ABSTRACT

Title of Dissertation: ANALYSIS OF THE ROLE OF *PRDM14* IN PLURIPOTENCY AND SPECIFICATION OF THE GERM CELL NICHE IN THE DOMESTIC PIG

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The generation of functional, mature germ cells in order to pass on its genetic information to the next generation is of paramount importance to a species' survival. As a result, primordial germ cells (PGC), the precursor cells that give rise to mature spermatozoa or oocytes, are one of the first cell types to be specified and induced in the early mammalian embryo. In mammals, this process happens during the primitive streak stage of development and PGCs continue to proliferate as they migrate from the posterior end of the embryo through the hindgut and settle in the future gonadal region. One gene in particular, *PRDM14*, is required for PGC specification in the mouse, but its function was unknown in the domestic pig (*Sus scrofa*). This dissertation project sought to determine the role of *PRDM14* in the porcine germ cell program as well as in early embryo development. The first study used the mouse as a model using CRISPR/Cas9 technology to direct pluripotent stem cells toward the

germ cell pathway by aggregation with a host embryo that was unable to form PGCs. Chimeras generated by this technique exhibited 100% occupation of the germline by donor control stem cells. In the second study, mRNA transcripts for a suite of germ cell-related genes were analyzed to determine their expression in various stages of the early porcine embryo: from zygote to blastocyst and embryonic day 28. Unlike in the mouse and human systems, several genes that were predicted to be expressed in the early embryo (*PRDM14*, *TET1*, and *PRDM1*, among others) were found to be extremely low in abundance. In the final study, the function of *PRDM14* in the pig was disrupted using gene editing, and the resulting phenotype was characterized. Loss of *PRDM14* does not seem to be critical for the specification of PGCs in the domestic pig, as evidenced by the existence of germ cells following knockout. Together, these studies have applications for spermatogonial stem cell transplantation technology in order to expand the genetic reach of superior animals by producing animals whose germ cells are derived from a genetically valuable donor.

ANALYSIS OF THE ROLE OF *PRDM14* IN PLURIPOTENCY AND SPECIFICATION OF THE GERM CELL NICHE IN THE DOMESTIC PIG

by

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5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine
6-DMAP	6-(Dimethylamino) purine
aa	amino acid
AAV	adeno-associated virus
AI	artificial insemination
BMP	bone morphogenetic protein
bp	base pair
CARM1	coactivator-associated arginine methyltransferase 1
Cas9	CRISPR-associated endonuclease 9
CER1	cerberus
COC	cumulus-oocyte complex
CRISPR	clustered regularly interspaced palindromic repeats
Cwc15	CWC15 spliceosome associated protein homolog
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidine-2-phenylindole
DAZL	deleted in azoospermia-like
ddPCR™	droplet digital PCR
DKK1	dickkopf-related protein 1
DMEM	Dulbecco's modified eagle's medium
dpc	days post-coitum
DSB	double strand break
E	embryonic day
ECC	embryonic carcinoma cells
EDTA	ethylenediaminetetraacetic acid
EGC	embryonic germ cells
ESC	embryonic stem cells
ESRRB	estrogen-related receptor beta
EUCOMM	European conditional mouse mutagenesis program
FHM	modified KSOM
GFP	green fluorescent protein
h	human
H&E	hematoxylin and eosin
H3K27me3	histone 3 lysine 27 trimethylation
H3K9me2	histone 3 lysine 9 dimethylation
hCG	human chorionic gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HOX	homeobox
HR	homologous recombination
HSV	herpes simplex virus
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IKMC	international knockout mouse consortium
indels	insertions or deletions

iPSC	induced pluripotent stem cells
IR	inverted repeats
IVF	in vitro fertilization
kb	kilobase
KO	knockout
KOMP	knockout mouse project
KSOM	potassium simplex optimization medium
LEFTY	left-right determination factor
LIF	leukemia inhibitory factor
MBCD	methyl-β-cyclodextrin
NANOS2	nanos C2HC-type zinc finger 2
PCR	polymerase chain reaction
PGC	primordial germ cell
PGCLC	primordial germ cell-like cell
PHA-L	phytohemagglutinin-L
PMSG	pregnant mare's serum gonadotropin
POU5F1	POU domain, class 5, transcription factor 1, also known as OCT4
PRDM1	PR domain-containing 1
PRDM14	PR domain-containing 14
PZM3	porcine zygote medium 3
RPS18	ribosomal protein S18
RT-PCR	reverse transcriptase polymerase chain reaction
RVD	repeat variable domain
SCNT	somatic cell nuclear transfer
SMGT	sperm-mediated gene transfer
SOX17	SRY-box 17
SOX2	SRY-box 2
STRA8	stimulated by retinoic acid 8
SV40	simian virus 40
TALEN	transcription activator-like effector nuclease
TBS-T	tris buffered saline containing tween
TET1	tet methylcytosine dioxygenase 1
TET2	tet methylcytosine dioxygenase 2
TFAP2C	transcription factor AP-2 gamma
TKX	a combination of telazol, ketamine, and xylazine
TL-HEPES	Tyrode's lactate N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
TYH	tryptone, yeast extract, HEPES media
VASA	vasa homolog, also known as DEAD-box helicase 4
WT	wild-type
ZFN	zinc finger nuclease

Chapter 1: Review of Literature

Background and Significance

The Domestic Pig as a Biomedical Research Model

Swine were among the first animals to be domesticated (along with goats, sheep, cattle, and dogs) as humans began to move toward more agrarian societies (Frantz, Meijaard et al. 2016). Fossil records indicate that domestication of pigs (*Sus scrofa*) occurred between 8,300-10,000 years ago independently at two locations: China and East Anatolia. Traditionally, the role of pigs in society has been for human consumption, especially as demand for pork meat increased around the time of the Industrial Revolution in the 18th and 19th centuries (Frantz, Meijaard et al. 2016, Groenen 2016). As such, breeding strategies for pigs have usually been focused on docility, size, growth rate, and meat characteristics in order to keep up with the demand for 115 million tons of pork produced globally each year (Frantz, Meijaard et al. 2016). However, in addition to their use as a meat source, a new use for swine in biomedical research has developed over the past two decades, further increasing their value and importance to humans.

Much of our knowledge of current human biology has been based on studying a variety of model species. In order to understand the development, diagnosis, and treatment of human diseases, it is important to have a relevant model species in place. While traditional laboratory model species (e.g. rodents, *Drosophila*, zebrafish, *C. elegans*) are informative for determining the function of single genes and proteins, it must be recognized that these model species do not always reflect the complexity of human biology (Bendixen, Danielsen et al. 2010). As mentioned previously, the

domestic pig has become increasingly important as a model species for biomedical research due to its many similarities to humans (Vodicka, Smetana et al. 2005).

Physiologically, swine are remarkably similar to humans in regard to gastrointestinal anatomy and function (Houpt, Houpt et al. 1979, Spurlock and Gabler 2008), cardiovasculature (Lelovas, Kostomitsopoulos et al. 2014), metabolism (Renner, Dobenecker et al. 2016, Zhang and Lerman 2016), and comparative organ size (Vodicka, Smetana et al. 2005). Pigs also have a much longer lifespan than other commonly used animal models, giving researchers the opportunity for longer term studies. Additionally, although its unique evolutionary background places it distinct from primates and rodents, transcriptomic analysis has determined that the pig has higher sequence conservation to the human than the mouse does (Wernersson, Schierup et al. 2005, Aigner, Renner et al. 2010). This similarity to the human genome is also true for protein coding sequences (Jorgensen, Hobolth et al. 2005). Combined, these characteristics make pigs a uniquely suitable model for applications of biotechnology and disease modeling for humans, especially as a bridge between traditional rodent models and nonhuman primates.

History of Genome Editing in Pigs

Over the past 20 years, the domestic pig has become an important animal species for modeling human diseases in addition to its traditional agricultural role. For human medical applications, the pig model has become especially useful for instances where rodent models do not accurately reflect the disease phenotype (Aigner, Renner et al. 2010). Since the first report of a genetically engineered pig

model of retinitis pigmentosa in 1997 (Petters, Alexander et al. 1997), at least 19 other human diseases now have pig models that have been fully characterized to show the disease phenotype, with countless others undoubtedly in progress (Rogers 2016). Notable among these reports are models generated for cystic fibrosis (Rogers, Hao et al. 2008, Rogers, Stoltz et al. 2008), diabetes mellitus (Umeyama, Watanabe et al. 2009, Renner, Fehlings et al. 2010, Renner, Braun-Reichhart et al. 2013), Huntington's disease (Yang, Wang et al. 2010, Baxa, Hruska-Plochan et al. 2013), Alzheimer's disease (Kragh, Nielsen et al. 2009, Jakobsen, Johansen et al. 2013), and Duchenne muscular dystrophy (Klymiuk, Blutke et al. 2013).

Even though the first report of a pig model for human disease was not reported until 1997, there were reports of genetically engineering livestock animals at least a decade prior via microinjection of a transgene into the pronuclei of superovulated sheep and pig oocytes (Hammer, Pursel et al. 1985). Indeed, the transgenesis and genetic engineering field was initially focused on developing techniques to improve production and agricultural traits in pigs and other livestock. The use of gene editing as a technique to generate human disease models was developed after these foundational studies (Laible, Wei et al. 2015). Some examples of improved agricultural traits developed via gene editing in pigs include: improved meat production (Pursel, Pinkert et al. 1989, Pursel, Hammer et al. 1990, Draghia-Akli, Fiorotto et al. 1999), improved milk proteins (Saeki, Matsumoto et al. 2004, Tong, Wei et al. 2011, Lu, Li et al. 2014), and disease resistance (Lo, Pursel et al. 1991, Weidle, Lenz et al. 1991, Lillico, Proudfoot et al. 2013, Burkard, Lillico et al. 2017). As new technological methods have been rapidly developed and applied,

especially over the last 10 years, scientists are now able to expeditiously generate animals with precise edits to the genome for a variety of applications. The recent ability to generate edited and transgenic swine with relative ease has led to an expanded increase of interest and productivity in a relatively short amount of time.

Genetic Engineering Techniques in the Domestic Pig

The term genetic (or genome) engineering is an umbrella term that covers a variety of techniques. It can refer to transgenic organisms, which have had an exogenous gene from another species inserted somewhere in their genome. It can also refer to knock-out (KO) animals which have had a gene functionally knocked out either by deleting the entire gene or important portions of the gene, rendering it nonfunctional. Another classification of genome engineering includes knock-ins, which have targeted insertion of a DNA sequence into the genome. And finally, gene editing can apply to instances where targeted nucleotides have been changed to affect the corresponding amino acid during translation. Each of these engineering methods has already been successfully reported in large animals, including pigs.

The field of livestock genetic engineering has rapidly evolved since the first report of fusion gene microinjection in 1985. However, most of the following techniques were perfected in the mouse model before being adapted for use in livestock species. This is due to several key advantages of the rodent model system: relative ease of superovulation and collection of oocytes, established *in vitro* fertilization (IVF) protocols, a well-developed embryo culture and embryo transfer system, and a short generation interval to quickly determine transgene expression and

penetrance. These characteristics of the rodent system allow for scientists to determine the viability of a new technique before starting the arduous task of validating the technique in a livestock species that requires more time and resources as compared to the rodent model. As expected however, some modifications are required to transfer these skills to the large animal system prior to their widespread use, and often these techniques have lower success rates in large animals. Regardless, the techniques for genome engineering in both rodents and livestock have been honed through the years, with scientists now able to target specific loci or single nucleotides for precise modifications.

Zygote Microinjection of DNA

Perhaps the simplest method of introducing foreign DNA into the mammalian genome is injection of a DNA construct into the cytoplasm or pronucleus of a zygote or an oocyte undergoing fertilization, respectively. This technique is performed using a DNA construct containing the gene of interest in order to generate a random integration of the DNA into the host genomic sequence. Because this method relies on random incorporation into the host's genome, it has a low yield: only 1-5% of microinjected embryos generate transgenic offspring (Engelhard, Hagen et al. 2009, Garrels, Ivics et al. 2012). This yield is even lower in other mammalian species, and extremely low in ruminants.

Pronuclear injection has some clear negative aspects that make its use less desirable for large animals such as the pig. It can lead to high rates of transgenic mosaic animals because the exogenous DNA may still be intact following the first

cleavage event. It can also lead to concatemer events, when the DNA insert consists of multiple copies of the same microinjected DNA sequence linked in series (Kues, Schwinzer et al. 2006, Jacobsen, Bawden et al. 2010, Garrels, Ivics et al. 2012). The pronucleus is also incredibly difficult, if not impossible, to visualize in large animal oocytes. Often, this means that the DNA may stay in the cytoplasm, reducing the rate of incorporation into the genome which leads to the low success rates of this method.

