]. Secondly, many cloned fetuses and newborns are physiologically or anatomically abnormal, i.e. stillborn or mummified [Walker 2002, Estrada 2007 and Kurome 2013]. Moreover, many live cloned piglets are weak and underweight due to unknown reasons [Estrada 2007]. In some cases, in spite of severe phenotypic abnormalities, this abnormal cloned pigs successfully produced normal offspring [Kurome 2013]. This same “transgenerational” correction has been reported in mice [Tamashiro 2002], suggesting that these abnormalities might be due to errors in epigenetic reprogramming by SCNT, rather than due to genetic modifications introduced during the SCNT process [William 2001 and Whitworth 2010]. Indeed, possible incomplete epigenetic reprogramming issues in pig SCNT have been reported; behavioral variations of cloned pigs [Archer 2003], sudden death caused by severe congestion of lung, and liver or neutrophilic inflammation in brain [Park 2005], increased incidence of intrauterine growth retardation [Estrada 2007], and enlarged tongue [Kurome 2013].

In spite of all the difficulties described above, SCNT still allows the generation of complex genetically modified animals by modification of donor cells in vitro followed by the SCNT procedure. In vitro modification and selection allows the identification of rare events and the use of multiple methods of enrichment, selection, and detection of correctly modified cells.
1.2 Gene Modification Methods for Generation of Transgenic Animals

1.2.1 Conventional gene modification by homologous recombination

For gene modification, researchers have utilized several different methods able to disrupt or modify gene function (Genome engineering). In general, transgenic animals can carry two types of genetic modifications. Those introduced at random locations of the genome (random modifications) and those introduced into precise and predetermined regions (targeted modifications).

Targeted modification introduced by conventional (unassisted) homologous recombination in mouse embryonic stem cells has been used for decades [Smithies 1985, Piedrahita 1992 and Soriano 1999]. Conventional gene targeting is defined as a method to introduce site specific modifications into the genome by homologous recombination in the absence of induced dsDNA breaks on endogenous genome [Krejci 2012]. Homologous recombination is a genetic recombination event able to achieve gene replacements or mutations. Two similar or identical DNA sequences are exchanged, and this occurs in the presence of double strand breaks (DSBs) [Krejci 2012]. Introduction of a targeting vector carrying similar or identical sequences to an endogenous DNA region can change the endogenous genetic sequences to the sequence being carried by the targeting plasmid [Doetschman 1987].

Even though the efficiency of homologous recombination is low in higher eukaryotic cells with rates of 1 in 1000 cells [Thomas 1986], the method has been used for decades to enable gene inactivation in a variety of research [Vasquez 2001]. The generation of stable knockouts by homologous recombination in embryonic stem cells is common in mice.
[Doetschman 1987, Thomas 1987, and Thompson 1989]. Embryonic stem cells are used as they can be manipulated in vitro and then used to generate a living organism. Selected transgenic embryonic stem cells are microinjected into the blastocoel cavity of a developing embryo to generate chimeric mice that are then used for the generation of homozygous mutant mice by breeding.

However, in large animal transgenic research, including pigs, gene modification by homologous recombination is commonly performed in somatic cells as ES cells are not available. While there are reports of modification of somatic fibroblast cells, the efficiency of this method is low and this has limited wide applicability of the technology. In addition, in order to obtain a living offspring, the somatic cells must then be used for SCNT, a difficult and inefficient procedure in swine.

1.2.2 Transcription activator-like effector nucleases (TALENs)-mediated gene editing

Genome editing using engineered nucleases is a highly efficient targeting technology that has been recently developed and rapidly evolving. As a tool for genome editing, programmable nucleases produce DSBs on a specific, pre-selected DNA site. The DSBs greatly enhances the process of homologous recombination [Rouet 1994]. In addition, the DSBs can be repaired by a non-homologous end joining (NHEJ) event that introduces random mutations in the DNA [Reyon 2012]. TALENs is one of the three most used methods, two of which are Zinc Finger Nucleases (ZFNs) and clustered regulatory interspaced short palindromic repeats associated Cas9 (CRISPR/Cas).
Each of two ZFN subunits consists of 3-6 zinc finger arrays which are combined by modular assembly process. DNA binding domain of an individual zinc finger module typically recognizes 3 base pair DNA sequence in the major groove of DNA (Pavletich 1991). The enzymes to cut the DNA are called FokI nucleases. In spite of the presence of off target effects (OTEs) (Cornu 2008), the strength of ZFNs is to be known as the most accurate gene modification tool for a high level of modification to completely alleviate symptoms for the therapy (Manjunath 2013). However, the success rate and mutation frequency of ZFNs are relatively low even though the two rates for all of the three methods are in the broad range depending on the cell type and method. Not as TALENs, there is no open source collection for all possible combinations (Bae 2003). Utilization of previously characterized ZFNs decreases time to create new ZFNs, but this method, called modular assembly, sometimes lead to the targeting failure (Ramirez 2008) or toxicity (Cornu 2008). Since, in order to successfully target, the target sites or zinc finger protein binding sites typically contain guanine rich repeat sequences, each of which has a guanine at 5’ end of each 3 base pair repeat, it gives less chance to find a pair of functional ZFNs within the target of your interest (Kim 2009). The chance to find the same target as yours is about one within 100 base pair sequence.

The targeting strategy of CRISPR is based on the processed target specific CRISPR RNA (crRNA) and the target independent trans-activating crRNA (tracrRNA) (Deltcheva 2011). The combination of crRNA and tracrRNA forms a single guide RNA. New CRISPR is easily created by designing 20 base pair guide DNA sequence that transcribes the single guide RNA. This is a unique feature for CRISPR/Cas method. Since it is quick and easy to
encode multiple guide sequences, the CRISP/-Cas method enables users to simultaneously edit several target sites (Cong 2013). It is another advantage that CRISPR/Cas can cleave methylated DNA (Hsu 2013). Despite the convenience of use, not all sequences are cleaved efficiently even in the presence of protospacer adjacent motif (PAM) (Koike-Yusa 2013). Unlike the other two methods, acting as monomers, the targeting strategy of CRISPR is highly dependent on about 20 base pairs of a single sequence even though there are some more rules, such as PAM or length of target sequence. In addition, some mismatches in the 5’ upstream region from the PAM sequence are tolerable for the guide RNA to recognize other similar target sites (Fu 2013). These two unique features potentially increase to provide the OTEs.

For this thesis, we utilized TALENs, given their relatively low cytotoxicity compared to ZFNs [Gaj 2013 and Kim 2014] and lower off-target effects compared to CRISPR/Cas [Mussolino 2011 and Kim 2014]. TALENs have promising high throughput genome editing potential and as such they have been used to successfully modify endogenous genes in many organisms; yeast [Li 2011], zebrafish [Sander 2011 and Huang 2011], rat [Tesson 2011], pig [Carlson 2012], cow [Carlson 2012] etc. Despite the highly successful editing events, off-target cleavages may occur on the sites with similar DNA sequence to the original targeting site which TALENs were designed for. Such off-target effects may produce non-specific DSBs, leading to unwanted mutations consequently resulting in cell death [Mussolino 2011]. Minimizing the off-target effect is crucial for the genome-wide specificities of TALENs.

A TALEN is composed of a TALE DNA binding domain and a DNA cleavage domain. The TALE DNA binding domain is a highly conserved proteins encoded by
Xanthomonas proteobacteria [Boch 2009 and Moscou 2009]. The bacteria use the TALEs to alter transcription in host plant cells, thereby yielding pathogenic bacterial colonization [Boch 2010]. One TALE binding domain is composed of TALE repeats in an array, and every single repeat in a domain contains a series of 33-35 amino acid repeat residues. The identity of each TALE repeat domain is determined by two repeat variable di-residues (RVD) found at positions 12 and 13 of the repeat [Boch 2009 and Moscou 2009]. Based on the determined identity by the two RVDs, an individual TALE repeat recognizes a different nucleotide. A single TALE repeat with NN, NJ, HD or NG of RVDs acts for the recognition of guanine, adenine, cytosine, or thymine, respectively [Streubel 2012 Cong 2012]. Hence, individual TALE repeats are linked together to recognize a stretch of DNA sequences.

The main feature of the DNA cleavage domain of TALENs is that it contains a FokI nuclease for DNA cleavage. The enzyme FokI is a type IIS restriction endonuclease naturally found in Flavobacterium okeanokoites [Sugisaki 1981]. FokI is composed of 587 amino acids and its molecular mass is 65.4 kDa. The components of FokI are an N-terminal DNA binding domain and a non-specific DNA cleavage domain at the C-terminal end. A FokI enzyme first binds to DNA as a monomer followed by the arrival of a second FokI monomer, with cleavage of both DNA strands occurring after FokI dimerization [Bitinatie 1998].

To generate a pair of TALENs, there are several major methods; Golden Gate cloning [Engler 2008, Engler 2009, and Engler 2011] its variant approach, MoClo [Weber 2011 and Werner 2012] and GoldenBraid [Sarrion-Perdigones 2011], high-throughput solid phase assembly [Reyon 2012 and Briggs 2012], and ligation independent cloning technique [Schmid-Burgk 2013]. The Golden Gate and its sister methods assemble multiple TALE
domains in an ordered fashion in a single reaction, using restriction enzymes to obtain individual TALE domains. The high-throughput solid phase assembly method performs a serial ligation of isolated TALE domains cut by restriction enzymes before subcloning. Ligation independent cloning is a restriction enzyme free method based on the production of each TALE domain by polymerase chain reaction.

1.3 High Mobility Group A2 (HMGA2)

The gene, *HMGA2*, encodes a non-histone chromosomal protein that modulate the interaction of transcription regulatory proteins with DNA by facilitating three dimensional changes in chromatin structure, which are so-called architectural transcription factors [Goodwin 1973]. Acting as an enhanceosome, HMGA2 protein bound onto the chromatin promotes the recruitment of regulatory protein complexes by inducing conformational changes of DNA structure. HMGA2 proteins contain the binding domain peptide with three of the AT-hook motif that preferentially recognizes AT-rich regions of minor groove of DNA. Of the three AT-hooks, the second motif is the most critical component for the function of HMGA2 protein and it is conserved among diverse organisms [Cattaruzzi G 2007], including pigs (Figure 1.1A). HMGA2 proteins are known to be regulated by cell cycle-dependent phosphorylation that alters their DNA binding affinity [Reeves 1991 and Tessari 2003].

*HMGA2* expression is necessary for the normal development required in the highly coordinated processes of cell proliferation and differentiation [Xiang 1990]. In mice, the expression of Hmga2 is found predominantly during fetal development beginning at 10.5
days post coitum (dpc) and ending at 15.5 dpc [Zhou 1995]. During embryonic development, 
*Hmga2* expression is detected at a high level throughout the whole embryo except in the 
embryonic brain, but it is not detected in normal adult tissues except in the testes [Zhou 1995, 
Hirning-Folz 1998, and Chieffi 2002]. In addition, HMGA2 proteins are extremely abundant 
in stem cells and many cancer cells. HMGA2 is highly expressed in undifferentiated human 
embryonic stem cells and globally affects the state of ES cell chromatin [Li 2006 and Li 
2007]. HMGA2 protein without its C-tail stimulates adipocyte cell growth and leads to 
human lipomas [Battista 1999]. Other reports demonstrated that HMGA2 overexpression 
causes pancreatic [Abe 2003] and lung carcinomas [Meyer 2007] and squamous carcinomas 
of the oral cavity [Miyazawa 2004]. Thus, as HMGA2 proteins are highly involved in cell 
growth and proliferation, misexpression or overexpression of HMGA2 proteins can 
transform cells to undergo malignant neoplasias [Zaidi 2006].

More relevant to this thesis, *HMGA2* has been implicated in affecting fetal and 
postnatal growth in a number of species. In dogs, single polymorphic biomarker analysis 
demonstrated that there was an association between the *HMGA2* gene and body weight 
[Jones 2008], and genome-wide association study showed the association of *HMGA2* with 
ear type [Boyko 2010]. Similar associations were also seen in Hmga2-deficient mice that had 
small ears and body size [King 1950 and Xiang 1990]. In humans, *HMGA2* genome-wide 
association studies showed that the variation in *HMGA2* gene affects human height [Weedon 
2007], and the identification of intragenic microdeletion in the *HMGA2* gene in short stature 
patients showed that *HMGA2* affects human growth [Lettre 2008 and Buysse 2009].
More direct evidence for the role of Hmga2 in growth regulation comes from observations in mice. MacArther described that certain matings produced undersized animals (runts) [MacArthur 1944]. It was reported that the pygmy locus is responsible for pygmy phenotype in mice [MacArthur 1944, King 1950, King 1955, Xiang 1990 and Zhou 1995]. In mice, homozygous pygmy mice, arisen spontaneously, were generated by mating of mice selected for small size, whereas transgenic homozygous mini-mice were generated by intercross of heterozygous offspring derived from chimeric transgenic founder (mosaicism) carrying mutations in hmgA2 locus [Xiang 1990 and Zhou 1995]. Genotypes of both hmgA2 homozygous mice were reported as same [Xiang 1990]. Compared to wild-type controls, adult mice lacking Hmga2 protein were 60% smaller, and adult heterozygous mutants were 20% smaller [King 1950, Xiang 1990 and Zhou 1995]. Study of rat pituitary implants to pygmy mice reported that growth reduction of Hmga2 null mice was not due to lack of growth hormone [King 1955]. In fact, the circulating levels of growth hormone and somatostatin were reported normal [Xiang 1990]. In addition, disruption of Hmga2 in mice has revealed that Hmga2 null mice have a deficiency in fat tissue and are resistant to diet-induced obesity [Anand 2000].

Hmga2 null mice have fertility issues. McArther described that fertility of spontaneous homozygous pygmy mice was almost normal while some were sterile [MacArther 1944], whereas transgenic homozygotes were infertile [Xiang 1990 and Chieffie 2002]. All of spontaneous heterozygous mutants [King 1950 and King 1955] and transgenic heterozygous mutants [Xiang 1990 and Zhou 1995] were fertile. While spontaneous Hmga2 null male mice with increased size were fully fertile, transgenic Hmga2 null male mice either
do not produce mature sperm or have an abnormal spermatogonia and spermatocyte morphology [Chieffi 2002].

In swine, HMGA2 is located on Chromosome 5 with 5 exons. The length of HMGA2 mRNA is 327 bp. HMGA2 protein consists of 108 amino acids and the predicted molecular weight of protein is 12 kDa. There is a pseudogene of HMGA2 located on Chromosome 1 [Kim 2004 and Li 2012]. The cDNA alignment of pseudogene and real gene of HMGA2 showed 91% homology degree (Figure 1.1B). As for dogs and mice, HMGA2 was suggested to be associated with ear size in pigs [Li 2012]. However, there have been no previous reports on the function of HMGA2 in growth regulation in pigs.