Transposon-mediated Gene Editing

Transposable elements, or transposons, are DNA sequences that are able to change positions within a genome. They were first discovered in other non-mammalian species, and have since been used for a variety of purposes including gene trapping, gene therapy, and transgenesis (Woodard and Wilson 2015). There are two main transposon systems currently in use: Sleeping Beauty and PiggyBac. The Sleeping Beauty transposon was discovered in medaka fish while the PiggyBac system was discovered in insect cells from the cabbage looper moth (Woodard and Wilson 2015).

These two DNA transposons use a “cut-and-paste” mechanism in which the transposon is removed from one area of the genome (or plasmid DNA) and inserted into another area with the correct flanking nucleotide sequence (Woodard and Wilson 2015). In this method, transposons must be accompanied by a transposase, the enzyme which facilitates the insertion of the transposon by nicking the DNA at the new insertion site. Possible insertion sites for transposons must contain flanking regions of inverted repeats and a specific nucleotide sequence that the transposase

recognizes. In the PiggyBac system, when the transposase is expressed, it binds to the inverted repeat flanking regions and induces a hydrophilic attack of the TTAA nucleotide sequence (TA in Sleeping Beauty system). The transposases join together and generate a hairpin loop that excises the transposon. Then the transposon is joined into the genomic DNA at a TTAA sequence (Woodard and Wilson 2015). A diagram of this process is shown in Figure 1.1.

Transposons have been favored in the genetic engineering field because of their relative ease of insertion and excision. If the transposase is expressed a second time, the transposon is excised from the DNA, leaving the same native TTAA without any exogenous DNA left behind (Ding, Wu et al. 2005). The transposon system is limited by the amount of DNA that can be integrated into the genome, however. While the PiggyBac system has been shown to integrate up to 100kb, increased cargo size has been correlated with decreased efficiency for both PiggyBac and Sleeping Beauty (Izsvak, Ivics et al. 2000, Li, Turner et al. 2011, Hudecek, Izsvak et al. 2017).

Like pronuclear injection, transposons are also microinjected into the embryo at the zygote stage, for incorporation into the DNA prior to the first cleavage. While transposons can integrate into a site containing the required TA or TTAA sequence > 95% of the time, efficiency is dependent upon the size of the DNA to be inserted; larger DNA sequences show reduced integration efficiency (Houdebine 2009). Due to the limitations of cargo size and low efficiency, transposons are not widely used in genetic engineering, although a 2017 study reported that the Sleeping Beauty system is currently in use in 10 gene therapy clinical trials (0.4% of gene therapy trials)(Hudecek, Izsvak et al. 2017).

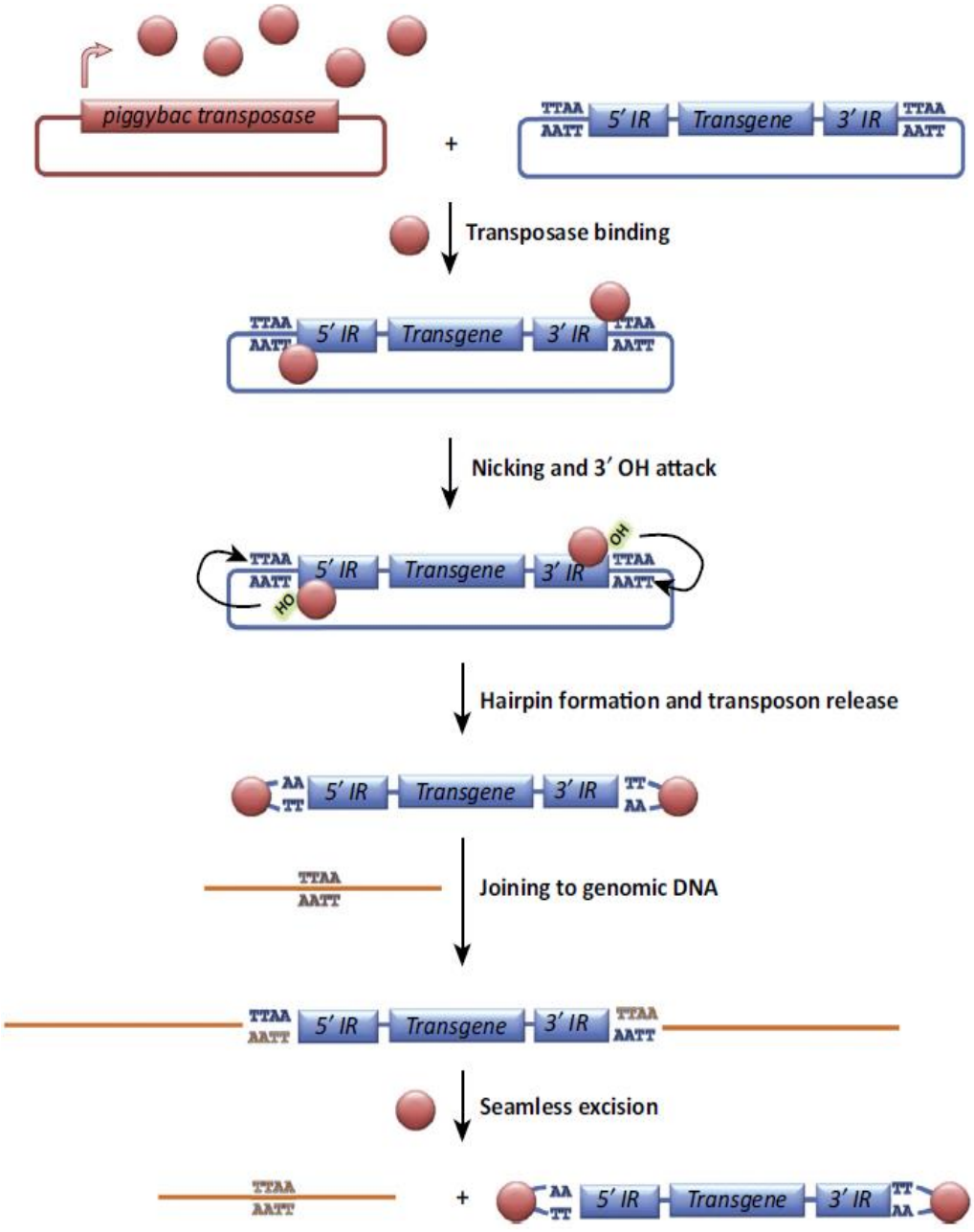

Figure 1.1 Steps of PiggyBac transposition. IR = inverted repeats. Figure reprinted from Woodard and Wilson (2015) with permission from Elsevier.

Viral Systems

A viral integration system takes advantage of the inherent ability of certain classes of viruses to incorporate themselves into the host organism's genome or express a particular protein inside of a host cell. It allows for the viral system to do the work of introducing the foreign DNA sequence into the genome via its suite of genes involved in initial infection and replication, but in a modified way so that the detrimental effects of the virus are not occurring inside the target cell. This can be done by deleting the genes necessary for viral replication (Strayer, Agrawal et al. 2006). A viral integration system such as the simian virus 40 (SV40) or the herpes simplex virus (HSV) was first used to show that they could infect the host animal's DNA and that the viral DNA could also be transmitted to their offspring, providing evidence for stable integration of exogenous DNA sequences through generations (Jaenisch and Mintz 1974, Brinster, Chen et al. 1981).

There are four main viral systems currently in use for the transfer of genetic material: retroviruses, adenoviruses, adeno-associated viruses (AAV), and herpes simplex virus (HSV). Each of these systems is unique in genetic capacity, expression level, ease of production, level of host immune response, and location within the host's genome (Howarth, Lee et al. 2010). An ideal viral gene delivery system is described as having the following characteristics: (1) The vector should be able to infect developing and mature cells, as well as dividing and non-dividing cells; (2) The vector should have a large packing capacity to incorporate transgenes of varying size; (3) It should not produce any toxicity or immune response in the host; (4) It should be easy to produce high titers of virus that infect with high efficiency; and (5) The vector

will contribute to stable, long-lasting expression (Osten, Grinevich et al. 2007, Howarth, Lee et al. 2010). To date, an ideal viral vector that meets all these criteria has yet to be demonstrated. Therefore, each of these systems described have distinct advantages and disadvantages, and the final decision of which viral system to use is made based on the specific needs of the research problem.

Retroviruses

Retroviruses are single-stranded RNA viruses that must be reverse transcribed to double-stranded DNA in the host-cell cytoplasm prior to transcription and translation to protein (Osten, Grinevich et al. 2007). Retroviruses can be naturally found in all vertebrates, and can cause chronic infections in addition to a variety of other serious conditions. These viruses consist of a lipid-enveloped virus particle that surrounds an inner core (Howarth, Lee et al. 2010). The genomes of these viruses are between 7-11 kb in size and contain three essential genes: *gag*, *pol*, and *env*. The *gag* gene codes for structural proteins that form the core of the virion particle. The *pol* gene encodes the viral enzymes reverse transcriptase, protease, and integrase; and the *env* gene encodes the envelope glycoproteins which are essential for mediation of entry of the virus into the host cell (Osten, Grinevich et al. 2007, Howarth, Lee et al. 2010). After the initial infection of the host cell, the viral RNA genome is reverse transcribed to double-stranded DNA and integrated into the host genome for replication.

Retroviruses have been an attractive candidate for use in gene transfer, gene therapy, and genetic engineering based on their ability to incorporate into the host

genome and have persistent expression for the lifetime of the host individual (Howarth, Lee et al. 2010). Retroviral vectors were the first genetic vectors to facilitate gene transfer efficiently and stably into mammalian cells (Cone and Mulligan 1984, Barquinero, Eixarch et al. 2004). In 1990, a retroviral vector was the first vector used in a gene therapy trial for adenosine deaminase deficiency (Blaese, Culver et al. 1995). Consequently, retroviruses have been in use for stable gene transfer into mammalian cells for over 30 years.

As a group, retroviruses have been a popular choice for genetic engineering and gene therapy experiments for two main reasons: they allow for lifelong expression of the gene of interest due to their complete integration into the host cell's genome, and they are relatively easy to produce (Howarth, Lee et al. 2010). However, with the exception of lentiviruses, retroviruses are unable to infect non-dividing cells because the complex which contains the double-stranded viral DNA is not able to cross the intact nuclear membrane (Barquinero, Eixarch et al. 2004, Osten, Grinevich et al. 2007, Howarth, Lee et al. 2010). For this reason lentiviruses, which are a subgroup within the retrovirus family, have been a popular choice for researchers because of their ability to infect both dividing and non-dividing cells.

Another disadvantage of the retroviral system is its limited size for packaging – it can only be used for inserts up to about 8-9 kb in size (Osten, Grinevich et al. 2007). They also require a membrane-bound receptor for entry into the cell. A cell which does not express the appropriate receptor will not be infected by the retrovirus (Barquinero, Eixarch et al. 2004). It can also be difficult to produce high enough viral titers for efficient use.

Adenoviruses

In contrast to retroviruses, adenoviruses are double-stranded DNA viruses. In humans, adenoviruses can cause respiratory illnesses, conjunctivitis, and gastrointestinal problems (Stewart, Fuller et al. 1993). They have a linear genome of approximately 36 kb in length (Buchen-Osmond 1997, Osten, Grinevich et al. 2007, Howarth, Lee et al. 2010). The genome of adenoviruses encodes a large number of viral proteins, estimated to be over 70 proteins in one serotype (Osten, Grinevich et al. 2007). These proteins are responsible for producing the protein capsid, fiber proteins, replication machinery, and other factors that are involved in reducing the host cell immune response and host cell lysis (Osten, Grinevich et al. 2007). In total, there have been over 100 adenoviral serotypes identified, with 51 in the human adenovirus family that can infect and replicate in a wide variety of organs including the gastrointestinal tract, liver, bladder, eye, and respiratory tract (Verma and Weitzman 2005). The most popular serotypes in use for generation of adenoviral vectors for use in transgenesis are the Ad2 and Ad5 serotypes (Verma and Weitzman 2005).

Adenoviral vectors have a size capacity of between 4.7-4.9 kb for a transgene insert. However, this capacity can be increased further by deleting dispensable sequences from the adenovirus genome, making room for up to 8.3 kb of transgene (Verma and Weitzman 2005). As the vectors were increasingly edited to change their properties, a third generation of vectors was introduced that generated large deletions using Cre-mediated excision. For example, one of these vector modifications

generated a 25-kb deletion that allowed for even larger transgenes to be incorporated into the adenoviral system (Lieber, He et al. 1996).

The adenovirus system for generating transgenic expression in mammalian cells is marked by several key advantages. First, much larger transgenes or multiple transgenes could be inserted into the vector due to their naturally larger genomic sequences. Second, the efficiency of the adenoviral system is quite high. Viral particles are made at high titers within a helper cell line such as HEK293 cells, and they are isolated rather easily (Howarth, Lee et al. 2010). Another advantage to this system is that adenoviruses do not integrate into the host cell's genome. Instead, they exist in the nucleus as episomal vectors that replicate independently of the host's genomic DNA (Hermens and Verhaagen 1998).

Adenoviruses as tools for gene transfer also have some major disadvantages that can be drawbacks for their use in certain experiments. As mentioned, they exist as episomal vectors so the expression of the transgene is usually limited to a short amount of time such as weeks or months. They also require a receptor system for initial infection of the host cell, which can prohibit some cells from receiving the virus. Lastly, adenoviral vectors seem to elicit a higher than usual immune response in the infected cells, although second and third generation vectors have been able to reduce the inflammatory response a bit, though not entirely (Hermens and Verhaagen 1998, Verma and Weitzman 2005, Howarth, Lee et al. 2010). Due to their extensive study and subsequent manipulation of the necessary genes required for infection and expression, there are a wide variety of adenoviral vectors available for use that make them an attractive transgenic system.

Adeno-associated Viruses

Adeno-associated viruses (AAV) are single-stranded DNA viruses that belong to the parvovirus family of viruses. They require the use of a helper virus to mediate their replication (Osten, Grinevich et al. 2007, Howarth, Lee et al. 2010). The helper virus is usually an adenovirus or herpes simplex virus (HSV). The AAV genome is quite small at 4.7 kb in size and consists of two open reading frames called *rep* and *cap* which are flanked by inverted terminal repeats (Buchen-Osmond 1997, Osten, Grinevich et al. 2007). The *rep* gene produces four proteins that are required for replication and packaging. The *cap* gene encodes for three structural proteins (Verma and Weitzman 2005, Osten, Grinevich et al. 2007).

There have been over 100 AAV serotypes identified from nonhuman primates, and 12 human serotypes that each have a different targeting capacity based on host species and cell type (Daya and Berns 2008). The AAV serotype 2 (AAV2) is a common choice of vector for many experiments due to its ability to infect a large number of cell types and species. In this vector system, it is common to replace the rep and cap genes with the transgene of interest and supply the Rep and Cap proteins in *trans* from a separate packaging plasmid in order to increase the DNA capacity of the vector (Verma and Weitzman 2005).

AAV have been a popular choice of vector for transgenic and gene therapy experiments due to their ability to transduce both dividing and non-dividing cell types. Additionally, expression from AAV vectors is stable long-term (years) and is non-pathogenic, eliciting an extremely minimal to nonexistent immune response from the host (Wright, Qu et al. 2003). The low immunogenicity offers a unique advantage

for their use in human clinical applications, as AAV have a low risk of recombination with wild-type virus (Koczot, Carter et al. 1973). AAV vectors also generate high titers for infection which are easily isolated.

The size of AAV vectors is a limiting factor in their use for transgenesis. They have the smallest capacity of the major viral systems in use, but have been engineered for more capacity by removing the rep and cap genes as previously discussed. They also integrate into the host cell's genome, which may exclude them from use in certain human trials unless researchers can find a way to cleanly excise them. While the limited immune response of AAV vectors is a major advantage, natural infections have resulted in a large prevalence of antibodies against the viruses. However, this obstacle may be overcome by combining factors from different serotypes, or modifications to the virus capsid (Huttner, Girod et al. 2003, Peden, Burger et al. 2004). On the whole, the AAV viral system is an excellent candidate for introducing transgenes, as long as the gene of interest is relatively small.

Herpes Simplex Virus

The last major group of virus to consider for transgene experiments is the herpes simplex virus (HSV). The genome of HSV consists of a linear double-stranded DNA molecule that is 152 kb in length (Verma and Weitzman 2005, Osten, Grinevich et al. 2007). The genome is arranged into unique long (U_L) and unique short (U_S) regions that are flanked by terminal repeats, and the genome encodes for over 80 proteins (Verma and Weitzman 2005, Osten, Grinevich et al. 2007). In humans, HSV primarily infects the skin and mucous membranes. After initial infection, the viral

particles can infect neurons that innervate the primary site and establish a latent infection that is able to become reactivated and spread years later (Hermens and Verhaagen 1998, Osten, Grinevich et al. 2007).

The main amplicon vector used for HSV infection in research is an HSV-1 vector that has particles that are identical to wild-type HSV-1 in a variety of aspects including structure, immunology, and host range. However, it differs from the wild-type HSV-1 in that it carries a DNA concatemer plasmid instead of the viral genome. Therefore, the HSV-1 amplicon vector contains no viral genes and thus it does not make viral proteins (Cuchet, Potel et al. 2007). This attractive setup generates a vector that is nontoxic for infected cells and nonpathogenic for infected organisms (Epstein 2005). The removal of virus genes also means that the vector can accommodate very large pieces of DNA due to the 153 kb capacity of the HSV-1 particle.

The HSV-1 system for introducing transgenes allows for the largest capacity of the four main viral systems, which can be useful for delivering complex genes or multiple copies of the gene when desired (Verma and Weitzman 2005). The HSV-1 genome also does not integrate into host DNA, as it remains episomal. However, due to the natural latency period that occurs in HSV-1 infection, it is usually the case that expression decreases after a matter of weeks. There is also a rather large immune response by the host cell which must be overcome for their potential use in gene therapy. With a few modifications that are likely on the horizon, the HSV-1 system may be the best candidate for transgene delivery by a viral system.

Sperm-mediated Gene Transfer

Sperm-mediated gene transfer (SMGT) is another method for introducing exogenous or edited DNA into animals in order to introduce genetic changes to an individual. Sperm of several species have been shown to have the ability to bind naked DNA, making them an easy target for DNA uptake (Lavitrano, Camaioni et al. 1989, Horan, Powell et al. 1991, Sperandio, Lulli et al. 1996). Additionally, DNA binding to sperm can also occur via the use of DNA-liposome complexes, although integration into the genome seems to be minimal (Bachiller, Schellander et al. 1991, Rottmann, Antes et al. 1992). SMGT has been reported successfully in a variety of species including mouse (Lavitrano, Camaioni et al. 1989), pig (Sperandio, Lulli et al. 1996), rabbit (Brackett, Baranska et al. 1971, Kuznetsov and Kuznetsova 1995), *Xenopus* (Kroll and Amaya 1996), cattle (Perez, Solano et al. 1991), chicken (Fainsold, Frumkin et al. 1990), and many others.

Often, SMGT is used in combination with artificial insemination although increasingly it is paired instead with *in vitro* fertilization systems. SMGT can be coupled with intracytoplasmic sperm injection (ICSI) in order to increase fertilization rate. ICSI is a technique in which a single spermatozoon is injected into the cytoplasm of an oocyte and is commonly used for in vitro fertilization in humans (Lotti, Polkoff et al. 2017). In addition to fertility applications however, this technique is also utilized in animal research to facilitate genetic changes by introducing edited sperm to the oocyte as part of the procedure.

In ICSI, the sperm is responsible for transmitting the edited or transgenic DNA directly into the egg. In combination with SMGT, this technique has been

performed in mice, rhesus monkey, and pig with varying success (Perry, Wakayama et al. 1999, Chan, Luetjens et al. 2000, Chan, Luetjens et al. 2000, Lai, Sun et al. 2001). The efficiency for this technique can be quite low, as little as 35% success rate (Smith and Spadafora 2005). It is theorized that the efficiency may be low because there are natural barriers against this method. Researchers have made the assumption that due to possible "free" DNA molecules that may be around due to natural cell death, it is reasonable to assume that sperm would be resistant to picking up these molecules and transferring them to potential offspring (Smith and Spadafora 2005).

In response to the low efficiency of SMGT, it has been paired with antibody fragments to allow the DNA to target cells and become internalized. This relies on receptor-mediated endocytosis and is coined "linker-based SMGT." This technique has had success in pigs and mice (Chang, Qian et al. 2002). SMGT is advantageous for use in generating transgenic and gene edited animals due to its simplicity but is not widely used because integration into the genome of the exogenous DNA is not stable and the efficiency is too low to reliably generate animals.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT; also known as cloning) is a technique in which a viable embryo is generated using genetic material from a somatic cell. This is performed by removing the nucleus of an oocyte (a process called enucleation) and fusing a somatic cell with the oocyte, generating a diploid cell whose genome is entirely from the donor somatic cell. The first reports using SCNT in mammals paved the way for the generation of genetically modified livestock animals (Campbell,

McWhir et al. 1996, Wilmut, Schnieke et al. 1997). After these first reports, SCNT has proven successful in many species including domestic, wildlife, and laboratory species (Galli, Lagutina et al. 2012). Shortly after the first reports of SCNT technology, the first genetically modified animals and knockout livestock were generated (Schnieke, Kind et al. 1997, McCreath, Howcroft et al. 2000).

Currently, SCNT using fibroblasts is the most widely used approach to generating genetically modified large animals, especially because true embryonic stem (ES) cells have not been established in livestock species (Tang, Gonzalez et al. 2015). However, somatic cells have a limited life span in culture which hampers the ability to establish a cell line with the exact genetic modifications desired. Transfection of fibroblasts may also induce senescence before they can be fully characterized (Laible and Alonso-Gonzalez 2009). To combat these problems, some researchers have attempted to serially clone embryos and re-derive the cell lines to save the genotype from senescence (Robl, Wang et al. 2007, Laible and Alonso-Gonzalez 2009).

While SCNT offers an excellent way to introduce predictable, targeted genetic change to an embryo, there are some drawbacks that are difficult to ignore. SCNT has different developmental competence, requiring large numbers of embryos to be transferred in order to establish pregnancy (Tang, Gonzalez et al. 2015, Whitelaw, Sheets et al. 2016). Higher than average pregnancy losses are most often due to placental abnormalities (Tang, Gonzalez et al. 2015). In addition to placental abnormalities, the offspring often have developmental abnormalities as well. Ruminants often present with large offspring syndrome and cloned piglets tend to be

of smaller size (Lee, Peterson et al. 2004, Schmidt, Winter et al. 2011). Despite the drawbacks, until true ES are established for livestock species, SCNT will most likely be the technique of choice for establishing precise genome modifications.

Pluripotent Cells

Pluripotent cells (also called stem cells) are characterized by their ability to individually contribute to all somatic lineages and the germ cells (Leitch, Blair et al. 2010). Cells exhibiting this unique capacity for differentiation can be generated by several methods: 1) by isolation of inner cell mass (ICM) cells from the blastocyst; 2) by reprogramming of somatic cells to a stem cell state; 3) by isolation of primordial germ cells (PGCs) to generate embryonic germ cells (EGC); or 4) by isolation from teratocarcinomas to generate embryonic carcinoma cells (ECC). Each of these types of stem cells share the defining properties of pluripotent cells, including the ability to contribute to germline-competent chimeras – animals generated from the combination of a diploid embryo with ESCs and whose germ cells at least partially arose from the ESCs. In mice and other animals, chimeras are made by injecting ESCs into the blastocyst stage embryo, where cells from both origins contribute to the animal.

Embryonic stem cells represent an immortal cell type. In culture, they can be passaged indefinitely with no reduction in capacity for self-renewal or differentiation (Evans and Kaufman 1981). This characteristic of stem cells makes them an ideal candidate for use genetic modification experiments because they can be passaged as many times as is necessary in order to confirm the desired genotype. Through the use of stem cells it is possible to insert a single copy of a transgene into a specific locus

via homologous recombination (HR), allowing for precise modifications to be characterized prior to their use in generating live animals (Capecchi 2005, Laible and Alonso-Gonzalez 2009). Unfortunately, ESC technology is only available in mice and rats as no true ESCs have been derived for livestock species (Tang, Gonzalez et al. 2015).