1.4 Stimulated by Retinoic Acid 8 (Stra8)

In mice, homozygous transgenic mice (mini-mice) were reported to be sterile [Xiang 1990 and Zhou 1995] while some homozygous spontaneous pygmy mice were reported to be fully fertile [MacAuther 1944 and King 1955]. Histological analysis demonstrated that germ cell differentiation was blocked in the tests of hmgalpha homozygous mice [Chieffi 2002]. Cellular damage of spermatogonia, degenerated spermatocytes, and no spermatozoa were identified [Chieffi 2002]. All of these results indicate that hmgalpha gene plays an essential role in male germ cell development. Due to the role of HMGA2 in meiosis during spermatogenesis and the previous reports of effects on male infertility in some male Hmgalpha mutant mice, we developed and approached to express HMGA2 only in the germ cells. This would, in theory, allow the reduced growth phenotype while rescuing the male infertility
phenotype. To accomplish this, we had to select a promoter that was germ cell specific and that, preferably, had been tested in swine.

*Stras8* gene encodes a cytoplasmic protein whose molecular weight is 45 kD [Oulad-Abdelghani M 1996]. Stra8 proteins have been known as a key player for the transition into meiosis in both female and male germ cells [Baltus 2006]. *Stras8* is expressed in premeiotic germ cells in both male and female gonads and required for the entry into meiotic divisions. In mammals, initiation time points of meiosis are different in male and female. Whereas meiosis starts during embryogenesis in female, the male embryonic germ cells arrest at the G0/G1 mitotic stage and resume after birth, entering meiosis [Menke 2003]. In mouse male gonads, Stra8 expression is restricted during embryonic development because retinoic acid is degraded by cytochrome p450 and thereby prevents Stra8 expression, eventually precluding initiation of meiosis [Koubova 2006].

Because *Stras8* expression is restricted to the premeiotic phase of spermatogenesis, the promoter of this gene has been used to identify germ cell [Sadate-Ngatchou 2008, Antonangeli 2009, and Sommer 2012]. In mice, a fusion construct of Stra8 promoter region and coding region of enhanced green fluorescence protein was used to select differentiated germ cells [Nayernia 2004]. In pigs, transgenic pigs that express mitochondrial localized enhanced yellow fluorescence protein driven by the mouse Stra8 promoter were produced to study male germ cell manipulation and development [Sommer 2012]. These transgenic studies provide evidence of specific localization of Stra8 expression to premeiotic germ cells. The infertility issue of *Hmga2* null mice could possibly be solved by introduction of *Hmga2* expression driven by Stra8 promoter in the mutant testes.
1.5 Reproductive Physiology in Pigs

Estrus or standing heat is the sign that indicates non-pregnant sows or gilts are ready to be bred (oestrus). Estrus lasts 36-48 hours in gilts and 48-72 hours in sows. Onset of estrus is characterized by behavioral (mounting or fence walking) and physical (vulvar swelling or vulvar color change or vaginal discharge) changes. The average time between the onset of one estrus to the onset of the next is 21 days. The length of estrous cycle is in range of 18-24 days. The estrous cycle consisting of two major phases, the follicular and luteal phases, is regulated by the hypothalamic-pituitary-gonadal axis. The initiation of the follicular phase is triggered by luteolysis that leads to the formation of the progesterone secreting corpus luteum (CL). Degenerating CLs result in decreased progesterone level in the blood, and this changes from the negative feedback to the positive feedback on the hypothalamus. Gonadotropin releasing hormone (GnRH) is then released at higher amplitudes and frequencies, causing higher concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) secreted from the anterior pituitary gland. The two hormones stimulate the follicular development to produce estrogen and inhibin. During estrus, the rising concentrations of estrogen, accompanied by low progesterone, positively feedback on the anterior hypothalamic area to release a large quantity of GnRH, while inhibin causes a negative feedback on FSH secretion from the anterior pituitary gland. The surge of GnRH turns on the anterior pituitary to secrete a surge of LH, inducing the ovulation of follicles in the ovaries. During ovulation, 15-24 ova are released for 1-4 hours. FSH does not surge with the same magnitude as LH because of the negative feedback by inhibin.

On the other hand, the luteal phase begins immediately after the ovulation. Once the
follicles are ovulated, the theca and granulosal cells of the follicles undergo a dramatic transformation, called luteinization. The luteinized follicles become CL functional to produce progesterone. The fully functional CL leads to progesterone plateaus. As an inhibitor, progesterone produced by CL reduces GnRH frequency from the hypothalamus and stops the LH surge from the anterior pituitary. Importantly, progesterone induces a strong positive influence on the endometrium of the uterus, which is responsible for secreting prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$). PGF$_{2\alpha}$ signals to the luteal cells to induce apoptotic effects that lead to the destruction of the CLs. This process, luteolysis, results in the cessation of progesterone secretion and the removal of negative feedback by progesterone on GnRH secretion, triggering new follicular phase to begin.

Unlike the reproductive cyclicity in sows, the hypothalamus in boars secretes GnRH in pulses that occur every several hours. The pulsatile secretions of GnRH cause discharges of FSH and LH in pulses, but the duration of FSH pulses is longer than LH due to relatively constant secretion of inhibin by the adult testes. LH in the blood is destined to act on the Leydig cells within the testes. Stimulated Leydig cells by LH synthesize progesterone, most of which is converted to testosterone, since high concentrations of testosterone are required for normal spermatogenesis in testes. Testosterone secreted by the Leydig cells is transported to the Sertoli cells and the blood. Acting of FSH on the Sertoli cells, transported testosterone is converted to estrogen also transported to the blood. Testosterone and estrogen in the blood exert a negative feedback on the hypothalamus.

In domestic breeds of pigs (Sus scrofa), females are sexually mature at 6-8 months of age and at about 230-280 pounds. Males start producing sperm at 4 months and become fully
fertile at 5-8 months [Knox 2003 and Senger 2012]. Gilts or non-pregnant sow have about 50 hours of estrus to be bred [Senger 2012]. Once they are pregnant after breeding or artificially insemination during the estrus, sow has 112-114 days of gestation. Sow farrows 13 piglets per litter in average and 10 piglets are weaned per litter after 3 weeks from birth [pigChamp 2011].

Upon the fertilization gametes, the embryos start developing by cell division. Porcine embryos enter the uterus by D4 and the migration of embryos occurs from D5 to D11 of gestation [Dhindsa 1967, Waite 1967]. Since porcine blastocysts are extremely motile, they even pass by neighboring littermates during migration. Embryo migration in utero results in the relatively uniform distribution of embryos within the available uterine environment [ThePigSite]. The uniform distribution of embryos throughout the uteri contributes to the equal distribution of uterine resources to each individual conceptus, which likely optimizes the individual survival chances of each conceptus. After the fertilization, around D12-14 of gestation, implantation of more than 4 viable embryos is required for pregnancy to continue [ThePigSite]. The embryonic loss occurs in pigs throughout the gestation. The greatest potential loss in litter size occurs at earlier gestation due to attachment failures of embryos [Geiert 2001] when the placenta starts developing.

1.6 Placental Development and Function

Porcine placenta shape is classified as a diffuse type, meaning that the entire surface of the allantochorion is involved in formation of the placenta [Senger 2012]. The diffuse placenta contains many chorionic villi that penetrate into the endometrium, forming the
epitheliochorial layer (fetal-maternal interface). The chorionic villi, that are small and finger-like projections, provide increased surface area to maximize an exchange of blood flow between fetus and sow. The specialized feature of diffuse placenta in pig is to have a number of microstructure, called areolae [Friess 1981 and Dantzer 1993], which is developed on D30 of gestation as a dome shaped formation. This structure plays an important role to transport resources from sow to fetus, by lining over the uterine glands [Friess 1981].

During the early critical stage for conceptus development (D10 to D30 of gestation), pig conceptuses interact with the uterine environment to modulate endometrial function and placental attachment for nutrient exchange [Stroband HWJ 1990]. Conceptus produces and secretes estrogen to the uterine lumen around D11 [Perry 1976, Ford 1982, and Pusateri 1990]. This provides the signal for sow to maintain luteal phase by production of progesterone during gestation [Geisert 1990]. To maximize delivery of fetal estrogen throughout the uterine surface, embryos migrate and space themselves between D8 and D11 of gestation [Dzuik 1985]. Following migration, rapid differentiation and expansion of conceptus trophoblastic membrane are initiated between D11 and D12 [Geisert 1982], rapidly elongating to a filamentous form and beginning of attachment to the uterine surface epithelium [Burghardt 1997]. By D16 of gestation, rapid expansion of the trophoblast of conceptus leads to placental growth as increasing the synthesis and secretion of estrogen [Perry 1962 and Ka 2001]. This rapid transition point of gestation stage represents one of the periods when the greatest embryonic loss occurs in pig [Pope 1985]. Between D13 and D18, trophoblastic attachment to the endometrial epithelium occurs [Burghardt 1997]. The embryo develops from the inner cell mass and the allantoic membrane develops to form the placental
vascular network for transport of resources [King 1982]

1.7 Summary

SCNT is a powerful tool to generate animal models for studying the effects of gene manipulation. Although the low rates of successful cloning with somatic cells have been reported, SCNT has been proven to work in many mammalian species [Baguisi 1999, Chesne P 2002, Wilmut I 1997, Cibelli JB 1998, and Wakayama T 1998], including pigs. Pigs are known as the best excellent models for biomedical research because of its anatomical and physiological similarities to human as well as the need for non-rodent based studies. Because pluripotent embryonic stem cells are not available yet in large animals including pigs, SCNT is the most useful technique to clone transgenic pigs. Since the first cloned pigs were reported [Polejaeva IA 2000], each step in the SCNT procedure was systematically optimized in pigs more than ten years ago [Betthauser 2000]. In addition, genetically modified pigs have been successfully generated by SCNT for more than a decade [Lai 2002, Rogers 2008, Klymiuk 2012, and Carlson 2012].

Gene targeting by homologous recombination is a conventional but widely used technique to modify genes in mammalian species [Thomas 1987, McCreath 2000, and Lai 2002]. Using a targeting plasmid carrying a reporter gene (transgene), conventional gene targeting by homologous recombination enables the site-specific introduction of a mutation to the genome. In swine transgenic research, there have been several genetically modified pigs produced by conventional gene targeting [Lai 2002, Dai 2002, Hao 2006, Phelps 2003, and Lai 2006]. However, the rate of homologous recombination is low and inactivation of
both alleles simultaneously is an extremely rare event. This forces investigators to generate the homozygous mutants through breeding. In contrast, TALENs allow us to specifically target genes of interest, are programmable to target any sites, and their efficiency is so high that inactivation of both alleles is seen at high rates (1-10%) [Miller 2011, Carlson 2012 and Reyon 2012].

*HMGA2* has been shown to be a crucial gene to encode architectural non-histone transcription factors that are necessary for cell growth and proliferation during embryonic development [Manfioletti 1991, Zhou 1995, and Rogalla 1996]. Variations in *HMGA2* locus were shown to be distinct heritable phenotypes in the development of mice and humans. In the study of insertional pygmy mutant mice, the size of adult mice with half expression of *HMGA2* was 20% smaller compared to wild-type controls, whereas adult homozygous mutants were 80% smaller than wild-type controls [Xiang 1995]. Genome-wide association studies in humans showed that genetic variants of *HMGA2* gene were associated with adult and childhood heights at high levels of statistical confidence [Weedon 2007, Buysse 2009 and Alyagoub 2012]. Despite HMGA2 proteins playing a critical role during embryonic development in mammals, HMGA2 null mice showed that the impairment of *HMGA2* gene function leads to the blockage of spermatogenesis [Chieffi 2002 and Di Agostino 2004] whereas several spontaneous pygmy mice with increased size were fully fertile [King 1955].

In mice, the expression of *Stra8*, in response to retinoic acid, is specifically localized at the premeiotic germ cells [Sommer 2012]. *Stra8* plays no role in the mitotic phases of embryonic germ cell development, but is required for the entry of germ cells into premeiotic phases [Baltus 2006]. Because of the tissue-specific and timing-specific expression of *Stra8*,
HMGA2 expression by Stra8 promoter provides a promising approach to rescue the impaired spermatogenesis in HMGA2 null mutants.
CHAPTER 2

Disruption of the *HMGA2* gene in swine reduces growth
2.1 Introduction

The high mobility group AT-hook 2 (HMGA2) has been shown to be a crucial gene for cell growth, proliferation, and apoptosis. HMGA2 is also a strong biological candidate for growth as mutations in this gene alter body size in mice and humans. Compared to wild-type controls, adult transgenic mice lacking HMGA2 are 60% smaller, and adult heterozygous mutants are 20% smaller [Xiang 1995 and Zhou 1995]. In humans, a microdeletion in HMGA2 locus resulted in short stature, with no dismorphologies, and normal puberty [Alyagoub 2012]. In order to determine the effect of HMGA2 on fetal and adult growth in pigs, we generated a transgenic pig line deficient in HMGA2 expression by gene trap approach, using conventional gene targeting with homologous recombination or nuclease mediated homology direct repair (HDR), in fetal fibroblasts (FF).

The porcine HMGA2 gene in FF was disrupted by gene trap approach and cell lines with mutated HMGA2 were used as nuclear donors in somatic cell nuclear transfer. We have generated transgenic cell line with hemizygotic expression of HMGA2 using homologous recombination mediated classical gene targeting, and also created homozygous male cell line from primary heterozygous male fetal fibroblasts by TALEN mediated gene editing technology. TALEN mediated HDR also allowed us to generate homozygous male cell lines that were able to express HMGA2 only in testes. The testes specific expression was driven by Stra8 promoter. Using the transgenic cell lines, we have produced and analysed mutant pigs that have absent or low expression of HMGA2. Our data demonstrated that disruption of HMGA2 gene reduces growth in swine. In addition, our reproductive characterization
revealed that cloned mutants generated by SCNT had reproductive physiological issues. We also demonstrated that male HMGA2 null pigs have undescended testes.

2.2 Materials and Methods

2.2.1 Generation of Transgenic Pigs

Plasmid construction

The pCR-Blunt II-TOPO plasmid (Thermo Fisher Scientific Inc., Rockford, IL) was used to generate pMCS (Figure 2.1A), containing five restriction sites, I-CeuI, SbfI, AscI, BbvCI, and I-SceI. The porcine *HMGA2* 5’ and 3’ homology arms were amplified from pig genomic DNA. The *HMGA2* 5’ homology arm was subcloned into pMCS using I-CeuI and SbfI, and the *HMGA2* 3’ homology arm was done with AscI and I-SceI. The loxp-βGeo-bpA component was amplified from pTV-Fbx15 [Tokuzawa 2003], and the βGeo-bpA-loxp-DTA-SV40 component was from pROSA26LAC [Soriano 1999]. The two components were fused by splicing by overhang extension (SOE) PCR. The loxp-βGeo-bpA-loxp-DTA-SV40 fragment was subcloned into the site between SbfI and AscI to complete the first targeting plasmid, p5’HA-βGeo-bpA-flox-DTA-SV40 polyA-3’HA (Figure 2.1B). This plasmid was used for conventional gene targeting in female cell lines.