Despite the lack of true ESCs for livestock species, an alternative cell type has been generated by reprogramming terminally differentiated fibroblasts into a pluripotent state. The reprogramming was done by exogenous expression of several key transcription factors leading to the establishment of induced pluripotent stem cells, known as iPSCs (Takahashi and Yamanaka 2006, Takahashi, Tanabe et al. 2007). Induced pluripotent stem cells have been generated for a variety of livestock species including pig (Telugu, Ezashi et al. 2011), cow (Han, Han et al. 2011), sheep (Sartori, DiDomenico et al. 2012), and goat (Ren, Pak et al. 2011).

iPSCs share many common characteristics with ESCs, but they differ in their epigenetic signature (Nowak-Imialek, Kues et al. 2011). While iPSCs have the same ability for self-renewal as ESCs, their germline competence in livestock has remained elusive, as has their contribution to chimeras (Nowak-Imialek, Kues et al. 2011, Telugu, Ezashi et al. 2011, Sartori, DiDomenico et al. 2012, Fujishiro, Nakano et al. 2013). The production of iPSCs is an exciting step in generating genetically modified livestock, because like ESCs they have unlimited self-renewal capacity, allowing for characterization of the desired genotype prior to use in SCNT. Although the full potential of iPSCs and ESCs has not yet been reached in livestock, stem cells are an exciting technique to produce genetically modified animals. However, with recent

advancements in gene targeting and editing techniques, the need for stem cells for use in genetic engineering may be waning.

Gene Editing Tools

The recent development of gene editing tools has made it relatively easier to make precise modifications to the genome. These tools, which include meganucleases, zinc-finger nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced palindromic repeats and CRISPR-associated (cas9) system induce a double-strand break (DSB) and allow the cell's own repair machinery to attempt a repair. Cells have two competing pathways to repair the DSB: through homology-directed repair or by non-homologous end joining. In homology-directed repair, a DNA strand with a homologous sequence is directed toward the DSB and used as a template for repair (Youds and Boulton 2011). This repair pathway can be exploited for generating knock-in animals by providing a homologous template with the desired genetic changes.

In non-homologous end joining, the two sides of the DSB are brought together for repair. Occasionally, errors are made during this process, leading to small insertions and deletions (indels) which can generate functional knockouts by generating premature stop codons, removing large portions of the gene, or creating frameshift mutations that lead to improper translation (Carroll 2017). This repair pathway is preferred when attempting to generate a knockout animal. The nucleases discussed below have different mechanisms to generate the initial DSB, but the end result is the same: modification of large animal genomes.

Meganucleases

A critical first step in the advent of genetic engineering techniques was the use of meganucleases. One such meganuclease is the I-*Sce*I yeast meganuclease. This nuclease contains a recognition site of 18 base pairs and is responsible for intron homing in yeast mitochondria via the induction of a DNA DSB and subsequent repair (Jacquier and Dujon 1985, Fernandez, Josa et al. 2017). After the discovery of this meganuclease, it was reported that I-*Sce*I meganuclease could promote mammalian homologous recombination at a much higher rate than spontaneous homologous recombination (Choulika, Perrin et al. 1995). Researchers were then able to introduce the I-*Sce*I recognition site at a locus of interest to significantly increase targeting efficiency by 100-fold (Cohen-Tannoudji, Robine et al. 1998). This meganuclease technology has been further honed by coupling I-*Sce*I sites to transgenes to increase efficiency of transgenesis in medaka (Thermes, Grabher et al. 2002), *Xenopus* (Pan, Chen et al. 2006), and even mammalian systems such as mouse and pig by adding a nuclear localization signal (Wang, Zhou et al. 2014). Although initially an extremely useful tool, genetic engineering via meganucleases has waned due to the development of nucleases that allow for recognition sequence customization which do not require the introduction of the recognition sequence into the locus of interest (Fernandez, Josa et al. 2017).

Zinc-Finger Nucleases

Zinc-finger nucleases (ZFNs) were among the first nucleases described for the purpose of site-specific genetic engineering (Kim, Cha et al. 1996). ZFNs are

composed of zinc finger recognition domains linked to a *Fok1* endonuclease domain (Tang, Gonzalez et al. 2015). The *Fok1* endonuclease domain was discovered as a DNA cleavage domain from a bacterial protein, and the zinc fingers were originally identified in sequence-specific eukaryotic transcription factors (Carroll 2017). A single zinc finger has approximately 30 amino acids, and several amino acids on the surface recognize three base pairs with varying levels of selectivity (Gaj, Gersbach et al. 2013).

In the ZFN targeting system, two zinc finger monomers must recognize and bind to the corresponding DNA sequence, allowing the *Fok1* domains to dimerize and induce the double strand break. The base pair triplets which the zinc fingers recognize can be changed to recognize many different DNA triplets, allowing for targeting at a desired sequence (Pabo, Peisach et al. 2001). However, the two zinc finger monomers must recognize sequences on opposite strands to allow the *Fok1* endonuclease to dimerize and cleave both DNA strands (Whitelaw, Sheets et al. 2016). Engineering of the zinc finger system has allowed for proteins that recognize up to 18 bp in length, conferring specificity within 68 billion bp of DNA (Gaj, Gersbach et al. 2013).

ZFNs provided the first system for site-specific targeting, but they have been overshadowed in recent years by the discovery of transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced palindromic repeats (CRISPRs), which are relatively easy to assemble and use. ZFNs are a bit cumbersome to assemble, as they require high GC content, recognize triplets instead of single base pairs, and require a short spacer sequence between the two zinc finger

monomers (Whitelaw, Sheets et al. 2016). ZFNs have been successfully used to generate knockout livestock, including cattle and pigs (Hauschild, Petersen et al. 2011, Yang, Yang et al. 2011, Yu, Luo et al. 2011, Luo, Song et al. 2014).

Transcription Activator-like Effector Nucleases

Transcription Activator-like Effector Nucleases (TALENs) were originally isolated from plant pathogenic *Xanthomonas* bacteria and are very similar to ZFNs in their mechanism. Like ZFNs, they use the *Fok1* nuclease and require dimerization around a spacer sequence to generate the DSB, but TALENs use a different recognition mechanism for sequence specificity (Isalan, Choo et al. 1997, Bhat, Malik et al. 2017).

TALENs contain DNA-binding domains that consist of 33-35 amino acid repeat domains. Each of these domains recognizes a single base pair. Within each series of amino acids (a TAL module), the amino acids at positions 12 and 13 confer DNA recognition and therefore sequence specificity (Whitelaw, Sheets et al. 2016). These two hypervariable amino acid residues are termed the repeat variable diresidues (RVDs) and it is these RVDs that are designed to recognize the desired DNA sequence to be targeted (Deng, Yan et al. 2012, Gaj, Gersbach et al. 2013).

TALENs offer an advantage over ZFNs because they recognize single base pairs instead of triplets, affording greater design flexibility (Gaj, Gersbach et al. 2013). However, the high number of identical repeat sequences can make assembly challenging and several methods of cloning have been devised to accelerate this process (Cermak, Doyle et al. 2011, Reyon, Tsai et al. 2012, Gaj, Gersbach et al.

2013, Schmid-Burgk, Schmidt et al. 2013). The binding site for TALENs must also start with a T base, which may limit the sequences available for targeting. Nevertheless, TALENs have been used to target pigs, sheep, and cattle (Bedell, Wang et al. 2012, Carlson, Tan et al. 2012, Tan, Carlson et al. 2012, Proudfoot, Carlson et al. 2015).

Clustered Regularly Interspaced Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) 9 System

The CRISPR/Cas9 system has recently emerged as an efficient alternative to the ZFN and TALEN systems for gene targeting. This system is naturally found in archaea and bacteria as an adaptive immune response to invading foreign DNA. It confers immunity to the organism by an RNA guiding system that escorts a nuclease to the invading viral DNA or plasmid DNA for cleavage (Wiedenheft, Sternberg et al. 2012, Gaj, Gersbach et al. 2013).

In this system, a guide RNA is complexed with the Cas9 nuclease (Figure 1.2). The guide RNA includes a short 20 nucleotide sequence that confers specificity to the DNA target sequence. The guide RNA forms a complex with the Cas9 nuclease and guides it to the complementary region to induce a double strand break (Tang, Gonzalez et al. 2015). As with ZFNs and TALENs, the cellular repair mechanism is activated, and knockins or functional knockouts can be obtained in the same manner as previously described. In addition to the target sequence on the guide RNA, the Cas9 protein also requires a conserved protospacer adjacent motif (PAM) that is immediately beside the complementary region of the target DNA (Jinek, Chylinski et al. 2012).

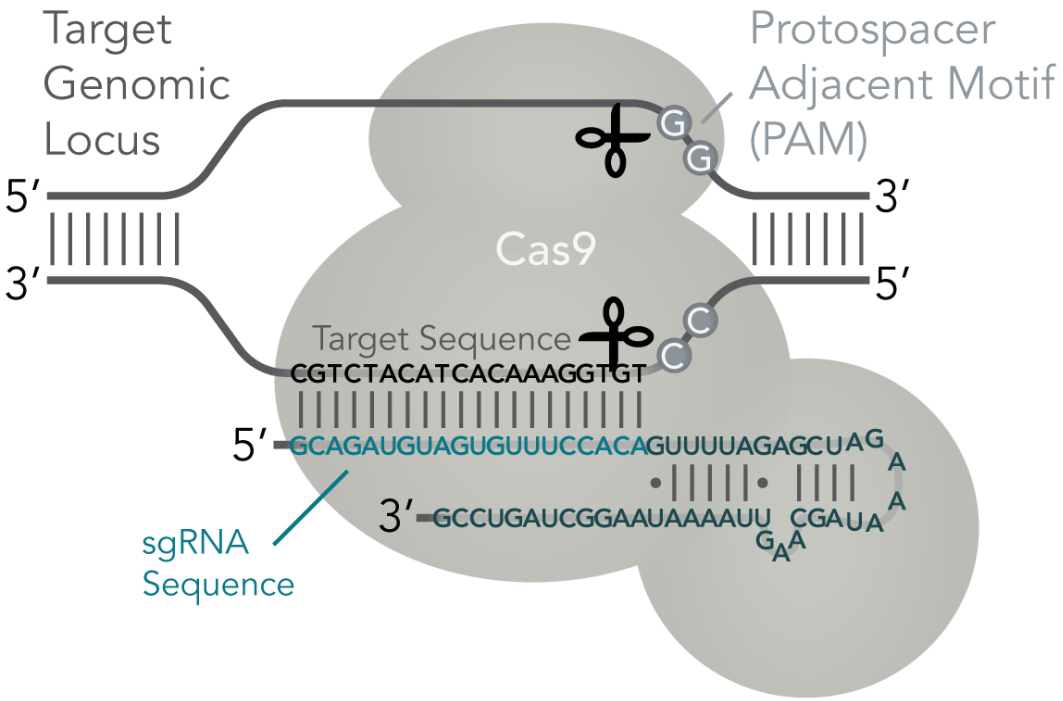

Figure 1.2 The CRISPR/Cas9 complex. Figure reprinted with permission from Ubiquigent.

The CRISPR/Cas9 system has become widely used in just a few short years. This is mostly due to its incredible ease of use. The CRISPR/Cas9 system only requires one recognition site instead of two, and its construction requires a simple oligo instead of large amino acid regions with the target sequence embedded within (Whitelaw, Sheets et al. 2016). Additionally, the Cas9 endonucleases have been recently converted into nickases, allowing for another method of precise genomic modification (Gaj, Gersbach et al. 2013). There are concerns regarding off-targeting DSBs that may occur, due to the single recognition sequence (Fu, Foden et al. 2013). These issues must be appropriately addressed in the future before this technology can be applied for human gene therapies.

Since its discovery, the CRISPR/Cas9 system has been used to generate over 300 gene edited pigs, cattle, sheep, and goats (Laible, Wei et al. 2015, Murray and Maga 2016, Park, Kaucher et al. 2017, Tan, Proudfoot et al. 2016). It has also become possible to target several genes at once, generating animals with changes at two or more loci, greatly increasing the speed at which complex gene edits can be produced (Cong, Ran et al. 2013, Ma, Shen et al. 2014). One publication even generated mice that had been mutated across five genes simultaneously (Wang, Yang et al. 2013). The CRISPR/Cas9 system has quickly become the most commonly used gene editing tool due to its simplicity, ease of use, and versatility.

Primordial Germ Cells

Primordial germ cells (PGCs) are specialized cells that are the precursors of gametes. PGCs are responsible for passing on genetic information from parent to offspring. These cells are specified very early in development from a subset of mesodermal cells which originate at the primitive streak. Due to their short generation interval and fast developmental timeline, many studies on PGC specification and development have been focused on the mouse model system, with only a few published studies on PGC specification and commitment events in other mammals (Johnson, Richardson et al. 2011).