To develop a plasmid with FRT flanked reporter gene, FRTwt-βGeo-bpA-FRT3 was generated with PCR primers that have FRT sites on their 5’ overhangs, and this fragment was subcloned into the first targeting plasmid using SbfI and AscI in order to create p5’HA-FRTwt-βGeo-bpA-FRT3-3’HA (Figure 2.1C). This second vector was used for conventional gene targeting in male cell lines.
To drive *HMGA2* cDNA from a germ cell specific promoter, the STRA8 promoter was amplified from gDNA extracted from mouse tail using primers described previously [Sommer 2012]. The complete coding sequence of *HMGA2* was amplified from cDNA synthesized from total mRNA extracted from porcine fetal fibroblast (PFF). The third targeting plasmid was completed as p5′HA-FRTwt-STRAT8-HMGA2 cDNA-bpA-FRT-3′HA (Figure 2.1D), which was used for TALEN mediated HDR to target a non-edited *HMGA2* allele in primary *HMGA2-*/+ male cells.

All PCR reactions for generating constructs were carried out using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., Rockford, IL). All PCR primers are listed in Table 2.1. All of plasmids created here were sequenced to ensure there was no mutation or frameshift in their DNA sequences. All sequencing primers are listed in Table 2.2.

**HMGA2 TALEN generation**

TALEN pairs to the porcine *HMGA2* were generated using the FLASH method [Reyon 2012]. To differentiate between the endogenous *HMGA2* and the homologous recombination modified *HMGA2* (transgenic allele), a unique site was selected that identified only the endogenous allele (Figure 2.3). Using this approach, TALENs that can induce DSB to the endogenous allele, but not the transgenic allele, were developed.
Genotyping assay to identify modified *HMGA2* alleles

To identify alleles that were modified by conventional homologous recombination or TALEN mediated HDR, PCR primers were designed as shown in Figure 2.4A and 2.4B. PCR conditions were the annealing temperature of 62°C for 5 sec and extension time of 2 minutes. To identify 5’ and 3’ ends of targeted sites, specific PCR assays were designed as shown in Figure 2.6, 2.7, and 2.9B and 2.9C. The annealing conditions for all of the PCR assays was 70.2°C for 5 sec, and the extension time was 2 minutes. All of PCR reactions for genotyping were performed with Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific Inc., Rockford, IL). Amplicons were purified by DNA clean & concentrator kit (Zymo Research, Irvine, CA) and sent for sequencing (Eurofins MWG Operon, Louisville, KY). All of the primers are listed in Table 2.2 and 2.3.

To identify the allele mutated due to DSB induced by TALENs, the same primer set used in Figure 2.4B was used for the assay in Figure 2.4C. The annealing step was 60°C for 5 sec but the extension time was run for 5 sec. Restriction enzyme digestion of amplicons was used to check if the BsaW1 restriction site had been modified (Figure 2.8C).

**HMGA2 detection by western blot in wild-type and *HMGA2* mutants**

Cell lines from all genotypes collected from D40 fetuses resulting from breeding of a HMGA2-/+ gilt to a HMGA2-/+ boar were used for analysis. In addition, D40 *HMGA2-/-* STRAS® fetuses were also analyzed. Total proteins were prepared by using RIPA (Sigma-Aldrich Co. LLC., St. Louis, MO) buffer mixed with protease and phosphatase inhibitor cocktails (Sigma-Aldrich Co. LLC., St. Louis, MO). The concentrations of isolated proteins
for each genotype were measured by BCA analysis following the manufacturer manual (Thermo Fisher Scientific Inc., Rockford, IL). Proteins were separated on Any kD Mini-PROTEAN TGX precast gel in Tris/Glycine/SDS buffer (Bio-Rad Laboratories, Inc., Hercules, CA). After protein transfer to a polyvinylidene difluoride membrane, the membrane was probed using anti-βactin and anti-HMGA2. The dilution factors of anti-βactin (Cat#ab8227 Abcam, Cambridge, MA) and anti-HMGA2 (Cat#ab97276 Abcam, Cambridge, MA) were 1:20000 and 1:100, respectively. Horseradish peroxidase (HRP)-conjugated secondary antibody (Cat#ab16284 Abcam, Cambridge, MA) was used by diluting it in 1:2000. Clarity Western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA) was used to detect protein bands.

**Generation of HMGA2-/+ and HMGA2-/-STRA8 animals**

For the HMGA2-/+ and HMGA2-/-STRA8 boar generation, D43 male HMGA2+/+ pig primary fibroblast cells and D40 male HMGA2-/+ pig primary fibroblast cells were used respectively. To generate HMGA2-/+ boar, the cell lines mutated by conventional homologous recombination with a targeting vector (Figure 2.1C) were used whereas the cell lines modified by TALEN mediated HDR with a donor plasmid (Figure 2.1D) were used to generate HMGA2-/-STRA8 boar. Transfection was performed with an Amaxa nucleofactor (Lonza, NJ) following its manufacturer manual. The amount of targeting plasmid (Figure 2.1C) transfected into 4 x 10^5 cells was 1.3 μg for the HMGA2-/+ boar generation. After overnight, the media was changed to selection media with 400 ~ 450 μg/mL of G418 for picking positive colonies. The amount of total DNA cotransfected into 4 x 10^5 cells was 1
μg; 250 ng of each TALEN vector, 250 ng of circular donor plasmid (Figure 2.1D) for knock-in, and 250 ng of pSSA reporter plasmid for fluorescence activating cell sorting (FACS: Porteus 2006).

To generate HMGA2-/+ gilts, D30 female wild-type pig primary fibroblast cells were grown in DMEM with 1g/L glucose (Mediatech, VA) with 15% FBS and 1% Penicillin Streptomycin. A classic transfection method was used to generate female heterozygous cell line. An electroporation solution was made of 75% cytosalt electroporation buffer (120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4, 6.5 mM MgCl2, pH 7) mixed with 25% Opti-MEM (Gibco). 1 x 10^6 cells were combined with 2 μg of targeting vector (Figure 2.1B) and electroporated with 1 ms of pulse length, 490 V, and 3 pulses. Selection method was same as for HMGA2-/+ boar generation.

**Oocyte culture and SCNT**

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. The experimental protocols used in this study were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC#: 11-145-B). The method used was essentially as described previously [Walker 1996]. Porcine mature ovaries were obtained at a local slaughterhouse and cumulus oocyte complexes (COCs) from 3-6 mm antral follicles were aspirated. COCs with uniform cytoplasm and at least two layers of compacted cumulus cells were selected. Approximately 50 to 70 COCs per well were cultured in 4-well Nunclon dish containing 500μl TC199-Hepes medium supplemented with 10% porcine follicular fluid (pFF), 5 μg/ml insulin, 10 ng/ml EGF, 0.6 mM cysteine, 0.2 mM
pyruvate, 25 µg/ml kanamycin and 5 IU/ml of each eCG and hCG, covered with mineral oil. The oocytes were matured for 40-42h at 38.5°C, 5% CO₂ in a humidified atmosphere. SCNT was completed as previously described [Walker 1996].

**Embryo transfer and pig management**

In the surgery preparation area, anesthesia was induced by administering the mixture of Xylazine and Ketamine (2-3 mg/kg each animal) (Henry Schein® Animal Health, Dublin, OH) via a marginal ear vein. In the surgery room, anesthesia was maintained by Isoflurane (Henry Schein® Animal Health, Dublin, OH) administered via a closed-circuit gas anesthetic unit. The mid-ventral abdominal area posterior to the sternum was disinfected by scrubbing thoroughly with iodine over the entire surgical field. A mid-ventral laparotomy was performed to expose the reproductive tract. A piece of tubing containing SCNT transgenic embryos connected to a syringe was placed into the oviduct and the embryos were expelled into the oviduct. The peritoneum, each layer of muscle, and skin were closed separately with absorbable synthetic suture material.

Recipient sow was located in a pen after the full recovery from surgery. When pregnancy was detected and maintained until a week before the expected due, sow was moved to a farrowing crate. If sow showed no signs of delivery three days after the due date, 1 cc of Lutalyse® (Pharmacia & Upjohn Company LLC, Kalamazoo, MI) was administered intramuscularly and additional 1cc was injected after 6 hours.

Transgenic newborn piglets were grown with control newborn piglets until weaning. While weaned piglets were raised in nursery barn, transgenic piglets were separately grown
from control piglets. During finishing stage, every two pigs were put in a pen. Table 2.4 summarizes the number of embryo transfers and pregnancies as well as the number of pigs farrowed or sacrificed during pregnancy.

2.2.2 Growth Measurement Analysis

Growth measurement and organ collection of wild-type and HMGA2 mutant pigs

Pig growth was measured by weight, crown-rump length, shoulder height, and abdominal cavity (Figure 2.2). Eight HMGA2-/- boars, their seven HMGA2+/+ SCNT littermate boars and five HMGA2+/+ naturally bred boars were measured every 2 weeks from birth to 30 weeks of age. One HMGA2-/- boar was also measured every 2 weeks until its death at 4 months. Six HMGA2-/- gilts and six HMGA2+/+ farm gilts were measured every 2 weeks from birth to 59 weeks of age. Organs of HMGA2 mutants and their equivalent aged controls were collected for analysis at the day of euthanasia. Boars were euthanized at 8.5 months of age whereas gilts were done at 2 years of age.

Statistical analysis for growth comparison

Nonparametric trend test (Friedman’s ANOVA) was performed to compare the growth curve (trend) of mutants to the one of wild-type. Parametric test (One-way ANOVA) was done for the weight comparisons.
2.2.3 Reproductive Characterization

**Superovulation and semen collection**

To stimulate the ovaries of *HMGA2-/+* gilts that at 1 year of age had not shown normal estrous behavior, we administered PG600® (Intervet Inc., Millsborough, DE), following manufacturer’s instructions. Estrus behavior was monitored by exposing *HMGA2-/+* gilts to *HMGA2-/+* boar daily for a month. Two *HMGA2-/+* boars were used for heat-check when they became 8 months of age (approximately 150 kg). To monitor estrus behavior, *HMGA2-/+* gilts were released toward the pens where wild-type farm boars were kept. For *HMGA2-/+* gilts that didn’t still show estrus behavior, one *HMGA2-/+* boar was introduced in the isolated hallway with *HMGA2-/+* gilts for 10 minutes.

Semen from two *HMGA2-/+* boars was collected starting at 6 months of age. Semen was collected for two consecutive days and the total number of sperm from each collection was determined using a Spermacue® (Minitube International, Verona, WI). Sperm mobility and morphology were examined by microscopy. Semen was used for in vitro fertilization and artificial insemination.

**Monitoring follicular development by trans-rectal ultrasound and serum progesterone level**

To determine if *HMGA2-/+* gilts have normal follicular growth patterns, we visualized follicular maturation using a transrectal ultrasound. In addition, serum progesterone level was measured using an enzyme-linked immunosorbent assay (ELISA). Two *HMGA2-/+* founder gilts and two *HMGA2+/-* control gilts were used. Follicular
dynamics were monitored twice a week for four weeks [Knox 1999]. On the same day ultrasound was performed, 2~3 ml of blood were collected from jugular vein using a spinal needle (Becton, Dickinson and Company, NJ). Blood was centrifuged (1,500 × g at room temperature for 15 mins), and the supernatant serum was frozen for future analysis. All ELISAs were performed using a microwell swine progesterone ELISA kit (Endocrine Technologies Inc., CA).

**Histological analysis of reproductive tissues from fetuses and offspring**

To determine the effect of the HMGA2 mutation on the reproductive tissues, male and female gonads were analyzed histologically at newborn and adult stages. Male samples at birth and 4 months old and female samples at 11 months and 30 months old were collected and fixed in 10% neutral buffered formalin (NBF) for a week at 4°C. All of tissues were carefully trimmed for the sites of interest before they were embedded in paraffin blocks. The embedded blocks were continuously cut until the full-face of tissues was seen, and then a 5µm histological section of each block was prepared on a slide. The histological sections were stained with hematoxylin and eosin (H&E) and analyzed.

**In vitro fertilization (IVF) to confirm germ line transmission**

For IVF was carried out essentially as described in [Zhang 2010]. Approximately 30 to 35 matured oocytes were transferred into 50-µL droplets of IVF medium. 1mL semen collected from HMGA2-/+ boar was centrifuged, washed twice and resuspended in fertilization medium to a concentration of 1 × 10^6 cells/mL. 50 µL of the sperm in the
fertilization medium was added to the fertilization droplets containing the oocytes, giving a final sperm concentration of \(0.5 \times 10^6\) cells/mL. Oocytes were coincubated with sperm for 4 to 6 h. After fertilization, oocytes were washed 3 times and cultured in 500 μL of NCSU-23 medium with 0.4% BSA at 38.5°C in 5% CO2 in air. LacZ staining of D6-D7 blastocysts was used to evaluate germ line transmission.

**Statistical analysis for goodness of fit**

Chi-square test and Fisher’s exact test were performed to validate the goodness of fit for the genotype ratio of piglets from three litters by breeding of \(HMGA2^{-/-}\) clones. Mating attempt of \(HMGA2^{-/-}\) gilt with \(HMGA2^{-/-}\) boar was performed only once to obtain two of the three litters. Each mating was performed once the estrus behavior of \(HMGA2^{-/-}\) gilts was detected. One litter was obtained by rebreeding of one of the \(HMGA2^{-/-}\) sows at 6 days after weaning. Mating was attempted only once too.

**Analysis of fetal loses at different stages of gestation in progeny from \(HMGA2\) mutants**

Conventional breeding between \(HMGA2^{-/-}\) gilts and boars indicated that HMG2 null fetuses were unable to make it to term. To determine the timing of fetal loss, one D40 and two D78 pregnancies were examined. After the confirmation of pregnancy by ultrasound, \(HMGA2^{-/-}\) sow for each gestational stage was euthanized by intravenous injection of sodium pentobarbital, named as Socumb (Henry Schein® Animal Health, Dublin, OH), at a rate of 1 ml per 10 lbs of body weight (IACUC#: 11-145-B). Fetal and placental tissues were collected for genotyping and morphological and histological analysis. Fetal weights were recorded.
Placental samples were fixed in 10% NBF for a week at 4°C, and trimmed and processed for histological sections. The sections were stained with H&E and examined for the chorioallantoic-endometrial contact areas. Rugae at the area were visualized for comparisons between genotypes.

2.3 Results

2.3.1 Generation of Transgenic Pigs by Knock-in HMGA2 Locus

Generation of HMGA2-/+ SCNT donor cell lines by homologous recombination

To study the effect of disruption of HMGA2 gene on fetal and adult growth in pigs, both HMGA2-/+ male and female primary fetal fibroblasts cell lines were generated by conventional gene targeting, using a gene trap approach, as HMGA2 is expressed in fetal fibroblast. This allows for enrichment of targeted cells by selection in G418 (Figure 2.5A). To confirm homologous recombination events, four different PCR-based screening assays, amplicon sequencing, RT-PCR and LacZ staining, were performed. As shown in Figure 2.5, evidence included the presence of two diagnostic PCR bands in HMGA2-/+ colonies (Figure 2.5B), sequencing confirming the presence of two different sequences (one endogenous and one transgenic) in a single sequencing reaction (Figure 2.5C), detection of β-geo mRNA (Figure 2.5D), and LacZ staining (Figure 2.5E). Overall, the evidence confirmed that we had been able to target the HMGA2 locus. In order to verify that the β-geo had been incorporated into the HMGA2 locus without frameshifts or mutations, we sequenced all of the four junctions as shown in Figure 2.6 and 2.7. Results confirmed that the transgene was correctly integrated into the HMGA2 exon 2 and joined to the upstream and downstream of
HMGA2 endogenous sequence, and that the whole sequence was in-frame from the start codon of HMGA2 gene to the stop codon of β-geo. No aberrant mutation was detected on the targeted region. Targeting rates were 20 out of 34 colonies (58.8%) for the male cell lines and 4 out of 28 colonies (14.3%) were for female cell line.

**Generation of HMGA2-/- SCNT donor male cell lines by TALEN mediated HDR**

To generate HMGA2-/- male cell lines, we disrupted the remaining allele in primary HMGA2-/+ male fetal fibroblast using TALENs. TALENs were designed to target the endogenous allele but not the β-geo targeted allele in the primary HMGA2-/+ fetal fibroblast cells. To test TALENs efficiency, the single strand annealing reporter plasmid (pSSA) with the binding sites of our TALENs was used [Porteus 2006]. GFP expression after TALENs and pSSA reporter plasmids were cotransfected was measured by FACS and microscopic observation. Results indicated that up to 16% cells were modified (11676 out of 73139 total electroporated cells) (Figure 2.8A and 2.8B). Sequencing demonstrated introduction of variable lengths of deletions at the endogenous HMGA2 locus (Figure 2.8C). Endonuclease assay for PCR amplicon of the target region confirmed the deletion events due to no more BsaWI site existed (Figure 2.8D). After single colony analysis of TALEN-modified colonies, we identified 26 out of 40 (65%) cell lines as mutant. In addition to generate HMGA2-/- cell lines, we also generated knock-in lines that contained the HMGA2 coding sequence under the Stra8 promoter. This was done to obtain a HMGA2 null pig capable of expressing the HMGA2 proteins only in the germ cell to reduce potential negative fertility effects. To generate the Stra8-cHMGA2 null cell lines for SCNT, TALEN-mediated HDR
was utilized. As Figure 2.9A shows, the targeting vector with STRA8 promoter-cHMG2 was co-transfected with TALENs into primary $HMG2^{-/-}$ fetal fibroblast cells. Positive mutant cells for $HMG2^{-/-}_{Str8}$ were identified by PCR reactions followed by sequencing (Figure 2.9B and 2.9C). 8 out of 40 (20%) cell lines had the Stra8 transgene at the correct $HMG2$ locus.

To confirm functional disruption of $HMG2$, western blot was performed on all genotypes that we created. As shown in Figure 2.10, $HMG2^{-/-}_{Str8}$ and $HMG2^{-/-}$ showed lack of $HMG2$ expression whereas $HMG2^{-/+}$ expressed less compared to $HMG2^{+/-}$. The confirmed mutant cell lines were used to generate pig by SCNT. Overall, we generated 8 $HMG2^{-/-}$ boars, 11 $HMG2^{-/-}_{Str8}$ boars, and 6 $HMG2^{-/+}$ gilts in order to examine the effect of $HMG2$ on their phenotype. Out of 11 $HMG2^{-/-}_{Str8}$ boars, only one was utilized for growth analysis due to the death of the remaining 10.

### 2.3.2 Growth Analysis of $HMG2$ modified Pigs

**Growth analysis of $HMG2$ mutant boars**

As reported in pygmy mouse [Xiang X 1990 and Zhou X 1995], disruption of one $HMG2$ allele in pigs results in growth reduction. Growth curve analysis revealed that both $HMG2^{-/+}$ and $HMG2^{-/-}_{Str8}$ boars were smaller from birth compared to their equivalent aged $HMG2^{+/-}$ controls. In contrasts, for the first three months, weights of $HMG2^{-/+}$ boars were not significantly different than $HMG2^{+/-}$ controls. However, during the next four months, $HMG2^{-/+}$ boars were 17% and 16% lighter in weight over $HMG2^{+/-}$ SCNT littermate and naturally bred boars, respectively (Figure 2.11A). Parametric test confirmed
that reduced weights for $HMGA2^{-/-}$ boars were significant ($p<0.05$) at 30 weeks of age. However, nonparametric trend test showed that the trends of three groups were not significantly different. A $HMGA2^{-/-}Stra8$ boar showed severe growth reduction during the early postnatal growth. At the age of 14 weeks, the weight of $HMGA2^{-/-}Stra8$ boar was 76.5% lighter than $HMGA2^{+/+}$ naturally bred boar (Figure 2.11B), the length was 34.7% shorter (Figure 2.11C), the height was 38.9% shorter (Figure 2.11D), and the body cavity was 38.9% smaller (Figure 2.11E).

At 25 weeks, $HMGA2^{-/-}$ boars weighted 82% as much as their littermate controls (Figure 2.12A). Analysis of 7 major organs confirmed that growth reduction was symmetrical with most of organs involved (Figure 2.12B). At 9 months of age, most of $HMGA2^{-/-}$ organ weights were in the range of 80-90% compared to $HMGA2^{+/+}$ controls (Table 2.5). Additional reduction in size was seen in the absence of $HMGA2$ (both alleles inactivated). Body weight of a $HMGA2^{-/-}Stra8$ boars was 25% of $HMGA2^{+/+}$ boars at 10 weeks (Figure 2.13A), and there was also reduction in weight of each $HMGA2^{-/-}Stra8$ organ (Figure 2.13B). $HMGA2^{-/-}Stra8$ organ weights were in the range of 20-40% compared to $HMGA2^{+/+}$ controls (Table 2.6).

**Growth analysis of $HMGA2$ mutant gilts**

As for boars, disruption of one allele of the $HMGA2$ gene in gilts resulted in 65% in weight of control gilts at 30 weeks of age (Figure 2.14A). Weight analysis of each organ revealed that $HMGA2^{-/+}$ organs were in the range of 60-80% compared to $HMGA2^{+/+}$ organs (Figure 2.14B and Table 2.7). There was a significant growth reduction for $HMGA2$-
/+ gilts until they reached stationary phase of growth at 59 weeks of age (Figure 2.14C).

Early in postnatal growth (two months), weights of \( HMGA2-/+ \) gilts were not different than \( HMGA2+/+ \) naturally bred gilts. However, mutants were 20-35% lighter during mid stages (six months) and 25-30% in late stages (three months) compared to controls. In contrast to \( HMGA2-/+ \) boars, nonparametric trend test showed that the trends of two groups were significantly different (\( p<0.05 \)). Parametric test confirmed that the weight reduction was significant at 59 weeks of age.

### 2.3.3 Reproductive Characteristics of \( HMGA2 \) Mutant Pigs

#### Fertility of \( HMGA2-/+ \) gilts, \( HMGA2-/+ \) boars, and \( HMGA2-/^{\text{Stra8}} \) boars

Despite daily monitoring for 18 months, four of six \( HMGA2-/+ \) SCNT gilts did not show any estrus behavior. Analysis of follicular development visualized by a trans-rectal ultrasound determined that, as control gilts normally had, all mutants had normal follicular growth and follicular cycles in spite of the absence of estrus behavior (Figure 2.15A).

However, analysis on serum progesterone levels revealed that \( HMGA2-/+ \) SCNT gilts had abnormally low progesterone concentrations compared to age-matched \( HMGA2+/+ \) naturally bred gilts (Figure 2.15B). To determine if the abnormal behavior of \( HMGA2-/+ \) SCNT gilts was due to the \( HMGA2 \) mutation or to the SCNT procedure, we monitored three \( HMGA2-/+ \) F1 gilts were obtained by natural mating of \( HMGA2-/+ \) SCNT founder sows bred to \( HMGA2-/+ \) SCNT founder boars. Interestingly, all three \( HMGA2-/+ \) F1 gilts showed normal estrus behavior at between 6 and 8 months, which is an expected pubertal range for normal gilts (Figure 2.15C). Ovaries of \( HMGA2-/+ \) SCNT founders macroscopically showed
vascularization was not fully developed than \textit{HMGA2+}/+ ovaries, and one of \textit{HMGA2-}/+ SCNT founders had severely abnormal cysts (data not shown). However, ovaries of \textit{HMGA2-}/+ F1 were morphologically normal when compared to \textit{HMGA2+}/+ ovaries (data not shown). Despite the abnormal morphology of \textit{HMGA2-}/+ SCNT founder ovaries, histological analysis on \textit{HMGA2-}/+ SCNT founder ovaries showed normal folliculogenensis (Figure 2.16A). Matured follicles and CLs were detected in \textit{HMGA2-}/+ SCNT ovary, supporting the follicular development seen by the transrectal ultrasound. \textit{HMGA2-}/+ F1 also had normal follicular growth as expected (Figure 2.16B).

In contrast, \textit{HMGA2-}/+ SCNT boars had normal puberty and sexual behaviors up to the present (2 years and 4 months) (Figure 2.15C). As shown in Table 2.8, sperm collected from \textit{HMGA2-}/+ SCNT boars were healthy and motile with qualified number that wild-type boar normally has at 7 months of age, and the majority of sperm had normal tail formation and cytoplasmic distal droplet as expected. LacZ staining assay on embryos generated by in vitro fertilization with collected \textit{HMGA2-}/+ sperm and \textit{HMGA2+}/+ oocytes confirmed germ line transmission of the β-geo transgene (Figure 2.15D). H&E histological analysis supported that \textit{HMGA2-}/+ boars reached normal puberty to produce sperm. Figure 2.16C shows that germ cell organization in a seminiferous tubule did not differ between mutants and controls.

While \textit{HMGA2-}/+ SCNT boars were normal, all HMGA2 null boars had undescended testes at birth (Figure 2.17A). \textit{HMGA2-}^{\text{stra8}} testes did not fully descent and lacked gubernaculum bulbs compared to control age-matched pigs (Figure 2.17B). A similar phenotype (lack of testes descent and lack of gubernaculum bulbs) was also seen in \textit{HMGA2-}
D78 fetuses generated by breeding of $HMGA2^{-/-}$ SCNT founders, while $HMGA2^{-/+} D78$ littersmates were normal (data not shown). In addition, neither HMGA2 null boar nor fetuses had distinct inguinal canals (data not shown). Histological analysis of undescended testes showed that internal structure and organization of seminiferous tubules in the neonatal $HMGA2^{-/-}^{\text{ Stra8}}$ testes were normal when compared to neonatal $HMGA2^{+/+}$ boars (Figure 2.17C). However, at 4 months of age, none of seminiferous tubules in a $HMGA2^{-/-}^{\text{ Stra8}}$ testes showed any evidence of spermatogenesis while a few tubules in a $HMGA2^{+/+}$ testis did. In summary, HMGA2 null leads to cryptorchid testes in boars, affecting future spermatogenesis.

**Fetal viability of HMGA2 null mutants**

Mating of $HMGA2^{-/+} X HMGA2^{-/-}$ founders resulted in 3 pregnancies to term. However, genotype of resulting offspring indicated the absence of the HMGA2 null genotype. As shown in Table 2.9, we performed chi-square test to validate the goodness of fit for the ratio of genotypes from the three litters. The test demonstrated our data did not fit a Mendelian ratio (p < 0.05). Fisher’s exact test confirmed the result of chi-square test (data not shown). To determine the timing of fetal loss and to identify a cause for the loss, one pregnancy at D40 as well as two pregnancies at D78 were examined. At both D40 and D78 litters, $HMGA2$ genotyping results confirmed the presence of all four different genotypes, including $HMGA2^{-/-}$ (Figure 2.18A and 2.18B). HMGA2 null fetuses could be detected at the expected Mendelian frequency of approximately 25% (3 of 12 in D40 litter and 4 of 19 in D78 litters). Fetal weights from D40 fetuses revealed a significant growth reduction for $HMGA2^{-/-}$ offspring (30% reduced) (Figure 2.18C). At D78 gestation stage, both $HMGA2^{-/}$
+/ and HMGA2-/ fetuses were significantly smaller (24% and 74% reduced in weight, respectively) than HMGA2+/+ fetuses (Figure 2.18D).

While no defects were detected in D40 and D78 fetuses per se (Figure 2.18E), placental abnormalities were seen only in HMGA2-/ conceptuses from both D78 litters at both the macroscopic and microscopic level. Macroscopically, HMGA2 null D78 placentas showed extensive degeneration (Figure 2.19A). This degeneration ranged from the whole placenta being affected (Figure 2.19A) to only some areas being affected (Figure 2.19B). At the histological level, placentas from HMGA2-/ fetuses had abnormal rugae at the contact area between chorionic membrane and uterine endometrium (Figure 2.19C). Despite the morphological difference at D78, placental weights of HMGA2-/ conceptuses from D40 and D78 litters were not significantly different from the other genotypes (Figure 2.19D).

To determine whether lack of HMGA2 or intrauterine resource depletion was responsible for lethality of HMGA2 null pigs after D78, we generated, by SCNT, a pregnancy carrying only the HMGA2 genotype. From this pregnancy, 10 live HMGA2 null clones were successfully generated (Figure 2.20A and 2.20B), indicating that HMGA2 deficiency does not cause fetal lethality. As expected, all HMGA2 null offspring had reduced body size (Figure 2.20C) and drastically reduced organ weights (Figure 2.20D and 2.20E). All of HMGA2 null organs were in the range of 20-50% compared to ones of wild-type control.