Specification of PGCs in the Mouse

In the mouse, progenitors of PGCs arise from the posterior region of the post-implantation epiblast (Figure 1.3). At the onset of gastrulation, precursors of PGCs are induced by BMP signaling (BMP2, BMP4, and BMP8b) from cells in the extraembryonic ectoderm (ExE; (Magnusdottir and Surani 2014). These BMPs act through SMAD1 and SMAD5 signaling to induce expression of PR domain containing 1 (*Prdm1*) and *Prdm14* in a dose-dependent manner, with the highest levels of BMP occurring in the posterior proximal epiblast (Ohinata, Ohta et al. 2009, Saitou and Yamaji 2012). Restriction of PGCs to the posterior epiblast location occurs due to BMP inhibitory signals such as left-right determination factor 1 (LEFTY1), cerberus 1 (CER1), and dickkopf homolog 1 (DKK1) which prevent posteriorization of the anterior epiblast (Ohinata, Ohta et al. 2009, Saitou and Yamaji 2012, Irie, Tang et al. 2014). Activation of *Prdm1* in precursor cells as early as E6.25

initiates a cascade of events including the induction of *Prdm14* and transcription factor AP2-gamma (*Tfap2c*) in PGC precursors that lead to PGC specification (Irie, Tang et al. 2014). Because the PGCs are derived from a mesodermal population, PGC precursors also initially express mesodermal transcripts such as homeobox (*Hox*) genes and brachyury (*T*). However, after the induction of *Prdm1*, *Prdm14*, and *Tfap2c*, the *Hox* genes are repressed and pluripotency genes *Pou5f1*, *Sox2*, and *Nanog* are expressed.

After PGCs are specified, PRDM1, PRDM14, and TFAP2C then coordinate a network which is able to repress the somatic program, induce genome-wide epigenetic reprogramming, and initiate the reacquisition of pluripotency (Figure 1.4) (Yamaji, Seki et al. 2008). Each of these three genes has a unique role in accomplishing these three goals. *Prdm1* is responsible for repression of the somatic program although its exact method of action is not clearly understood (Ohinata, Payer et al. 2005). *Prdm14* is absolutely essential in PGC specification and is involved in epigenetic reprogramming as well as initiating and maintaining pluripotency, even in mouse and human ESCs in culture (Yamaji, Seki et al. 2008, Ma, Swigut et al. 2011, Grabole, Tischler et al. 2013, Yamaji, Ueda et al. 2013). *Tfap2c* is believed to function downstream of *Prdm1* and is known to be important for migration of PGCs to the gonad because knockouts show reduced cell number and PGCs fail to migrate (Schafer, Anschlag et al. 2011). *Tfap2c* mutants are able to specify the initial PGC population but further germ cell differentiation is impaired, and somatic differentiation is initiated (Weber, Eckert et al. 2010).

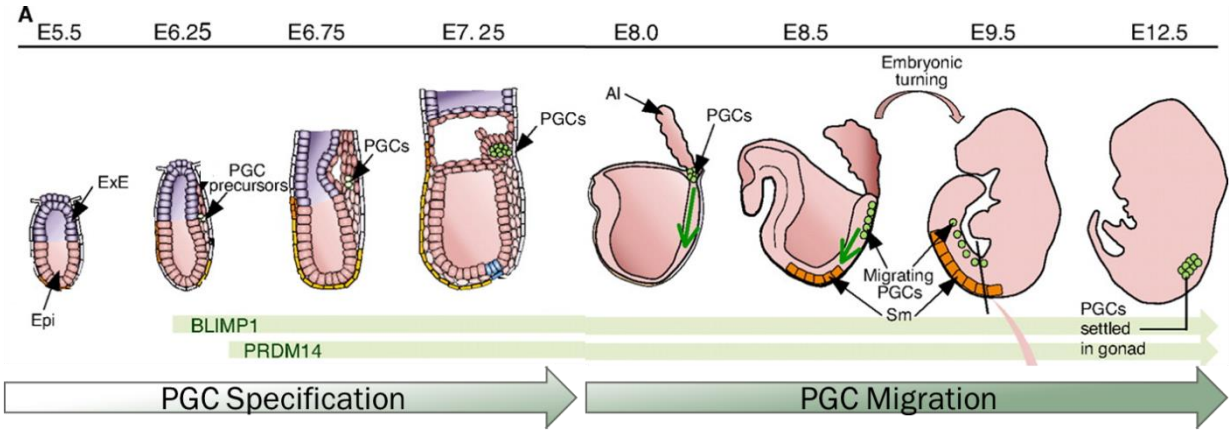

Figure 1.3 Specification and migration of mouse primordial germ cells. Al: allantois; Epi: epiblast; ExE: extra-embryonic ectoderm; Sm: somite. Figure reprinted from Saitou, Kagiwada et al. (2012) with permission from Company of Biologists.

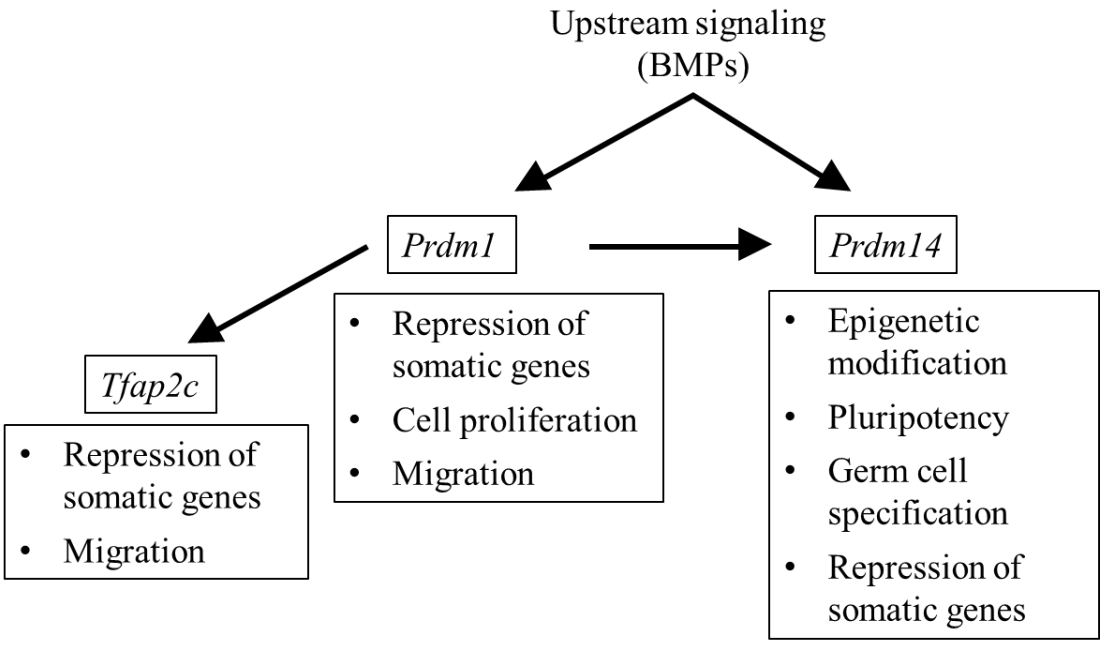

Figure 1.4 The tripartite signaling network for PGC specification in the mouse. Adapted from Saitou and Yamaji, *Cold Spring Harbor Perspectives*, 2012.

Prdm14 belongs to a family of transcriptional regulators that contains 16 members. These genes are so named because they each contain a PR domain, which is a domain that has diverged from the SET domain originally identified in the *Drosophila* Trithorax protein (Nakaki and Saitou 2014). The SET domain defines a large group of histone methyltransferases, and some PRDM family members have histone methyltransferase activity, although *Prdm14* does not. Most PRDM family members also have zinc finger domains; the PR domain and zinc finger domains are highly conserved among vertebrates and the N terminal half of the protein is more divergent (Nakaki and Saitou 2014). The structure of Prdm14 includes the PR domain as well as 6 zinc fingers (Figure 1.5).

Figure 1.5 The motif organization of *PRDM14* in humans.

Migration and Other Events

Following specification, PGCs must migrate from the posterior ExE region through the hindgut and colonize in the genital ridges. During this migration which occurs from E7.75-E11, several epigenetic processes occur which serve to establish the PGC as a future germ cell: erasure of imprints, reactivation of the X chromosome, and global DNA hypomethylation and histone modifications (Irie, Tang et al. 2014). It is imperative that PGCs undergo extensive reprogramming toward genome-wide

hypomethylation in order to allow for normally silenced genes to be induced at the correct developmental time point. This occurs through the loss of histone H3 lysine 9 dimethylation (H3K9me2) and an increase in histone H3 lysine 27 trimethylation (H3K27me3). At the same time, pluripotency genes are being upregulated to prepare cells for a pluripotent profile (Barrios, Irie et al. 2013). Imprints are also beginning to be erased during the migration period so that by E13.5, parental imprints and CpG methylation in promoter regions of germline-specific genes are almost entirely erased. After E12.5, the genital ridges begin sexual differentiation and it is at this time that PGCs receive signals from these tissues that lead them away from the PGC program towards a more specialized germ cell.

Specification of PGCs in the Pig

In the pig, progenitors of PGCs arise in the caudal third of the embryo scattered around the primitive streak at day 12 of embryonic development (Figure 1.6) (Petkov and Anderson 2008). By day 13, the progenitors are still in the area of the primitive streak though some have appeared in the extra-embryonic yolk sac wall, forming a cluster of PGCs (Hyttel, Kamstrup et al. 2011). These progenitor cells are characterized by continued expression of POU5F1 after the epiblast has ceased its expression of POU5F1. They also express SOX17, and most cells within the cluster also express PRDM1. In cells that express both SOX17 and PRDM1, NANOG expression is also retained from the early epiblast (Kobayashi, Zhang et al. 2017).

Between E12.5 and E13.5, PGCs exhibit co-expression of a variety of pluripotency and PGC factors: SOX17, PRDM1, NANOG, and POU5F1, as

determined by immunohistochemical staining (Kobayashi, Zhang et al. 2017). At this stage of development, porcine PGCs do not express the mesodermal factor T. Surprisingly, PRDM14 expression is weak during this specification period, and appears cytoplasmic at E14. As in mice, the initial PGC cluster (E12) contains few cells (~60) which soon increase to more than 300 cells by E 15.5 (Kobayashi, Zhang et al. 2017). During E14-15, the yolk sac folds under the posterior portion of the embryo to form the ventral wall of the hind gut. The PGCs then become restricted to this area at E15 and can be found in the entire length of the hind gut (Hyttel, Kamstrup et al. 2011). After the sharp increase in PGC number, they enter quiescence prior to migration, similar to the mouse system (Seki, Yamaji et al. 2007, Kobayashi, Zhang et al. 2017).

Migration of PGCs in the Pig

Following specification in the posterior embryo, PGCs must migrate to their eventual location in the genital ridge. Migration occurs between days 14 and 24 of embryonic development. Most PGCs remain in the dorsal hind gut until at least day 18, with some having already reached the genital ridge at this time. By day 20, most PGCs have migrated dorsally toward the genital ridge and have taken residence in that tissue (Hyttel, Kamstrup et al. 2011). Also by this time the mesonephric tissue has bulged, forcing the genital ridges toward the midline. By day 23-24 all PGCs have reached the future gonads, integrating into the genital ridge tissue (Hyldig, Ostrup et al. 2011).

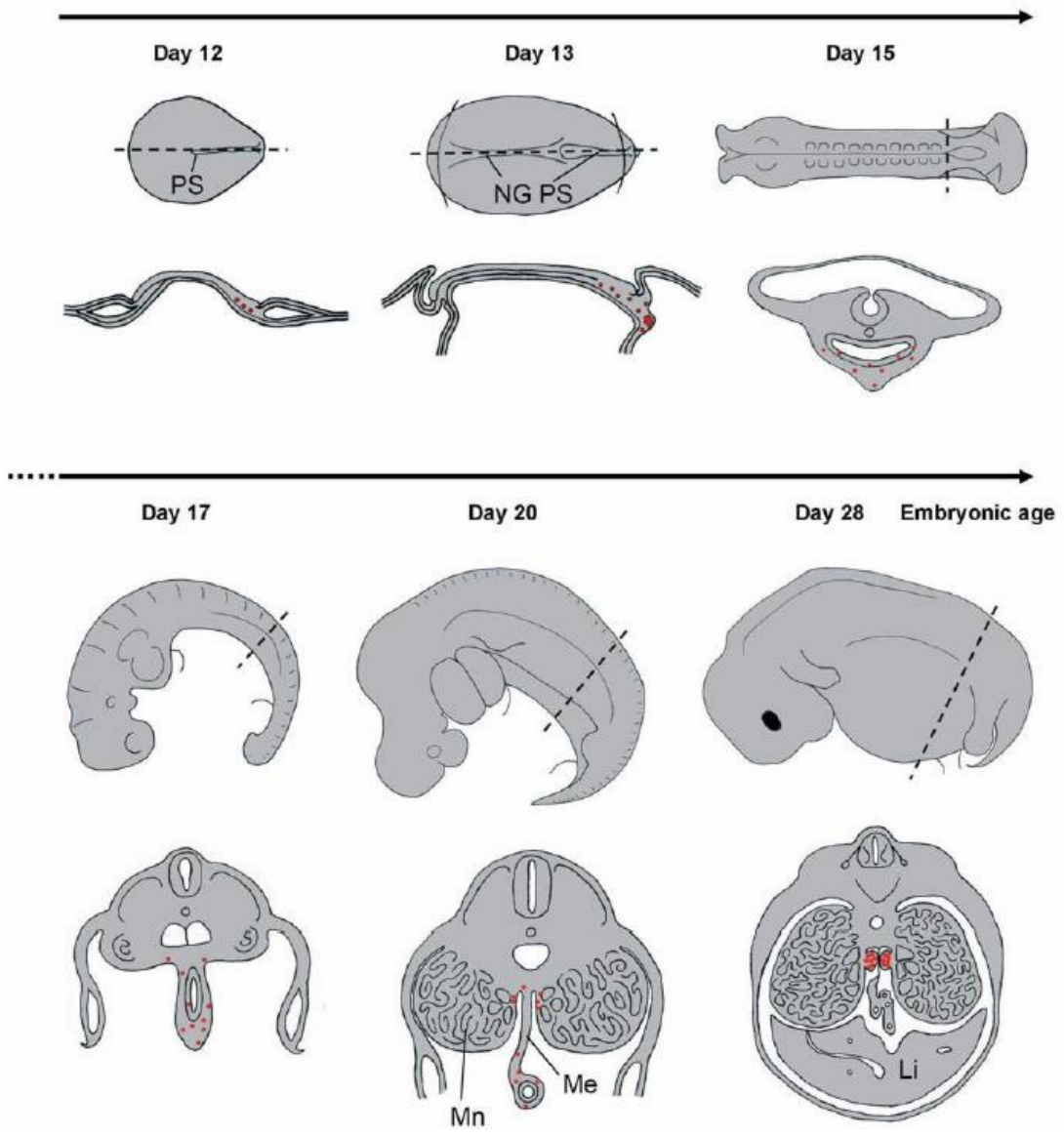

Figure 1.6 Schematic of the porcine germ line during early development. Sections of the embryo are presented below drawings of the whole embryo. Broken lines indicate the section site. Red dots represent PGCs. PS: primitive streak; NG: neural groove; Mn: mesonephros; Me: mesenterium; Li: liver. Figure reprinted from Hyttel, Kamstrup et al. (2011) with permission from Acta Scientiae Veterinariae.

Methylation Patterns of Porcine PGCs

As in the epiblast progenitor cells, newly specified PGCs exhibit high levels of DNA methylation. By the time they reach the genital ridge however, the DNA is largely hypomethylated, similarly to the mouse system. Demethylation of PGC DNA is initiated around E15, during the initial period of migration through the hind gut to the genital ridge (Hyldig, Croxall et al. 2011). Demethylation of imprinted genes in the pig is comparable to levels seen in the mouse, although it is not known whether the same genes in both mouse and pig have the same demethylation pattern. The demethylation process is completed by day 28-31, with remethylation occurring soon after (Byskov, Hoyer et al. 1986, Takagi, Talbot et al. 1997, Hyttel, Kamstrup et al. 2011). After establishing residency in the genital ridge, PGCs begin the differentiation program toward gametogenesis.

The Role of PRDM14 in Human PGCs

Recent studies regarding human PGC specification have demonstrated a diminished role for *PRDM14* in the human (h) germ cell program. PGC-like cells (termed PGCLCs) represent an important cell population for research, as obtaining native PGCs from humans is understandably difficult. PGCLCs have been differentiated from human ESCs, and in this population of cells *SOX17* is the earliest marker of the PGCLC lineage. Importantly, *SOX17* is upstream of *PRDM1*, and it works to repress somatic genes during *in vitro* hPGCLC specification (Irie, Weinberger et al. 2015). Human PGCLCs express lower levels (both transcript and protein) of *PRDM14* as compared to hESCs, indicating a reduced role for *PRDM14* in

human PGC specification (Kobayashi, Zhang et al. 2017). While it does retain low expression in PGCLCs and PGCs, *PRDM14* is only gradually expressed after *PRDM1* and *SOX17* upregulation, and is enriched in the cytoplasm in contrast to the nuclear localization in the mouse system (Irie, Weinberger et al. 2015).

The relationship of *PRDM14* with *SOX2* is also different between the mouse and human. In murine PGCs, *Sox2* is regulated by *Prdm14* whereas *SOX2* is repressed in human PGCLCs. This may be due to the divergence between the 2 proteins, potentially resulting in functional differences (Irie, Weinberger et al. 2015). Additionally, RNA sequencing and immunofluorescence studies have found that human PGCs express low levels of *PRDM14* and a knockdown of *PRDM14* had no effect on the ability for human ESCs to differentiate to PGCLCs (Gkountela, Zhang et al. 2015, Guo, Yan et al. 2015, Irie, Weinberger et al. 2015, Sugawa, Arauzo-Bravo et al. 2015, Tang, Dietmann et al. 2015, Gell, Zhao et al. 2018). Collectively, these recent data demonstrate a reduced role for *PRDM14* in the specification of PGCLCs following differentiation, and an increased role of other germline factors such as *PRDM1* and *SOX17*.

Summary and Objectives

While much is known about murine primordial germ cell specification, development, and signaling, very little is known about these topics in non-rodent models. It has only been in recent years that attention has turned toward determining this process in non-rodent "bridge" models such as pigs. Researchers have begun to characterize the expression profile of porcine PGCs during specification, as a first step in relating this process to humans. Because so little is known about this process

in pigs, it was the goal of this dissertation research to determine the genes and pathways that may be of importance for pig PGC specification. Specifically, to determine (1) what, if any, germ cell factors including *PRDM14* are expressed during preimplantation embryo development in the pig; and (2) the role, if any, of *PRDM14* in pig primordial germ cell specification by generating a functional knockout for this gene via gene editing technologies. In addition to these studies in the pig, this research sought to determine if the *Prdm14* knockout phenotype could be rescued in the mouse via blastocyst complementation, generating a way for pluripotent stem cells to efficiently contribute to chimeras. These projects together sought to increase understanding of the role of *PRDM14* in two commonly used animal models.

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Chapter 2: Direction of Pluripotent Cells Toward a Primordial Germ Cell Fate in Chimeric Mouse Embryos

Abstract

The generation of a genetically engineered line of animals requires modifications in the germline to be successfully transmitted to the next generation. However, aggregation of genetically modified pluripotent stem cells from another embryo or ESCs often results in a lack of occupation of the germline and a failure to contribute to the germline in the chimeric offspring. The overall objective of this study was to test the hypothesis that ablation of the endogenous germline in the host embryo will lead to exclusive occupation of the gonad and contribution by donor pluripotent cells (ESCs or embryonic cells) using the chimeric mouse as a model. To achieve this objective, the CRISPR/Cas9 system was used to target *Prdm14*, a gene required for PGC development. The Cas9 protein, alongside a guide RNA (sgRNA) targeting *Prdm14*, were injected into surrogate embryos, and were aggregated with a blastomere (or 10 ESCs in embryo/stem cell aggregations) from a GFP-expressing donor embryo and transferred to a pseudopregnant recipient for lineage tracing analysis. Because the homozygous mutant for *Prdm14* has previously been shown to be sterile and completely devoid of PGCs, any functioning PGCs, and later germ cells, in the resulting mice were expected to be of donor origin. This technique resulted in offspring chimeric for GFP and produced gametes arising solely from the donor-derived R1 cells, as determined by mating founder animals with wild-type partners and analyzing the offspring. The gene-edited ESC line, $Cwc15^{+/-}$ did not

result in chimeras that had ESC contribution to the germline. In the future, other gene-targeted embryonic stem cell lines which have previously failed to contribute to the germline in the chimeric offspring should be tested using this methodology.

Introduction

Validation of iPSCs and ESCs as fully pluripotent requires a multitude of *in vitro* and *in vivo* assays to confirm that the stem cells contribute to all germ layer lineages, including germ cells. *In vitro* assays generally require differentiation of the stem cells into cell types representing the three germ layers, as confirmed by transcript and protein data. *In vivo* assays are more stringent, as they rely on physiological signals from the animal itself to drive differentiation. For human stem cells, the most rigorous assay is the generation of teratomas: a procedure wherein cells are injected into immunocompromised mice and are allowed to grow for several weeks (International Stem Cell Banking 2009, Nelakanti, Kooreman et al. 2015). After this time, the tumor is excised and analyzed to determine if there are cell populations within the teratoma that represent cell types from all three germ layers, using morphological and immunohistochemical means.

In characterizing stem cells from species other than humans, there are two additional embryonic tests to determine their potency: chimera formation and tetraploid complementation. In order to generate chimeric animals, stem cells are injected into a blastocyst, transferred into a surrogate, and allowed to develop to term (Daniel and Anderson 1978). The resulting offspring are assessed for the contribution of stem cells to the whole animal. Most often, this is easily done by coat-color

chimerism using two different coat colors. For example, the host embryo could be from a white mouse while the injected stem cells are from a black/agouti mouse, giving the offspring a patchy or mottled black/gray and white coat. These chimeras are then mated to white mice to determine if the stem cells contributed to the germline of the chimeric individual. If the stem cells did contribute to the germline, the resulting litter should contain one or more black mice.

Tetraploid complementation is the most rigorous assay for determining the developmental potential of a population of stem cells in the mouse. Tetraploid cells are able to contribute to the extraembryonic membranes, but are unable to develop into a fetus. In this assay, cells from a two-cell stage embryo are fused together, generating a tetraploid cell (Tarkowski, Witkowska et al. 1977). This cell has the ability to divide normally until the blastocyst stage. As in the case of chimeras, stem cells are then injected into the blastocyst. Since the tetraploid cells are unable to contribute to the fetus proper, the fetus is entirely composed of cells from the injected stem cell population.

Most often, stem cell research uses the generation of chimeric animals as the standard for assessing pluripotency, and also as a first step in generating a knockout or gene-edited mouse line. Due to the short generation interval of mice, it is relatively easy to use a stem cell intermediate to generate animals with edits to their genomic sequence, because a targeted homozygous individual can be created in as little as four months (Singh, Schimenti et al. 2015). For simple edits, the CRISPR/Cas9 system can be used to generate knockouts or knock-ins, without the need for stem cells and the use of chimeras at all.

The ease of this process of generating chimeras has led to a global effort to generate knockout cell lines for thousands of genes, and subsequently characterize the resulting phenotype in the mouse. Recently, an organization known as the International Knockout Mouse Consortium (IKMC) has spearheaded a project that allows investigators to collaborate on generating a bank of mESC lines that are targeted for knockout or conditional knockout. As part of this organization, it is the goal of the Knockout Mouse Project (KOMP) to establish a knockout ESC line for each gene in the mouse genome, greatly easing the ability to generate mice with more complex genotypes and increasing access to these ESC lines through a multinational collaborative effort. In addition to the KOMP, IKMC also oversees the European Conditional Mouse Mutagenesis program (EUCOMM), which is a similar collaboration that focuses on conditional gene trapping and gene targeting (Bradley, Anastassiadis et al. 2012).

Since the KOMP was established, investigators around the world working with ESCs have been using these validated cell lines in their own experiments, albeit with varying degrees of success. Both published and anecdotal evidence have demonstrated that germline transmission of validated alleles is highly variable and can be problematic (Pacholczyk, Suhag et al. 2008). For example, one group using purchased EUCOMM cells produced mice carrying germline transmission of the knockout allele only 1.4% of the time (5/354 pups) (Coleman, Brennan et al. 2015). The germline transmission rate may also vary by ESC genetic background, producing variable rates up to 95% as determined by another study but in general aggregate

germline transmission rates are around 50% across all lines, with fewer showing the correct knockout allele (Cotton, Meilak et al. 2015).