2.4 Discussion

This study demonstrated that HMGA2 mutation cause the growth reduction in pigs. It
has been reported that lack or reduced expression of *hmga2* results in growth retardation in mice [Xiang 1990, Benson 1994 and Zhou 1995]. Adult homozygous mutant mice (mini-mice) were 40% the size of their wild-type littermates whereas adult heterozygous mutant mice only attain 80% the size of their controls [Xiang 1990 and Zhou 1995]. Our results are in agreement with the findings. As in mutant mice, our weight data for *HMGA2/-+* mutant boars and gilts revealed that adult heterozygous mutant boars were approximately 80% the size of wild-type controls, and the similar amount of body reduction was also seen for adult heterozygous gilts. Although the trending of heterozygous mutant boars was appeared differently from that of controls, nonparametric trend test for heterozygous mutant boars showed that trending for 30 weeks was not significantly different. For heterozygous mutant gilts, trending was significantly different. The reason for different statistical results of boars and gilts was because we have weight data of heterozygous mutant boars only for 30 weeks. At the early postnatal stage (10 weeks), our pre-pubertal *HMGA2* null boar was only 25% the size of wild-type control. Although no data was collected from the late postnatal development of *HMGA2* null boar, our data showed promising evidence that the reduction in growth of *HMGA2* null boar would be maintained or increased during later development, as shown in *HMGA2/-+* pig growth.

Growth reduction in pigs was not only due to reduced body fat but also due to the reduced weight of organs commensurate with the reduced body weight. At 9 months of age, most of *HMGA2/-+* organ weights were in range of 80-90% compared to *HMGA2/+/+* controls, as previously reported in the characterization study of heterozygous mutant mice [Benson 1994]. At 10 weeks of age, most organs of *HMGA2* null boar were severely small in
weight (20-40% of wild-type control) as in adult mini-mice (40-60% of wild-type littermates) [Benson 1994 and Zhou 1995]. Therefore, disruption of HMG2 gene results in the growth reduction for both boar and gilt.

To further our understanding of what other effect HMG2 mutation made in transgenic pigs, reproductive characterization was performed since the fertility is an important feature in transgenic animal model [Vasudevan 2010]. While two of six HMG2-/+ SCNT gilts were bred, pregnant, and come back in-heat 5 days after weaning, four HMG2-/+ SCNT gilts showed no estrus behavior and abnormally low progesterone level while they had normal follicular development. Abnormally low level of sex hormone during a month is one of reasons for HMG2-/+ SCNT gilts without estrus behaviors. Despite daily mating attempts, the four mutants were not able to be bred.

In mice, homozygous mini-mice carrying transgenes were not generated by SCNT but by intercross of heterozygous offspring derived from chimeric transgenic founder (mosaicism) carrying mutations in hmg2 locus [Xiang 1990 and Zhou 1995]. All of heterozygous mutants were fertile and bred normally [Xiang 1990 and Zhou 1995]. It was also reported that heterozygous mutants that were arisen spontaneously during breeding of selected mice were fertile and normally bred [King 1955]. Since generation method of heterozygous mutants in mice was different from one of ours, abnormal estrus behavior of HMG2-/+ gilts was thought to be from the method difference. Behavioral variations or phenotypic abnormalities of clones generated by SCNT have been reported in pigs [Archer 2003 and Kurome 2013]. According to Niemann group, the most critical factor is epigenetic reprogramming of the transferred donor cells during SCNT cloning [Niemann 2008]. Partial
reprogramming on epigenetic marks may lead to aberrant gene expressions for future
development. Inconsistent reproductive behaviors between HMG2-/+ cloned gilts (two with
estrus behavior but none of four) suggest that each of them had different extent of epigenetic
reprogramming during SCNT procedure. Previous studies reported that cloned animals with
phenotypic abnormality were able to produce normal offspring [Tamashiro 2002 and
Cho2007]. This was also proved when we monitored HMG2-/+ F1 offspring. Lack of estrus
behavior was recovered when HMG2-/+ F1 gilts were generated by breeding of HMG2-/+ SCNT founders. HMG2-/+ gilts from F1 litter of HMG2-/+ founder sow had normal estrus
behaviors at the expected pubertal age. This suggests that no estrus behaviors and low
progesterone levels might be caused by epigenetic errors during SCNT procedure. This
means that the variable epimutations, occurred to donor cells when culturing in vitro, were
erased during germ line transmission and the epigenetic marks that were partially
reprogramed through SCNT procedure were completely reprogramed by breeding
[Humpherys 2001 and Hydig 2011]. Therefore, no estrus behavior shown by HMG2-/+ founders was not due to HMG2 mutations but due to SCNT effect.

All of our HMG2 null boars (HMG2-/-Stra8) had bilateral cryptorchid testes. The
cryptorchidism was also seen in spontaneous pygmy mice (Small Pygmy Line) [King 1950
and King 1955] and mini-mice [Benson 1994]. It was reported that testes of mini-mice were
located at inguinoscrotal testis position [Klonisch 2004]. In addition, hmg2 homozygous
mini-mice revealed that spermatogenesis was disrupted [Chieffi 2002 and Di Agostino 2004]
whereas several spontaneous pygmy mice with increased size (Large Pygmy Line) were fully
fertile [King 1955]. The fertility issue in male mice was most likely not only because of
genetic mutation of *hmga2* but also because of cryptorchidism. Regarding with two conditions, possible explanation can be made for the fertility issue in HMGA2 null mutants. First, the main phenotype of *hmga2* KO was the reduced cell number [Zhou 1995]. Lack of *hmga2* expression is likely to make Hmga2 null mice to be subfertile due to the reduced number of primordial germ cells. Second, since testes are very sensitive to temperature, exposure of testes to the intraabdominal temperatures prior to adolescence is disturbed, which leads to impaired fertility [Senger 2012]. In the two conditions together, the subfertility in mini-mice would be reduced down to the infertility when the increased temperature environment is exposed to undescended testes, resulting in block of spermatogenesis [Kazusa 2004]. Evidence of possible blockage of spermatogenesis was also seen in our HMGA2 null boar. As shown in Figure 2.17C, no evidence of spermatogenesis was detected in the mutant boar. Therefore, it is possible that combination of the two conditions synergistically contributes to sterility and testicular failure in male mice and most likely did to our HMGA2 null boars too.

Two *HMGA2-*/+ SCNT sows bred by *HMGA2-*/+ SCNT boars were able to term three litters with lack of *HMGA2-* piglets. All of three litters consistently had mummified piglets, which were likely *HMGA2-* genotype (data not shown). Interestingly, we were able to detect *HMGA2-* genotype in the litters at early (D40) and late (D78) gestation stages, getting all fetuses healthy at both gestations (Figure 2.18E). No placenta abnormality was seen in D40 litter, but in D78 litter. *HMGA2-* placentas from one of two D78 litters were completely degenerated whereas one from the other was not fully degenerated (Figure 2.19). This tells us that the gestation stage around D78 is the critical turning point for *HMGA2-*
fetuses to fail to survive. It is interesting to note that in cloned mice generated by nuclear injection, placental defect (placentomegaly) was identified [Wakayama 1999 and Ogura 2000] and in genetically modified cattle generated by whole cell injection, abnormal placenta was characterized as having hydroallantois, enlarged placentomes, and anedermatous chorioallantois and amnion [Cibelli 1998] which lead to embryonic mortality in the first-trimester of gestation. In addition, examination on bovine placenta of somatic cell cloned fetuses revealed that placentas of small fetuses (small for age) were abnormal at the early gestation [Hill 2000]. The abnormal phenotype of bovine cloned placenta was similar to that of our degenerated placenta of $HMGA2^{-/-}$ fetus, which were flat, cuboidal chorionic epithelium with a marked decreased vascularity, or lacked normal cotyledon development and number [Hill 2000]. It is also reported that placentas from bovine NT fetuses did not appear to be able to produce normal cotyledons, so the decrease in fetomaternal exchange area cause starvation to fetal death in utero around D60 [Stice 1996]. Even though reduced number of placentomes possibly occurred in third-trimester and term cloned transgenic bovine fetuses were reported [Hill 1999], all of the reports are not the case for placenta degeneration only occurred for our $HMGA2^{-/-}$ porcine fetuses at the late gestation (D78) since the $HMGA2^{-/-}$ embryos were not generated by SCNT but by breeding. Therefore, it must be other factors than SCNT effect to cause the placental abnormality of $HMGA2^{-/-}$ fetuses.

Pig models were established to study the effect of uterine crowding to conceptus development and survivability, since uterine capacity is one of important factor affecting fetal survivability. There are four different methods to study uterine crowding; unilateral
hysterectomy and ovariectomy (UHO), unilateral oviduct ligation (UOL), superovulation, and superinduction. Despite similar ovulation rate due to compensatory ovarian hypertrophy, UHO gilts that have only half of the uterine endometrial surface area available for conceptus development produced smaller litter size at D86 of gestation and farrowed litters, indicating greater fetal loss by uterine crowding compared to control [Christenson 1987]. In UOL model, placental weight (D30 and D90) and fetal weight (D90) from non-ligated sows were lower compared to ones from tubal ligated sows, suggesting uterine crowding negatively affecting placental and fetal development at D30 and D90 [Town 2004]. It was also reported that uterine space is not necessarily associated with the embryonic mortality up to D30 but is after D30 in the presence of large litter or restricted uterine space [Webel 1974]. Increasing ovulation rate (superovulation) significantly increase litter size at D25 to D40 [Longenecker 1968] but does not at term [Bazer 1968], and embryo transfer (superinduction) method may limit litter size by uterine capacity set prior to D25 [Bazer 1969b], so no significant litter size was increased at term [Bazer 1969a]. Both of these two models suggest uterine capacity negatively affects litter size. However, none of the model studies have reported the placenta degeneration only occurred for small fetuses or runts as the ones we saw in our $HMGA2^{-/-}$ SCNT gilts at D78. Nevertheless, concept of uterine crowding leading to limited uterine capacity makes a possible explanation for lack of $HMGA2^{-/-}$ piglets.

$HMGA2^{-/-}$ sow had smaller uterus compared to $HMGA2^{+/+}$ control (Figure 2.14B). In pigs, uterine capacity only affects litter size after D30 [Frenton 1972 and Ford 2002] even though it is set as early as between D11 and D13 [Ford 2002]. It was reported that there is a significant positive correlation between total length of the uterus and total number of fetuses
in pigs during early (D30) [Holness 1982] and late (D77) gestations [Wu 1987]. There is a negative correlation between uterine size and prenatal mortality [Wu 1987]. A long and heavy uterus has a better potential to accommodate fetuses, making them for easy development until farrowing [Bennett 1989 and Van der Lende 1990]. Regarding of uterine capacity, smaller size uterus of \textit{HMGA2}-/+ gilts, which is subjected to have relatively lower capacity, contributes to set much higher competitive environment for developing fetuses at D78. Even relatively modest uterine crowding negatively affects fetal development at D30 and D90 [Town 2004], and a reduced uterine space available for each fetus increased the number of mummified fetuses [Père 1997]. An increased occurrence of mummified fetuses was also observed when the size of uterine was reduced [Wu 1988]. Positive correlation between uterine capacity and fetal survival [Rillo 2001] was proved by successful generation of HMGA2 null piglets by SCNT. Cloned HMGA2 null piglets were successfully born from \textit{HMGA2}+/+ sows that possess uterus with relatively bigger capacity, which is more than enough for small cloned fetuses (\textit{HMGA2}-/-) in the absence of \textit{HMGA2}-/+ or \textit{HMGA2}+/+ fetuses.

Placenta degeneration might be triggered because \textit{HMGA2}-/- fetuses were lost at the competition against other genotype fetuses, whose sizes dramatically increases during the final half of gestation [Knight 1997]. For rapidly growing fetuses during the late gestation, a lot more metabolic needs must be satisfied through the blood flow from sows [Ford 1995 and Wu 2006]. However, low uterine capacity of \textit{HMGA2}-/+ sows provides limited resources and sets much higher competitive environment for the \textit{HMGA2}-/- fetuses to struggle against littermates. It is the environment that \textit{HMGA2}-/- fetuses are unable to compete for resources
in crowded uteri carrying $H_MGA2^{-/-}$ and $H_MGA2^{+/+}$ fetuses. It has been known that uterine capacity begins to critically affect litter size after D30 gestation in pigs when a competition by littermates is increasingly necessary for limited uterine space and resources [Frenton 1972, Huang 1987, and Wu 1987]. This is supported by other studies about uterine capacity.

The uterine blood flow per fetus deceases with increasing litter size [Père 2000] and the increased litter size and weight are associated with increased placental vascularity [Biensen 1998]. Therefore, lost at the competition, $H_MGA2^{-/-}$ fetuses may not be able to maintain vascular density of placenta and a number of areolae (ultrastructures in all over placenta, which help for the resource transfer, such as diaplacental iron transport, from sow to fetus [Friess 1981 and Dantzer 1993]) as shown in Figure 2.19A, which leads to lack of blood flow and nutrient transport, eventually resulting in the placental abnormality and fetal death.

Blood flow through placental vascular network as well as nutrient transfer through ultrastructure of areolae play a critical role for fetal growth and development [Biensen 1998 and Friess 1981] since they are the only ways for fetuses to communicate with sow. In fact, porcine conceptuses begins producing embryonic signals on D11-12 of gestation [Perry 1976, Ford 1982, and Pusateri 1990], and they secretes significant amounts of estrogen into the uterine lumen through the blood flow. This leads to endometrial secretion of growth factors, including insulin-like growth factor-I (IGF-I), which affect fetal development [Simmen 1989 and Wilson 1997]. Therefore, lethality of $H_MGA2^{-/-}$ fetuses was due to lost in mutual signals (placental degeneration) between conceptus and sow, which was triggered by lost in competition by littermates. This means that no $H_MGA2^{-/-}$ piglets in the litters from $H_MGA2^{-/+}$ founders breeding was not due to the mutations on the $H_MGA2$ loci. According
to Zhou group, they found that no expression of \( hmg\Delta2 \) was detected in wild-type placenta of mice during any of gestation stages [Zhou 1995], supporting our explanation that lack of \( HMGA2 \) expression was not a direct reason for the placenta degeneration leading to \( HMGA2^{-/-} \) fetal loss at the late gestation. Although a positive correlation between placental weight and fetal weight at D70 of gestation was reported [Vianna 2004], D78 mutant fetuses were significantly smaller than wild-type (Figure 2.18D), while placental weight for all genotypes were compatible at D78 of gestation (Figure 2.19D). This means that \( HMGA2 \) gene mutations are not involved in the placental development, so that do not directly lead to embryonic lethality.
CHAPTER 3

Conclusions and future directions
3.1 Conclusions

The overall objective of this study was to determine the effect of HMGA2 on fetal and adult growth in pigs without deleterious effect. As in mice, our results were able to demonstrate that the deficiency of HMGA2 expression caused growth retardation in both male and female pigs. Lack of HMGA2 proteins caused even more reduction of body size in a male pig during the early postnatal development. Reproductive characterization identified that heterozygous cloned gilts showed no estrus behavior and abnormal hormonal level despite the presence of follicular development. Our data indicated that abnormal reproductive characteristics of heterozygous cloned gilts were due to SCNT procedure, since we identified that heterozygous naturally bred gilts had normal estrus behavior. In contrast, heterozygous cloned boars had normal reproductive. We have also seen embryonic lethality of homozygous piglets from breeding of heterozygous clones. Our data demonstrated that competition for intrauterine resources by littermates was responsible for lack of homozygous piglets. In conclusion, our results demonstrate that disruption of HMGA2 gene reduces growth in both boar and gilt without leading to reproductive abnormalities or embryonic lethality but causing cryptorchidism for HMGA2 null boars.

3.2 Future Directions

Decreasing degree of competition by unilateral oviduct ligation

Uterine crowding increases embryonic mortality, affects fetal and placental development [Town 2004, Tse 2008 and Bernard 2010]. In pigs, unilateral oviduct ligation has been popularly used to study the effect of uterine crowding, resulting in lower numbers
of relatively healthy embryos and higher placental weights [Webel 1974, Pere 1997, Town 2004, Tse 2008 and Pardo 2013]. The method allows embryos to have double capacity since they enter the horn opposite the one of origin and continue migrating for between D9 and D12 [Dziuk 1985]. So, unilateral oviduct ligation (Tied and Cut) on left side oviduct was performed to reduce litter size in the uterus of HMGA2-/+ F1 gilts. This will potentially reduce uterine crowding and provide less competitive uterine environment for HMGA2-/- fetuses to be better able to compete for resources. Currently, mating has been attempting to obtain pregnancies that may give a birth of HMGA2-/- piglet.

Rescue of cryptorchidism for HMGA2 null boars

One possible solution for our HMGA2 null cryptorchid boars would be to force to develop the growth of gubernaculum that is mainly related to testes’ descent before birth. Researchers who have studied on the cause of cryptorchidism in mice have demonstrated that the expression of Insl3 was crucial for the testes’ descent [Nef 1999 and Huang 2012]. The overexpression of Insl3 in female mice also made ovaries’ descent [Adham 2002]. In addition, cryptorchidism in the Insl3 knock out male mice was rescued by the pancreas specific expression of Insl3 gene driven by rat Insl2 promoter [Adham 2002]. In this way, the β-cells in the pancreatic islets are able to make a faster action to convert pro-Insl3 to bioactive Insl3 gene products. The results and technical strategies in cryptorchid mice study may apply for us to solve the fertility issue of our HMGA2 null boars.
Table 2.1. Oligonucleotide used for plasmid construction

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<td>27</td>
<td>Sequencing primer for checking 5' end of 5' homology arm (Seq1)</td>
<td>ACTTGCTGCTGCTTCCCTGG</td>
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<td>Sequencing primer to check pβGeo-bpA-flox-DTA-SV40</td>
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<td>Sequencing primer to check pβGeo-bpA-flox-DTA-SV40</td>
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<td></td>
<td>Sequencing primer to check left or right TALEN plasmids</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>49</td>
<td>AACAGGCAAGGCTTGACCG</td>
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<td>50</td>
<td>AGTAACACGCGTAGAGGCAGTGAAC</td>
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<td>Sequencing primer to check left TALEN plasmid</td>
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<td>54</td>
<td>Sequencing primer to check left or right TALEN plasmids</td>
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<td>56</td>
<td>Sequencing primer to check left or right TALEN plasmids</td>
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<tr>
<td>57</td>
<td>Sequencing primer to check left or right TALEN plasmids</td>
<td>ACGCCACAGATGTCATTC</td>
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<td>58</td>
<td>Sequencing primer to check right TALEN plasmid</td>
<td>AGTTTAGCGATTGCGAATAACAAATG</td>
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<tr>
<td>59</td>
<td>Sequencing primer to check right TALEN plasmid</td>
<td>TGTAGCGATTGCGAACAATTG</td>
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Note: Parenthesis is the word used in figures.
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<th></th>
<th>Description</th>
<th>Oligonucleotide Sequence (5’ to 3’)</th>
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<tr>
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<td>FWD PCR primer to check 5’ end of βGeo or STRA8 allele (FWD1)</td>
<td>TGGGGGACGGGACTCAGAAAAAC</td>
</tr>
<tr>
<td>61</td>
<td>FWD PCR primer to check 5’ end of βGeo or STRA8 allele (FWD2)</td>
<td>AAGGGGACAAATCCACTCCAGTCTCT</td>
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<tr>
<td>62</td>
<td>FWD PCR primer to check 5’ end of βGeo or STRA8 allele (FWD3)</td>
<td>TCCTACCTCCCAATCTCCCGAAAG</td>
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<tr>
<td>63</td>
<td>REV PCR primer to check 5’ end of βGeo allele (REV)</td>
<td>ACAGTACGCGCCTACAGGAAGACGKG</td>
</tr>
<tr>
<td>64</td>
<td>FWD PCR primer to check 3’ end of βGeo allele (FWD)</td>
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</tr>
<tr>
<td>65</td>
<td>REV PCR primer to check 3’ end of βGeo or STRA8 allele (REV1)</td>
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</tr>
<tr>
<td>66</td>
<td>REV PCR primer to check 3’ end of βGeo allele (REV2)</td>
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<td>67</td>
<td>REV PCR primer to check 3’ end of βGeo or STRA8 allele (REV3)</td>
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<td>REV PCR primer to check 3’ end of STRA8 allele (REV_S)</td>
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<td>69</td>
<td>FWD PCR primer to check 3’ end of STRA8 allele (FWD_S)</td>
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<td>70</td>
<td>FWD primer for sexing</td>
<td>CCAGAGTGTTAACCTACACG</td>
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<tr>
<td>71</td>
<td>REV primer for sexing</td>
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<td>72</td>
<td>FWD PCR primer to detect the presence of transgene</td>
<td>TGACCTACAGGCGATATACGAAATGTC</td>
</tr>
<tr>
<td>73</td>
<td>REV PCR primer to detect the presence of transgene</td>
<td>TGCGGTAACCCGTCAGATTGG</td>
</tr>
<tr>
<td>74</td>
<td>FWD multiplexing PCR primer1 binding to intron1 of HMGA2</td>
<td>AAGTTTCCGCGCCTGGCCTAGAC</td>
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<tr>
<td>75</td>
<td>FWD multiplexing PCR primer2 binding to exon1 of HMGA2</td>
<td>ACTTCAGCGCGGCGAGACACC</td>
</tr>
<tr>
<td>76</td>
<td>REV multiplexing PCR primer binding to exon2 of HMGA2</td>
<td>TCGCCTGGATTGGGGGCGACTC</td>
</tr>
<tr>
<td>77</td>
<td>REV multiplexing PCR primer binding to βgeo</td>
<td>ACAGTATCAGGCCCTACAGGAAGATGCG</td>
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<td>78</td>
<td>FWD PCR primer for endonuclease assay</td>
<td>TGACCTACAGGCGATATACGAAATGTC</td>
</tr>
<tr>
<td>79</td>
<td>REV PCR primer for endonuclease assay</td>
<td>TGAGCGGTAAACGTCGATGCG</td>
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Table 2.4. Information for animals generated

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<thead>
<tr>
<th>Sex</th>
<th>Method of Generation</th>
<th>Genotype</th>
<th># of Total Embryo Transfer</th>
<th># of Pregnancy</th>
<th># of Fetal Collection</th>
<th># of Pigs Euthanized during Pregnancy</th>
<th># of Delivery</th>
<th># of Pigs Farrowed</th>
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<tbody>
<tr>
<td></td>
<td>Boar SCNT</td>
<td>HMG A2+/+</td>
<td>8</td>
<td>5</td>
<td>2 (at D43)</td>
<td>1</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMG A2-/-</td>
<td>17</td>
<td>6</td>
<td>1 (at D40)</td>
<td>6</td>
<td>4 (full term) &amp; 1 (C-section)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Breeding HMG A2+/+</td>
<td></td>
<td>-</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Breeding HMG A2-/-</td>
<td></td>
<td>-</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
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<tr>
<td></td>
<td>Gilt SCNT</td>
<td>HMG A2-/-</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Breeding HMG A2+/+</td>
<td></td>
<td>-</td>
<td>6</td>
<td>3</td>
<td>8</td>
<td>3</td>
<td>8</td>
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<tr>
<td></td>
<td>Breeding HMG A2-/-</td>
<td></td>
<td>-</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
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Table 2.5. Organ weight comparison; *HMGA2-/+* vs *HMGA2+/+* boars at 9 months of age

<table>
<thead>
<tr>
<th></th>
<th>Mean weight for <em>HMGA2-/+</em> (n=5)</th>
<th>Mean weight for <em>HMGA2+/+</em> Litter Mate (N=6)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body</td>
<td>165.2 ± 37.3 kg</td>
<td>221.73 ± 3.6 kg</td>
<td>74.5</td>
</tr>
<tr>
<td>Heart</td>
<td>504.4 ± 27.5 g</td>
<td>638.5 ± 15.6 g</td>
<td>79.0</td>
</tr>
<tr>
<td>Lungs</td>
<td>830.2 ± 85.6 g</td>
<td>926.8 ± 49.0 g</td>
<td>89.6</td>
</tr>
<tr>
<td>Livers</td>
<td>3056.6 ± 67.2 g</td>
<td>3145 ± 193.4 g</td>
<td>97.2</td>
</tr>
<tr>
<td>Kidneys</td>
<td>671.6 ± 23.2 g</td>
<td>656.3 ± 30.2 g</td>
<td>102.3</td>
</tr>
<tr>
<td>Brain</td>
<td>86.8 ± 3.2 g</td>
<td>98.5 ± 2.9 g</td>
<td>88.1</td>
</tr>
<tr>
<td>Testes</td>
<td>1081.8 ± 65.5 g</td>
<td>1282.2 ± 56.9 g</td>
<td>84.4</td>
</tr>
<tr>
<td>Adrenal Glands</td>
<td>5.8 ± 0.2 g</td>
<td>6.8 ± 0.7 g</td>
<td>84.9</td>
</tr>
</tbody>
</table>
Table 2.6. Organ weight comparison; $HMG\text{A}2^\text{- Strauss}$ vs $HMG\text{A}2^+/+$ boars at 15 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>Weight for $HMG\text{A}2^\text{- Strauss}$ (n=1)</th>
<th>Weight for $HMG\text{A}2^+/+$ (N=1)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body</td>
<td>11.6 kg</td>
<td>62 kg</td>
<td>18.7</td>
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<tr>
<td>Brain</td>
<td>57 g</td>
<td>87 g</td>
<td>65.5</td>
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<tr>
<td>Heart</td>
<td>69.58 g</td>
<td>242 g</td>
<td>28.8</td>
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<tr>
<td>Lungs</td>
<td>314.34 g</td>
<td>480 g</td>
<td>65.5</td>
</tr>
<tr>
<td>Livers</td>
<td>314.83 g</td>
<td>1138 g</td>
<td>27.7</td>
</tr>
<tr>
<td>Kidneys</td>
<td>71.56 g</td>
<td>205 g</td>
<td>34.9</td>
</tr>
<tr>
<td>Adrenal Glands</td>
<td>1 g</td>
<td>4 g</td>
<td>25.0</td>
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<tr>
<td>Testes (w/ epididymis)</td>
<td>44.28 g</td>
<td>206 g</td>
<td>21.5</td>
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Table 2.7. Organ weight comparison; *HMGA2*−/+ vs *HMGA2*+/+ gilts at 2 years of age

<table>
<thead>
<tr>
<th></th>
<th>Weight for <em>HMGA2</em>−/+ (n=1)</th>
<th>Weight for <em>HMGA2</em>+/+ (N=1)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body</strong></td>
<td>218 kg</td>
<td>292 kg</td>
<td>75%</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td>447 g</td>
<td>765 g</td>
<td>58%</td>
</tr>
<tr>
<td><strong>Lungs</strong></td>
<td>646 g</td>
<td>1772 g</td>
<td>36%</td>
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<tr>
<td><strong>Livers</strong></td>
<td>1923 g</td>
<td>3142 g</td>
<td>61%</td>
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<tr>
<td><strong>Kidneys</strong></td>
<td>322 g</td>
<td>745 g</td>
<td>43%</td>
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<tr>
<td><strong>Brain</strong></td>
<td>115 g</td>
<td>110 g</td>
<td>105%</td>
</tr>
<tr>
<td><strong>Ovaries</strong></td>
<td>20 g</td>
<td>25 g</td>
<td>80%</td>
</tr>
<tr>
<td><strong>Adrenal Glands</strong></td>
<td>6 g</td>
<td>9 g</td>
<td>67%</td>
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Table 2.8. Semen quality from two pubertal *HMGA2-/+* founder boars 7 months after birth:

Data from two consecutive collection days

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<tr>
<th>ID</th>
<th>Day</th>
<th>Motility</th>
<th>Volume (ml)</th>
<th>Concentration (x10^9 cells/ml)</th>
<th>Total #/ejaculate (x10^9 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HMGA2-/+</em> boar #1</td>
<td>1st day</td>
<td>good</td>
<td>96</td>
<td>0.560</td>
<td>53.8</td>
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<tr>
<td></td>
<td>2nd day</td>
<td>good</td>
<td>74</td>
<td>0.468</td>
<td>34.6</td>
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<td><em>HMGA2-/+</em> boar #2</td>
<td>1st day</td>
<td>good</td>
<td>78</td>
<td>0.867</td>
<td>67.6</td>
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<td></td>
<td>2nd day</td>
<td>good</td>
<td>78</td>
<td>0.504</td>
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Table 2.9. Chi-square test on litters resulting from *HMGA2/-* × *HMGA2/-* mating