Germline transmission of stem cells and their associated mutated alleles is of paramount importance. In routine culture, ESCs from the C57BL/6J background tend to be difficult to maintain and are more likely to become aneuploid than cells from other genetic backgrounds, such as 129S5 (Hughes, Qu et al. 2007, Gertsenstein, Nutter et al. 2010). In addition to the potential pluripotency of stem cells, the chromosomal makeup of the ESC population has been shown to contribute to the success or failure of chimera production, the degree of chimerism, and the potential for the ESCs to contribute to the germline (Longo, Bygrave et al. 1997). A higher percentage of euploid cells in the ESC population results in chimeras with larger degrees of chimerism and successful germline transmission. This suggests that the inability for ESCs to go germline may more likely be explained by chromosomal abnormalities rather than a loss of pluripotency (Longo, Bygrave et al. 1997, Suzuki, Kamada et al. 1997, Carstea, Pirity et al. 2009).

One example of an aneuploidy event is trisomy 8; these cells tend to grow faster than euploid ESCs in culture and do not generate chimeras that show germline transmission (Liu, Wu et al. 1997, Liu, Zhang et al. 1998). As a result of these common chromosomal abnormalities, most labs purchasing ESC lines from KOMP or EUCOMM routinely buy multiple clones containing the same mutation and subject them to karyotyping to determine percent aneuploidy. Each lab determines a minimum threshold of euploidy, below which they will not use that ESC line for chimera production.

Due to the difficulties in generating mouse chimeras that show germline expression from otherwise validated ESC lines, this project sought to establish a method which would guarantee germline transmission in the first generation by taking advantage of the PGC specification pathway. In mice, PGCs originate in a subset of mesodermal precursors in the epiblast stage embryo via BMP signaling and induction of a tripartite signaling network led by *Prdm1*, *Prdm14*, and *Tfap2c* (Gunesdogan, Magnusdottir et al. 2014). *Prdm1* is the first gene expressed at E6.25 and is followed shortly by *Prdm14* and *Tfap2c* (Saitou and Yamaji 2012). These factors are responsible for genome-wide epigenetic reprogramming, re-acquisition of pluripotency, and repression of the somatic program in the PGC subset of cells within the mesoderm (Yamaji, Seki et al. 2008). Among these genes, *Prdm14* has been shown to be an essential transcription factor for PGC specification. *Prdm14* also has a critical role in maintaining naïve pluripotency and establishment of the epigenome in mESCs (Nakaki and Saitou 2014). Importantly, ablation of *Prdm14* is not embryonic lethal and results in the loss of PGCs in mouse models while still maintaining an otherwise wild-type phenotype (Yamaji, Seki et al. 2008).

The approach for this project took advantage of the principles for lineage specification; that is, directing a multipotent cell toward a specific cell fate. For this objective, it was necessary to direct the ESCs toward a PGC fate so that they would be fully incorporated into the germline. This would overcome one of the problem areas discovered by users of and contributors to the KOMP, allowing for higher rates than usual of germline transmission by validated pluripotent ESCs. This technique also has potential applications for other organ and cell culture systems, as it may be

useful to direct stem cells down a differentiation pathway prior to transplantation to a recipient, for example. For this project, it was hypothesized that by preventing the wild-type embryo from entering the PGC lineage by knocking out the function of *Prdm14*, the ESCs would have uninhibited access to fill the germ cell niche, establishing a chimera that has exclusively donor-derived germ cells.

This project utilized two approaches in order to test this hypothesis: embryo-embryo aggregation and blastocyst complementation of stem cells into embryos. In embryo-embryo aggregation, one blastomere of a 4-cell stage donor embryo was injected into a 4-cell host embryo of a different strain background. In blastocyst complementation, donor stem cells were injected into the host embryo at blastocyst stage, as is the usual technique for generating chimeras. The resulting animals were then mated to determine contribution of donor cells to the host animal's germline.

Materials and Methods

Generation of Prdm14 chimeric mice via embryo-embryo aggregation

C57BL/6J (referred to as wildtype (WT); The Jackson Laboratory, Bar Harbor, ME) females were superovulated using intraperitoneal injections of 7.5 IU PMSG (pregnant mare's serum gonadotropin; Sigma, St. Louis, MO) followed by 7.5 IU hCG (human chorionic gonadotropin; Sigma, St. Louis, MO) 46 hours later. WT embryos were collected 10-12 hours post mating with WT males using KSOMaa (potassium simplex optimization medium containing amino acids; Zenith Biotech, Guilford, CT) Evolve media containing HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Zenith Biotech, Guilford, CT).

Zygotes from WT females were moved to FHM handling media (modified KSOM, EMD Millipore; Billerica, MA) and microinjected with a CRISPR guide RNA targeting exon 1 of the *Prdm14* (Figure 2.1) gene using 25 ng of Cas9 protein (PNA Bio; Thousand Oaks, CA) and 12.5 ng of guide RNA transcribed in vitro (Ambion MEGAshortscript T7; Austin, TX) and cultured at 37°C in KSOMaa Evolve (Zenith Biotech; Guilford CT) under 5% oxygen and 5% carbon dioxide.

Figure 2.1 Diagram of the structure of murine *Prdm14* and guide RNA target site. Gray boxes: non-coding regions; Green boxes: open reading frame.

Embryos from matings between C57BL/6J-GFP (GFP: green fluorescent protein; The Jackson Laboratory, Bar Harbor, ME) males and females were collected on E1.5 at the 2-cell stage. At 2 days post-coitum (dpc), the zona pellucida was removed from GFP embryos and the four blastomeres were separated using a combination of acidic Tyrode's solution (Sigma; St. Louis, MO) and gentle pipetting. One blastomere from the GFP embryo was injected into the 4-cell stage *Prdm14* CRISPR-injected WT embryo. Reconstituted embryos were incubated in phytohemagglutinin PHA-L (Sigma; St. Louis, MO) for 20 minutes to facilitate blastomere aggregation and returned to culture. Embryos were cultured overnight and

transferred into the oviduct of day 0.5 pseudopregnant CD1 (Charles River Laboratories; Frederick, MD) females as described below and allowed to go to term.

Generation of Prdm14 chimeric mice via blastocyst complementation (stem cell injection)

C57BL/6J-GFP females were superovulated using intraperitoneal injections of 7.5 IU PMSG followed by 7.5 IU hCG 48 hours later. GFP cumulus-oocyte complexes were collected 14-16 hours post-hCG and placed into a 200 µL *in vitro* fertilization (IVF) drop of high calcium HTF medium (human tubal fluid; Table 2.1) containing 0.25 mM reduced glutathione (Sigma; St. Louis, MO). IVF was chosen in order to generate a higher percentage of zygotes as compared to natural matings.

Cauda epididymides from ≥ 8-week old C57BL/6J-GFP males were dissected and gently squeezed to release spermatozoa from the epididymides. Spermatozoa were incubated in TYH (modified Kregs-Ringer bicarbonate medium) containing MBCD (methyl-β-cyclodextrin; Table 2.2) at 37°C under 5% oxygen and 5% carbon dioxide. After 1 hour of incubation, 3-5 µL of sperm from the edge of the medium drop were collected and transferred to the IVF drops containing the fresh cumulus-oocyte complexes. Fertilization dishes were then incubated at 37°C under 5% oxygen and 5% carbon dioxide for 3.25-4 hours.

Presumptive zygotes were microinjected with a CRISPR guide targeting exon 1 of the *Prdm14* gene using 25 ng of Cas9 protein (PNA Bio; Thousand Oaks, CA) and 12.5 ng of guide RNA and cultured at 37°C in KSOMaa Evolve (Zenith Biotech; Guilford, CT) under 5% oxygen and 5% carbon dioxide until blastocyst stage 4 days later. At blastocyst stage, 10-12 embryonic stem cells (R1 control or $Cwc15^{-/-}$ AD7

clone experimental cell line) were injected into the blastocoele of each blastocyst. These blastocysts were transferred into the uterus of day 2.5 pseudopregnant CD1 (Charles River Laboratories; Frederick, MD) females and pregnancies were allowed to go to term.

Table 2.1 Composition of high calcium HTF medium

Reagent Name	mg/100 ml
NaCl	593.8
KCl	35.0
$MgSO_4 \cdot 7H_2O$	4.9
KH_2PO_4	5.4
$CaCl_2 \cdot 2H_2O$	75.5
$NaHCO_3$	210.0
Glucose	50.0
Na-lactate (ml)	0.34
Na-Pyruvate	3.7
Penicillin G	7.5
Streptomycin	5.0
BSA (Fraction V, Fatty Acid-Free)	400.0
Phenol Red (0.5% solution)	0.04 (ml)

Table 2.2 Composition of sperm incubation medium (TYH + MBCD)

Reagent Name	mg/100 ml
NaCl	697.6
KCl	35.6
$MgSO_4 \cdot 7H_2O$	29.3
KH_2PO_4	16.2
$NaHCO_3$	210.6
Na-Pyruvate	5.5
Glucose	100.0
$CaCl_2 \cdot 2H_2O$	25.1
Methyl-β-cyclodextrin	98.3
Penicillin G	7.5
Streptomycin	5.0
Polyvinylalcohol	100.0

Stem cell culture

Mouse stem cells for blastocyst injection were cultured in a standard embryonic stem cell culture consisting of 80% DMEM/F-12 (Dulbecco's Modified Eagle Medium; Gibco, Grand Island, NY), 20% fetal calf serum (Atlanta Biologicals; Flowery Branch, GA), 2 mM L-alanyl-L-glutamine dipeptide (Gibco; Grand Island, NY), 0.1 mM non-essential amino acids (Gibco; Grand Island, NY) 1 mM sodium pyruvate (HyClone; Pittsburgh, PA), 0.02 mM β-mercaptoethanol (Gibco; Grand Island, NY), and 1000 U/ml LIF (Leukemia Inhibitory Factor; EMD Millipore, Billerica, MA). Stem cells were passaged every 2-3 days using 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid; Gibco, Grand Island, NY).

Embryo transfer into recipient females

Embryo transfer was performed on either day 0.5 or day 2.5 pseudopregnant CD1 (Charles River Laboratories; Frederick, MD) females, depending on the stage of embryo development. Embryos up to morula stage were transferred into the oviduct of day 0.5 pseudopregnant females and embryos at blastocyst stage were transferred into the uterine horn of day 2.5 pseudopregnant females.

Recipients were weighed using a scale (Sartorius BP610, Sigma; St. Louis, MO) to determine dosage for buprenorphine analgesic (Par Pharmaceuticals; Spring Valley, NY). Buprenorphine was administered subcutaneously at 0.1 mg/kg of body weight. The animal was then moved to the induction chamber of the SomnoSuite Small Animal Anesthesia System (Kent Scientific; Torrington, CT) and induced at a flow rate of 250 mL/min and a concentration of 3.0% isoflurane (VetOne; Boise,

Idaho) until the mouse was completely limp. After induction, the mouse was moved to a warming plate and a nose cone placed over its nose. The flow rate was reduced to 200 mL/min with a concentration of 2.0-2.2% isoflurane for the remainder of the procedure. The area just below the distal end of the rib cage down to the top of the knee was shaved on either side of the mouse. The shaved area extended from the dorsal-ventral boundary to the spine. The shaved surgical area was then treated with betadine using a circular scrubbing motion from the central area to the outer edge, and then rinsed with 70% ethanol in the same manner. Eye gel (CLC Medica; Waterdown, ON, Canada) was placed onto the eyes of the animal to reduce drying during the procedure.

Using a stereomicroscope, an initial incision was made roughly 1/3 of the way distally from the ribcage and 1/3 of the way ventrally from the spine. A second incision through the fat and muscle layer was made and the ovarian fat pad located. The ovary and cranial end of the uterine horn was pulled outside the body cavity and placed onto sterile gauze. For oviductal transfer, a small cut was made to the oviduct cranial to the swollen ampulla and 10-20 embryos were transferred using a glass pipette, as well as an air bubble to prevent backflow of embryos out of the oviduct. For uterine transfer, a hole was made in the proximal end of the uterine horn using a 26-gauge needle and 10-15 blastocyst transferred. Once the transfer was complete, the reproductive tract was guided back into the body cavity. The muscle incision was closed using one Halsted suture (Ethicon; Cincinnati, OH) and skin stapled (Roboz; Gaithersburg, MD).

After surgery, mice were placed in a new cage that was pre-warmed at 37°C until the animal recovered and moved freely. Staples were removed after 10 days and the mice were weighed to determine pregnancy status.

Caesarian section of surrogate mothers and cross-fostering to foster mothers

The number of offspring produced by the blastocyst complementation technique was low (see Results), and the pups were often cannibalized by the dam as a result. In order to combat this outcome, Caesarian sections (C-sections) were performed to allow for cross-fostering to mothers with larger litters. First, the mother was sacrificed in the afternoon prior to the morning she was due to give birth. As quickly as possible, the reproductive tract was exposed, excised from the mouse, and moved to a warming plate. Next, the uterine horns were dissected open, exposing the placental membranes of each fetus. The membranes were opened using a fine pair of forceps, and pups removed from the amniotic sac. The pups were stimulated to take their first breath by rubbing a cotton swab near their mouths. The umbilical cord was then clamped with hemostats for 1-2 minutes, and cut close to the pup's body. The pups remained on the warming plate for 10 minutes until they became pink and rosy in color.