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<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
<th>(O-E)</th>
<th>(O-E)^2</th>
<th>(O-E)^2 / E</th>
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<tbody>
<tr>
<td><em>HMGA2+/-</em></td>
<td>6</td>
<td>4.75</td>
<td>1.25</td>
<td>1.56</td>
<td>0.33</td>
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<tr>
<td><em>HMGA2-/-</em></td>
<td>13</td>
<td>9.50</td>
<td>3.50</td>
<td>12.25</td>
<td>1.29</td>
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<tr>
<td><em>HMGA2--</em></td>
<td>0</td>
<td>4.75</td>
<td>-4.75</td>
<td>22.56</td>
<td>4.75</td>
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<tr>
<td>Totals</td>
<td>19</td>
<td>19</td>
<td>0</td>
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<td>X^2 = 6.37</td>
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P value 0.025 < p < 0.05
Figure 1.1. cDNA and protein sequences for *HMGA2* gene. (A) *HMGA2* mRNA and protein sequences. Red box indicates 2\textsuperscript{nd} AT hook that is also conserved in swine. Protein sequence is shown above the mRNA sequence. (B) Alignment of cDNA sequences from real and pseudo genes of *HMGA2*. Only 28 base pairs were mismatched. The degree of homology between them was 91%. NCBI Blast was used to make the alignment.
Figure 2.1. Plasmid constructs used for HMGA2 gene editing. (A) Plasmid with MCS was used for the generation of targeting plasmids. (B) Targeting plasmid to generate HMGA2-/+ female cell line. β-Geo is a reporter gene of LacZ fused with neoR. It contains 5’ and 3’ homology arms. β-Geo-flox flanked by DTA is between the homology arms. 5’ homology arm (2.7 kb) contains from exon1 through partial exon2 of HMGA2 sequence whereas 3’ homology arm (4.2 kb) does from remaining of exon2 to intron2. (C) Targeting plasmid to generate HMGA2-/+ male cell line. It also contains 5’ and 3’ homology arms, and β-Geo flanked by FRT sites between the homology arms. 3’ end of 5’ homology arm was moved towards upstream of exon2 by 12 bp. (D) Donor plasmid for TALEN-mediated HDR to generate HMGA2-/- male cell line. It contains Stra8 promoter followed by HMGA2 coding sequence. The fragment of Stra8 promoter-chMGA2 flanked by FRT sites was plugged in between the homology arms. The same homology arms were used as in (C).
Figure 2.2. A diagram for pig size measurements. Pig size was determined by measuring (A) crown-rump length, (B) shoulder height, and (C) abdominal cavity.
**Figure 2.3. Schematic diagram for TALEN induced DSB in \textit{HMGA2}-/+ cells.** The figure shows two different alleles in an \textit{HMGA2}-/+ cell. One allele is the endogenous unmodified allele whereas the other allele is the one that contains the transgene. Since a pair of TALENs cannot form a dimer on the transgenic allele due to the distance between the two TALENs, only the \textit{HMGA2} endogenous allele is modified. E2S indicates the \textit{HMGA2} exon2 separated by a transgene.
Figure 2.4. Genotyping strategies for *HMGA2* heterozygosity and homozygosity. (A) The transgenic allele I was generated by conventional homologous recombination. Three different PCR primers were used to identify *HMGA2-/+* events. One forward primer binds to the upstream of exon1. One of two reverse primers binds only to the endogenous allele whereas the other reverse primer specifically binds to the transgene. (B) The transgenic allele II was generated by TALEN mediated HDR with a targeting donor plasmid. One set of primers (one forward and one reverse) can bind to both transgenic alleles, giving two different PCR bands due to the shorter transgene (blue box) than the other (green box). (C) The same PCR primer set was used as used in (B), but the length of extension time was only for 5 sec. The primers cannot amplify the transgenic allele I.
Figure 2.5. Generation and validation of HMG A2/-+ male or female fetal fibroblast cell lines. (A) Schematic process of HMG A2 gene targeting by homologous recombination. (B) gDNA PCR screening assay. Inserted transgenic allele was only detected in HMG A2/-+ colony. (C) Sequencing confirmation for gDNA PCR screening assay. Purified PCR bands were sequenced. (i) In silico DNA sequence alignment of the transgenic allele in female cells (TfA), the HMG A2 endogenous allele (EA), and the transgenic allele in male cells (TmA). Three sequencing results indicates (ii) heterozygous female cells (TfA+EA), (iii) wild-type female or male cells (EA only), and (iv) heterozygous male cells (EA+TmA). Black box shows that the three sequencing results were obtained from the same locus in HMG A2 gene. (D) cDNA RT-PCR for β-Geo (1st lane), HMG A2 (2nd lane), and GAPDH (3rd lane) expressions. Expression of reporter gene was only detected in HMG A2/-+ colony (E) LacZ staining assay to test the functionality of reporter gene. β-geo was expressed only when it was under the control of HMG A2 promoter as shown on the right panel.
Figure 2.6. PCR and sequencing diagnosis for both ends of the β-Geo targeted site in *HMGA2-/+* female cells. (A) PCR band indicated by blue arrow was amplified by FWD3 (blue arrow) + REV (black arrow). PCR band indicated by red arrow was amplified by FWD2 (red arrow) + REV (black arrow). PCR band indicated by red arrow was amplified by FWD1 (green arrow) + REV (black arrow). Multiple Sequence Alignment (MSA) was generated with sequencing results by sequencing primers (Seq1 and Seq2). The two assays showed that the presence of 5’ end of transgene at HMGA2 locus was confirmed. HMGA2 indicates the reference sequence of endogenous HMGA2 gene, PCR indicates sequencing results of PCR products from the left panel, and Vector indicates the sequence of targeting vector. (B) PCR band indicated by blue arrow was amplified by FWD + REV1. PCR band indicated by red arrow was amplified by FWD + REV2. PCR band indicated by green arrow was amplified by FWD + REV3. The two assays showed that the presence of 3’ end of transgene at HMGA2 locus was confirmed.
Figure 2.7. PCR and sequencing diagnosis for both ends of the β-Geo targeted site in *HMGA2-/+* male cells. (A) PCR band indicated by blue arrow was amplified by FWD3 (blue arrow) + REV (black arrow). PCR band indicated by red arrow was amplified by FWD2 (red arrow) + REV (black arrow). PCR band indicated by red arrow was amplified by FWD1 (green arrow) + REV (black arrow). Multiple Sequence Alignment (MSA) was generated with sequencing results by sequencing primers (Seq1 and Seq2). The two assays showed that the presence of 5’ end of transgene at HMGA2 locus was confirmed. HMGA2 indicates the reference sequence of endogenous HMGA2 gene, PCR indicates sequencing results of PCR products from the left panel, and Vector indicates the sequence of targeting vector. (B) PCR band indicated by blue arrow was amplified by FWD + REV1. PCR band indicated by red arrow was amplified by FWD + REV2. PCR band indicated by green arrow was amplified by FWD + REV3. The two assays showed that the presence of 3’ end of transgene at HMGA2 locus was confirmed.
Figure 2.8. TALEN system for the generation of HMGA2-/-.

(A) Efficiency of TALENs to repair pSSA. FACS was performed to sort cells with eGFP expression. pSSA is the eGFP vector that has stop codons in the middle of eGFP coding sequence. TALENs target to introduce DSB to the site of stop codons. Then, self-homologous recombination occurs and repairs the coding sequence so as to express eGFP. The only cells, mutant candidate, carrying the correctly repaired pSSA vector were sorted (15.96%; HMGA2-/- candidates). Stra8 indicates pStra8-cHMGA2 donor vector. (B) GFP expression from transfected cells. Each panel indicates no DNA introduced (Neg), the co-transfection of Stra8-cHMGA2 donor vector and pSSA reporter plasmid (Stra8+pSSA), GFP plasmid only transfected as a positive control (GFP+), and all four plasmids co-transfected (TALEN+Stra8+pSSA). Upon the co-transfection of TALENs and pSSA, repaired pSSA expresses eGFP in possibly mutated cells (right bottom panel). (C) Sequence alignment of the mutated sequences in HMGA2-/- colonies (Mutant_ID#) and HMGA2 reference sequence (HMGA2 Ref.). This assay was done by TALENs without HDR. This shows that generated TALENs worked to introduce DSB at the specific target of our interest. Length of deletion was varied from 7 bp to 431 bp. (D) Endonuclease assay for the target sites. gDNA PCR was performed with primers just bound to the flanking regions of TALEN binding sites. U indicates uncut PCR amplicon. C indicates PCR amplicon cut by BsaWI. The uncut PCR band was 743 bp and the PCR bands cut by BsaWI were 504 bp and 243 bp. BsaWI was not able to cut PCR amplicons from HMGA2-/- colonies since BsaWI restriction site was deleted in HMGA2-/- colonies.
Figure 2.9. PCR and sequencing diagnosis for both ends of the Stra8 targeted site. (A) Strategy to produce $HMGA2^{-/-}$ Stra8 by using TALEN mediated HDR. There is only one $HMGA2$ endogenous allele in the $HMGA2^{-/+}$ primary cell line. TALENs was used to inactivate the endogenous allele by TALEN mediated HDR. The donor plasmid for knock-in contained Stra8 promoter and $HMGA2$ cDNA. mHMGA2 stands for mRNA sequence of HMGA2. (B) PCR band indicated by blue arrow was amplified by FWD3 (blue arrow) + REV_S (black arrow). PCR band indicated by red arrow was amplified by FWD2 (red arrow) + REV_S (black arrow). PCR band indicated by red arrow was amplified by FWD1 (green arrow) + REV_S (black arrow). Multiple Sequence Alignment (MSA) was generated with sequencing results by sequencing primers (Seq1 and Seq2). The two assays showed that the presence of 5’ end of transgene at HMGA2 locus was confirmed. HMGA2 indicates the reference sequence of endogenous HMGA2 gene, PCR indicates sequencing results of PCR products from the left panel, and Vector indicates the sequence of targeting vector. (C) PCR band indicated by blue arrow was amplified by FWD_S + REV1. PCR band indicated by red arrow was amplified by FWD_S + REV2. PCR band indicated by green arrow was amplified by FWD_S + REV3. The two assays showed that the presence of 3’ end of transgene at HMGA2 locus was confirmed.
Figure 2.10. *HMGA2* expression level in wild-type and mutated D40 fetal fibroblasts.

(A) *HMGA2* expression level for *HMGA2*+/+, *HMGA2*−/+, *HMGA2*−/−, and *HMGA2*−/−*Stra8* were detected by Western blot. Proteins were extracted from primary D40 fetal fibroblasts of *HMGA2*+/+, *HMGA2*−/+, *HMGA2*−/− generated from the breeding of *HMGA2*+/+ founders. Proteins for *HMGA2*−/−*Stra8* were isolated from primary D43 fetal fibroblasts generated by SCNT. One allele from *HMGA2*−/+ only express half of *HMGA2*+/+ expression, suggesting there was no compensated expression from the other allele. There was no expression detected from *HMGA2*−/− or *HMGA2*−/−*Stra8*. Predicted band size of HMGA2 was 12 kDa, but observed band size on the blot was bigger than that due to posttranslational modification. β-actin (internal control) band size was 42 kDa as expected. HEK293 lysate was used as positive control. (B) Semi-quantitative assay for relative HMGA2 protein levels normalized to β-actin. Compared to *HMGA2*+/+, relative HMGA2 protein level in *HMGA2*−/+ was less than half and none in *HMGA2*−/−. Relative HMGA2 protein level was undetectable in *HMGA2*−/−*Stra8*. 
(A) Western blot analysis of HMGA2 and β-Actin proteins.

(B) Bar graph showing the relative protein level of HMGA2 (normalized by β-Actin).
Figure 2.11. Growth curve comparison of $HMGA2^{-/-}_{\text{Stra}^8}$, $HMGA2^{-/+}$, and $HMGA2^{+/+}$ boars. (A) Weight curve comparison of $HMGA2^{-/+}$ SCNT founders, $HMGA2^{+/+}$ SCNT littermates and $HMGA2^{+/+}$ naturally bred boars. They were weighed every two weeks for 30 weeks. Nonparametric trend test showed that the trend of three groups were not significantly different, whereas Parametric test showed that $HMGA2^{-/+}$ SCNT founders were significantly smaller at 30 weeks of age. At 30 weeks old, $HMGA2^{-/+}$ SCNT founders were 80% (p<0.05) and 77% (p<0.05) in weight over $HMGA2^{+/+}$ SCNT littermates and $HMGA2^{+/+}$ naturally bred boars, respectively. (B) Weight curve (C) Crown-Lump length curve (D) Shoulder height curve (E) Body cavity curve of $HMGA2^{-/-}_{\text{Stra}^8}$ SCNT founder was compared to previous data of $HMGA2^{-/+}$ SCNT founders and $HMGA2^{+/+}$ SCNT littermates. Compared to $HMGA2^{+/+}$ littermates, $HMGA2^{-/-}_{\text{Stra}^8}$ SCNT founder was 23% in weight, 65% in length, 61% in height, and 61% in body cavity at 4 months of age.
Figure 2.12. Representative weight and organ size phenotypes of *HMG2-/+* SCNT founder boar to *HMG2++* SCNT littermate. (A) At 25 weeks of age, the body weight of the *HMG2-/+* founder was 82% of its *HMG2+/+* littermate. (B) Organ size comparisons confirmed the growth reduction in *HMG2-/+* founder. At 9 months of age, animals were euthanized and major organs were collected; Brain (not shown), Heart, Lung (not shown), Liver (not shown), Kidney, Adrenal gland, Testes were analyzed. Most of transgenic organs were in the range of 80-90% compared to wild-type organs. Examples for heart, kidney, adrenal gland and testes are shown. The tissue indicated by asterisk is significantly smaller.
Figure 2.13. Representative weight and organ size phenotypes of $HMGA2^{-/-}$ Stra8 SCNT founder and $HMGA2^{+/+}$ naturally bred boars. (A) At 10 weeks of age, the body weight of $HMGA2^{-/-}$ Stra8 founder was 25% of $HMGA2^{+/+}$ naturally bred boar. (B) At 15 weeks of age, animals were euthanized and major organs were collected; all of the mutant organs analyzed, brain, heart, lung, liver, kidney, adrenal gland, and testes were in the range of 20-40% compared to wild-type organs.
Figure 2.14. Growth analysis of \( HMG\text{A}2-/+ \) SCNT founder and \( HMG\text{A}2+/+ \) naturally bred gilts. (A) At 30 weeks of age, \( HMG\text{A}2-/+ \) founders were 65% of \( HMG\text{A}2+/+ \) gilts. (B) Reproductive organs were collected from \( HMG\text{A}2-/+ \) founder sow and \( HMG\text{A}2+/+ \) naturally bred gilt at 2 and half years of age; as for the other major organs (not shown), mutants were 60-80% in size of wild-type controls. (C) \( HMG\text{A}2-/+ \) founders and \( HMG\text{A}2+/+ \) naturally bred gilts were weighed every two weeks for 59 weeks. Nonparametric trend test showed that the trends of two groups were significantly different (p<0.05). Mutants were 80% in weight over wild-type controls at 59 weeks of age. Parametric test confirmed that the reduction was significant at 59 weeks of age (p<0.05).
Figure 2.15. Reproductive characteristics of gilts and boars carrying *HMGA2* mutations. (A) *HMGA2-*/+ SCNT founder females did not show any estrus behavior by two years of age. Examination of follicular growth by transrectal ultrasound showed that *HMGA2-*/+ SCNT founders were cycling and ovulating. However, no estrus behavior was observed. (B) ELISA test for progesterone level. Progesterone levels of two *HMGA2*+/+ naturally bred gilts and two *HMGA2-*/+ SCNT founders were measured at every three to four days for 33 days. Both *HMGA2-*/+ SCNT founders had abnormally low levels of progesterone, explaining why they didn’t have estrus behaviors. (C) *HMGA2-*/+ female offspring (F1) born from the breeding of *HMGA2-*/+ SCNT founders, with either paternally (left panel) or maternally (right panel) inherited transgenic allele, showed normal estrus behavior at between 6 and 8 months of age, suggesting that lack of estrus behavior and, in contrasts to *HMGA2*+/+ naturally bred gilts, abnormally low hormone level of *HMGA2-*/+ SCNT founders are likely to be SCNT-related phenotype but not to be HMGA2-related phenotype. (D) *HMGA2-*/+ SCNT founder males showed normal puberty and sexual behavior up to the present (2 years and 4 months). Semen quality and germ line transmission were tested. Wild-type oocytes and *HMGA2-*/+ sperms were used for IVF. LacZ staining of resulting embryos confirmed the transgene transmission.
Figure 2.16. Histological analysis of ovaries and tests of *HMGA2-*/+ SCNT founders or *HMGA2-*/+ F1. (A) At 30 months of age, *HMGA2-*/+ founder gilt had normal oocyte maturation and corpus luteum (CL) as did control gilt. Arrow indicates an oocyte in an antral follicle, and arrowhead a CL. (B) At 11 months of age, *HMGA2-*/+ F1 also had normal folliculogenesis. (C) At 9 months of age, *HMGA2-*/+ SCNT founder boar (left panel) had normally organized seminiferous tubules same as its wild-type littermate boar (middle panel) and naturally bred control boar (right panel). Scale bar is 100 µm.
Figure 2.17. Cryptorchid testes of HMGA2/-^Stra8 SCNT founder boar. (A) Autopsy showed cryptorchidism in a 4 months old HMGA2/-^Stra8 SCNT boar (left panel) whereas testes normally descent in a age-matched HMGA2+/+ naturally bred boar. (B) Whole reproductive organs were removed for the comparison. HMGA2/-^Stra8 SCNT founder has no gubernaculum bulbs when compared to wild-type control has (black arrows). (C) H&E histology sections of HMGA2/-^Stra8 testis at 4 days of age showed that cell structure and arrangement in seminiferous tubules looked normal as in age-matched HMGA2+/+ testis. However, at 4 months of age, none of seminiferous tubules in HMGA2/-^Stra8 testis showed germ cell differentiations while in a few tubules in HMGA2+/+ testis there were signs of spermatogenesis. Scale bar is 100 µm.
Figure 2.18. Characterization of D40 and D78 fetuses produced by breeding of $HMG A2$-/+ SCNT founders. (A) PCR genotyping assay for D40 and D78 fetuses. M indicates maternally inherited transgenic allele, P paternally inherited transgenic allele, and E endogenous allele. (B) Sequencing result of $HMG A2$ null fetuses. Two transgenic sequences were aligned together in silico (upper panel). Both of parental transgenic sequences were present in the homozygous fetuses (bottom panel). MiA indicates the sequence of maternally inherited allele whereas PiA does the sequence of paternally inherited allele. (C) Healthy fetuses with four different genotypes were detected in an early gestation litter (D40) at the expected Mendelian frequency. All fetuses were from $HMG A2$-/+ SCNT founder sow. All four different genotypes were present in the litter, having consistent phenotypes. While the weight of $HMG A2$-/+ fetuses were compatible to $HMG A2$+/+ fetuses, $HMG A2$-/- fetuses were significantly smaller (30% reduced) compared to the other two genotypes. (D) Healthy fetuses with four different genotypes were also present in late gestation litters (D78). Comparison of D78 fatal weights from two litters confirmed the genotyping assay. Compared to $HMG A2$+/+ fetuses, $HMG A2$-/+ fetuses were 24% smaller and $HMG A2$-/- were 74% smaller. (E) All of fetuses with four different genotypes at D40 and D78 were morphologically normal and healthy.
(A) 

(B) 

(C) 

(D) 

(E)
Figure 2.19. Placenta abnormality detected in the HMG2-/- fetuses at D78 of gestation. (A) HMG2+/+ placentas (not shown) and HMG2-/+ placentas (left panel) were still healthy and well vascularized at D78. However, HMG2-/- placentas (right panel) showed lack of absorptive dome-shaped structures (areolae) and vascularization. (B) Some areas of HMG2-/- placenta from a different litter at D78 were still healthy and vascularized (arrow), while most areas were degenerated. (C) Histological analysis of placenta and endometrium from HMG2+/+, HMG2+/+ runt, HMG2-/-+, and HMG2-/- fetuses. Villi were severely degenerated in HMG2-/- placenta compared to other genotypes. Furthermore, morphologically poor contact of villi between uterine endometrium and chorionic membrane was detected for HMG2-/- (arrow), suggesting the connection between sow and HMG2-/- fetuses is deficient. Endo indicates the endometrium of uterus, and Chorio indicates the chorionic membrane of placenta. Scale bar is 100 µm. (D) Placental weights for D40 and D78 conceptuses. No significant difference between genotypes was detected at either D40 or D78.
**Figure A**

Images of tissue samples labeled as HMGA2-/-, HMGA2-/-, and HMGA2-/-

**Figure B**

A close-up of a tissue sample labeled as HMGA2-/-

**Figure C**

Microscopic images of tissue sections labeled as Endo, Chorio, HMGA2+/+, and HMGA2+/- runt

**Figure D**

Bar graph showing placental weight with categories D40 and D78 (Two Litters), comparing HMGA2+/+, HMGA2-/-, and HMGA2-/-.
Figure 2.20. Characterization of HMGA2 null piglets generated by SCNT. (A) Genotyping PCR assay confirmed that the 10 piglets were $HMGA2^{-/-}$. E indicates endogenous allele. βGeo indicates a transgenic allele with βGeo reporter gene and Stra8 does the other transgenic allele with Stra8 promoter. (B) Birth weights of $HMGA2^{-/-}$ piglets were compared to the ones of $HMGA2^{+/+}$ naturally bred boars and $HMGA2^{-/+}$ SCNT founder boars. $HMGA2^{-/-}$ were significantly smaller than either $HMGA2^{+/+}$ wild-type controls and $HMGA2^{-/+}$ founders. HMGA2 null boars were 45% of $HMGA2^{+/+}$ wild-type controls. (C) HMGA2 null piglets ($HMGA2^{-/-}$) were successfully generated by SCNT, indicating that the $HMGA2$ gene mutations were not lethal. The picture was taken at 1 day of age. (D) At 4 days of age, major organs were collected. $HMGA2^{-/-}$ and wild-type control showed drastic differences in organ size. All of the mutant organs were in the range of 20-50% compared to wild-type organs. (E) Testis comparison is shown as an example.
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APPENDIX
Appendix A

Lack of genomic imprinting of DNA primase, polypeptide 2 (PRIM2) in human term placenta and white blood cells.


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Introduction

Genomic imprinting is an epigenetic process that leads to allelic gene expression in a parent-of-origin specific way, influencing development, growth and behavior in mammals[1-3]. Over 100 genes have been conclusively demonstrated to be imprinted in mammals [3-7], however, both in silico prediction studies and massive parallel sequencing suggest that there may be as many as 1,000 imprinted genes [8-10]. PRIM2, encoding a subunit of primase, is involved in purine metabolism, pyrimidine metabolism, and processes such as DNA replication, and transcription, all of which are crucial for normal growth and development [11-13]. PRIM2 expression has been detected in most of tissues in mammals including brain, liver, blood, and placenta [14-15] and has been reported as an imprinted, maternally-expressed gene in human white blood cells (WBC) [16]. We recently identified PRIM2 as a gene up-regulated in small for gestational age (SGA) placentas (data not shown). In order to further understand the role of PRIM2 in the human placenta we examined whether the PRIM2 gene was imprinted in the placenta, as reported for WBC. Here we report lack of PRIM2 imprinting in the human placenta and WBC.
**Materials and Methods**

**Tissue collection.** Term placentas were from women who had consented to be part of a Thrombophilia and Intrauterine Growth Restriction at Duke University Medical Center. The placental samples were obtained and stored as previously described [17]. Whole blood samples from healthy donors were collected at Durham Regional Hospital. All protocols were approved by the Intuitional Review Boards of Duke University Health System, North Carolina State University and the Centers for Disease Control and Prevention.

**Isolation of DNA and RNA, and first-strand cDNA synthesis.** Genomic DNA was extracted from term human placenta and blood tissues using the Wizard Genomic DNA Purification Kit (Promega). RNA from placenta was isolated as previously described [17]. For isolation of total RNA from WBC, PAXgene blood RNA system (PreAnalytix) was used. DNase was used post-isolation to remove trace amounts of genomic DNA contamination. cDNA was synthesized from the isolated pure total RNA using Affinity Script™ Multi Temperature cDNA Synthesis Kit (Stratagene).

**DNA/cDNA amplification and analysis.** Based on the alignment of the pseudogene and the transcribed gene, primers specific to each target were designed. Primers used were F3693 5’- GTGTTGCACTCTGTGGTGTAATTGTGA-3’ and R3694 5’-AGTCTCGTACCTCAAACCTGCT-3’ for the pseudogene and F3695 5’-GTCACCAAGGCTTAGTGCAGTGA-3’ and R3696 5’-AGTCTCTGTGTACCTCAAACCTCCT-3’ for thePRIM2 transcribed sequence. PCR
conditions were; during the first 20 cycles, the annealing temperature was decreased each cycle by 0.5°C from 65°C to 55°C, followed by an additional 25 cycles with 55°C annealing temperature. Amplicons were then sequenced by Eurofins MWG (Operon). The amplicons for heterozygous samples were subcloned into pCR-Blunt II-TOPO vector (Invitrogen). Transformed TOP10 clones were sequenced to confirm. For the confirmation assay only with cDNA, three additional primers were used; F46235’-
TTCCCGAGTTTTGAATTCTTGCAGGTG-3’, F3484 5’-
TTCCCGAGTTTTGAATTCTTGCAG-3’, and R3535 5’-
GTGCATGCCTGTAGATGGATTGG-3’. PCR conditions were; 40 cycles with 55°C annealing temperature.

Results and Discussion

PRIM2, presently annotated as NC_000006.11 (NCBI Genome Build 37.1) is mapped to chromosome 6 whereas a known PRIM2 pseudogene is mapped to chromosome 5 [18-19]. Our results indicate that the National Center for Biotechnology (NCBI) annotation is incorrect. In an initial sequencing analysis it was noted that the mRNA for Exon 14 of PRIM2 (ref|NM_000947.2) had a higher degree of homology to the region annotated as the PRIM2 pseudogene (ref|NW_001838929.1) than to the region annotated as the actual PRIM2 (ref|NT_007592.15). Using this information primers were developed that could successfully amplified either the PRIM2 pseudogene, where the gDNA was amplified but not the cDNA, or the PRIM2 transcribed gene, where both the gDNA and cDNA were amplified (Figure 1A and 1B). Sequencing of both products confirmed the missannotation of the transcribed PRIM2 in the NCBI database. In addition, amplification of the whole length of PRIM2
mRNA strongly supported the evidence of the missannotation of PRIM2 gDNA sequence (Figure 1C). These evidences allowed us to develop an assay that could examine imprinting at this locus in humans.

To examine imprinting, genomic DNA and cDNA were isolated from 21 placenta samples and the transcribed PRIM2 was amplified, sequenced and two single nucleotide polymorphisms (SNPs) identified (rs71214002; A/G and rs62402991; A/G). Additionally, 6 heterozygous individuals within the 21 samples were identified. As shown in Figure 2A, none of the placentas heterozygous for rs71214002 or rs62402991 showed evidence of imprinting as both alleles are clearly expressed. As the only known report of imprinting of PRIM2 in humans was in WBC, imprinting was also examined in this tissue and no evidence for imprinting detected (Figure 2B). Sequencing results on subcloned amplicons for heterozygous individuals of both tissues showed 50% composition of each allele for both SNPs, confirming no evidence of imprinting. In an attempt to understand how others could have reported PRIM2 as imprinted, primers that simultaneously detect both the pseudogene and the transcribed gene were utilized and the amplified DNA analyzed. As shown in Figure 2C this can lead to the incorrect classification of an individual as a heterozygous at the transcribed gene locus, while homozygous at the cDNA level, resulting in evidence of imprinting. What is happening in reality is amplification of two targets, the pseudogene and the transcribed gene, that differ in a few nucleotides (thus the "heterozygote" call). When cDNA is examined, however, only the transcribed "allele" is detected resulting in what appears to be mono allelic expression.
In summary, while the role of PRIM2 in placental function still needs to be elucidated, our results indicate that imprinting disregulation at this locus should not be considered as a plausible explanation for its overexpression in diseased states. This missannotation, in addition to affecting the analysis of imprinting at this locus, affects the mapping of known SNPs to that region due to incorrectly assigning the pseudogene sequence data to the transcribed locus. In other words, some of SNPs in the RefSNP may not be PRIM2 polymorphic alleles among the population or between two parental chromosomes, but between the gene and its pseudogene. This may lead to miscalculations of PRIM2 allelic frequencies in a population and lead to misinterpretation of results [20].

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Figure 1. Ability to discriminate between the PRIM2 pseudogene and the PRIM2 gene. PCR primers were designed to differentially amplify the PRIM2 pseudogene (F3693+R3694) and the transcribed PRIM2 sequence (F3695+R3696). Additional PCR primers were designed to confirm the discrimination. **A.** Detection of the PRIM2 pseudogene showing amplification from the placental genomic DNA but not from the placental cDNA indicating that this sequence is not transcribed. **B.** Detection of the PRIM2 gene showing presence in both the genomic DNA and the cDNA indicating that it is being transcribed. Amplicon identity was confirmed by sequencing as described in text. # refers to sample number.
Figure 2. Lack of imprinting in the human PRIM2 gene. Individuals with two polymorphisms (rs71214002 and rs62402991) within the coding sequence of the PRIM2 gene were identified and their DNA and cDNA examined to determine whether mono allelic expression indicative of imprinting could be detected. A. Placental samples from informative heterozygotes were collected and DNA and cDNA used to amplify PRIM2 using primers that can differentiate between the transcribed PRIM2 locus and the PRIM2 pseudogene. In all cases examined (n=6) sequencing results showed that both alleles were transcribed and present in the cDNA when compared to the DNA. This was true for both polymorphisms. B. The same assay but carried on white blood cells (WBC), again showing lack of imprinting of the human PRIM2. C. Sequencing results obtained from amplicons generated by PCR primers that can simultaneously amplify the PRIM2 gene and its pseudogene. The sequencing of the genomic DNA identifies three potential heterozygotes. In the cDNA, however, all heterozygotes disappear suggesting that imprinting is occurring at this locus. This, however, is an artifact caused by amplification of both the PRIM2 gene and its pseudogene, and the presence of polymorphisms between the PRIM2 and the PRIM2 pseudogene.
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