After the pups gained sufficient pink color, they were cross-fostered to a CD1 female who had given birth the morning of the same day by mixing in the delivered pups with her own pups. Usually 3-4 pups were cross-fostered and the foster mother's litter size reduced to 8-9 pups to ensure she produced enough milk for all pups. The litters were monitored for 10-15 minutes before being returned to the cage rack.

Production of offspring from chimeric animals

To confirm the phenotype of founder chimeras, all founder mice were grown to puberty (5-8 weeks) and then bred with WT animals (in case of embryo-embryo aggregation founders) or with GFP animals (in case of blastocyst complementation founders). These pregnancies were taken to term to generate F1 progeny. The resulting offspring were analyzed for GFP expression. All GFP animals were photographed using a 500 nm emission filter with the NIGHTSEA™ dual fluorescent protein excitation light (Electron Microscopy Sciences; Hatfield, PA) 2-5 days after birth, and WT pups of the same age were photographed as controls under the same lighting conditions. Where time did not permit pregnancies to go to term, females were sacrificed at E1.5 and 2-cell embryos were flushed from the oviducts and cultured in KSOMaa Evolve (Zenith Biotech; Guilford, CT) at 37°C under 5% oxygen and 5% carbon dioxide until blastocyst stage when they were imaged under a microscope to determine GFP status.

Results

Aggregation of GFP blastomere with PRDM14$^{-/-}$ embryos can generate chimeric mice

Blastomeres from GFP embryos were aggregated with putative *Prdm14*$^{-/-}$ mouse embryos. Preliminary experiments were performed to confirm the ability of the guide RNA to generate edits to the gene. Prior to performing an embryo transfer, embryos were cultured to the blastocyst stage and analyzed for GFP expression. Every embryo showed chimeric GFP expression, indicating that the aggregation was

successful and the embryos were capable of developing to the blastocyst stage (Figure 2.2).

For embryo transfer, the aggregated embryos were transferred the day after aggregation (8-cell to morula stage) to 0.5 dpc pseudopregnant females and pups were born approximately 20 days later. Fourteen embryo transfers were performed, with 8 resulting in pregnancies. Of those 8 pregnancies, only 2 resulted in offspring that survived until weaning, resulting in 9 animals. Upon birth, animals were analyzed for GFP expression. From two litters 2 true chimeras were generated, demonstrating that the aggregation was successful due to the appearance of patchy expression of GFP on the individual (Table 2.3; Figure 2.3). Seven other animals were generated that were characterized as 100% GFP-expressing, suggesting that the donor blastomere was responsible for giving rise to a majority of the mouse's cells, as seen from GFP expression externally.

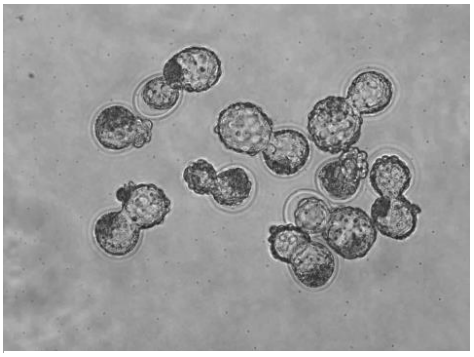
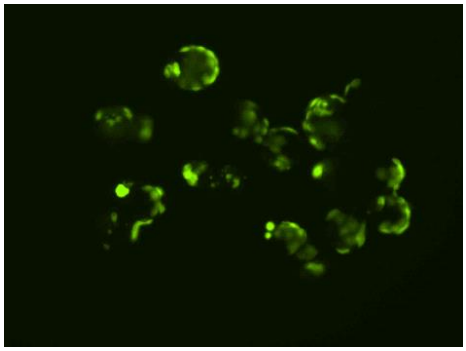
A  B 

Figure 2.2 Chimeric blastocysts were generated after embryo aggregation. A) Bright-field image; B) GFP image.

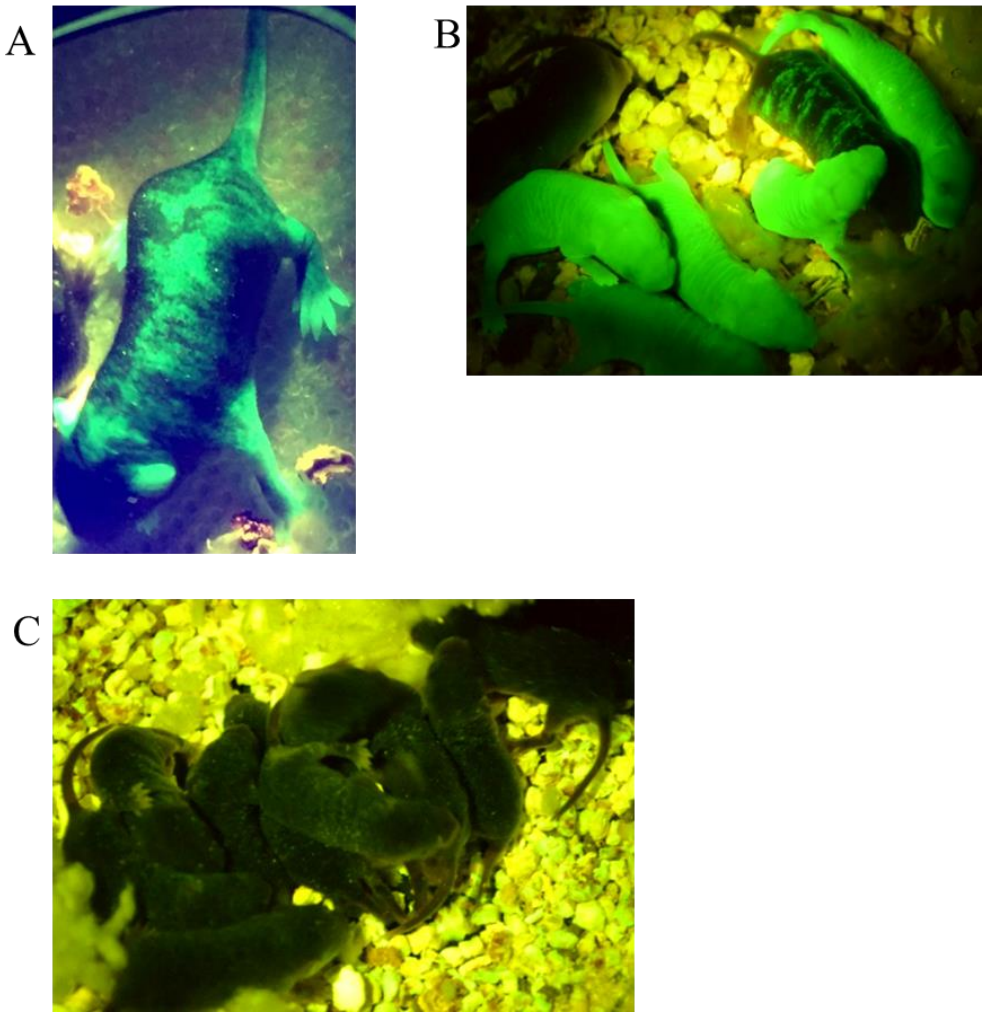


Figure 2.3 Chimeric founder (F_0) pups born from embryo-embryo aggregations. A) Chimeric pup from replicate 1; B) Chimeric pup from replicate 2; C) Wild-type, age-matched control pups.

Table 2.3 Summary of generation of chimeric founders (F_0) from *Prdm14*$^{-/-}$ and GFP embryo aggregations.

Replicate	# Chimeras	Litter Size	Percentage Chimeric
1	1	7	14.29%
2	1	9	11.11%
Total	**2**	**16**	**12.50%**

Founder chimeric individuals of embryo-embryo aggregations have germline originating from donor blastomeres

The founder animals from embryo-embryo aggregations were then raised until puberty (5-8 weeks), when they were mated with WT individuals. This mating was performed to determine if the germ cells of the founder animals arose completely from the GFP donor blastomere as expected. If the germ cells were all generated from the GFP donor embryo, then after mating, subsequent offspring should all be GFP positive, indicating 100% occupation of the germline by the GFP embryo donor lineage. Each founder individual (including those founders assessed to be 100% GFP) was mated to produce at least 1 litter for analysis. Across all litters, every F1 pup born was externally 100% GFP positive, indicating that all germ cells from the chimeric parent were of donor origin. The results of each litter can be found in Table 2.4.

Blastocyst complementation generates chimeras from R1 ESCs

Using this technique, 10 embryo transfers were performed, all of which resulted in pregnancies generated from embryos that were injected with the robust R1 control stem cell line. However, after delivery by the surrogate recipient mother, only 5 animals survived until weaning age. Of those 5 animals, 3 were chimeric founders. In this experiment, the host embryo was GFP-expressing while the donor stem cells were not. This was the opposite expression pattern as the previous embryo-embryo aggregation experiment. However, founder animals still retained the classic look of a chimera, with a patchy coat including GFP positive and GFP negative areas (Figure 2.4).

Table 2.4 Summary of GFP offspring from founder chimeric individuals (F_1).

Parent ID	% Chimerism	# GFP Pups	# Total Pups	Percentage
1	50	16	16	100
2	>90	19	19	100
3	>90	8	8	100
4	>90	8	8	100
5	>90	9	9	100
6	>90	8	8	100

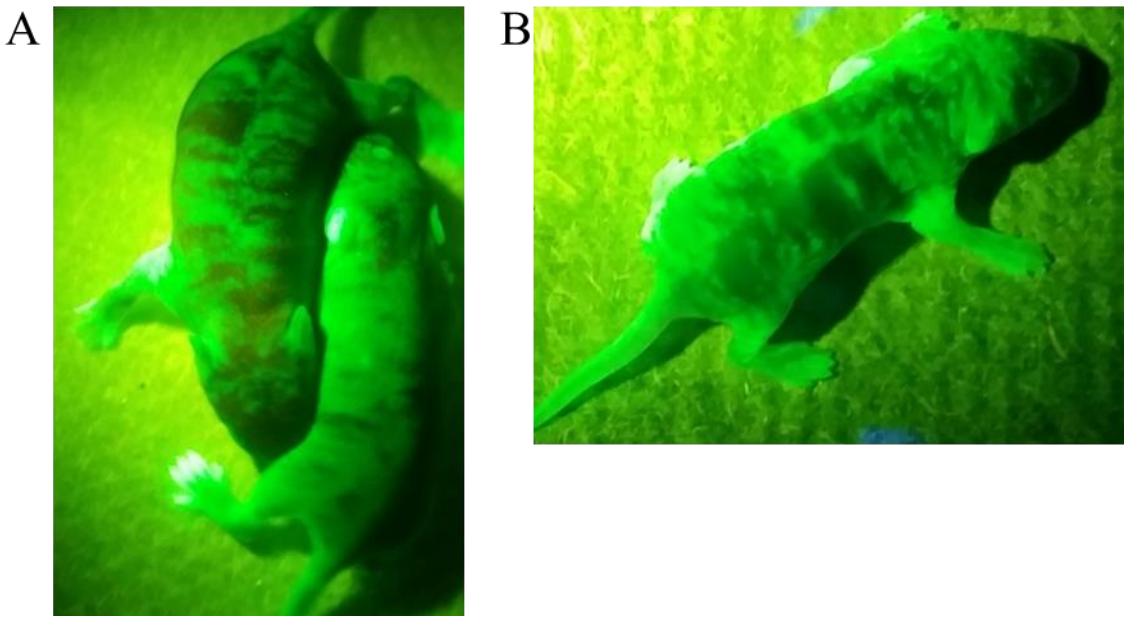


Figure 2.4 Chimeric founder (F_0) pups born from blastocyst complementation with R1 cells. A) Chimeric pups from replicate 1; B) Chimeric pup from replicate 2.

R1 founder chimeras have germline originating from ESC background

The founder animals from blastocyst complementations using R1 control ESCs were raised to puberty and then mated to WT mice. In this experiment, the donor R1 stem cells did not express GFP but were injected into a host embryo that did express GFP. Therefore, if the R1 stem cells contributed to the germ cell lineage, they would not express GFP, and resulting offspring from mating to a WT individual should also be GFP negative. Each founder animal was mated to produce at least 3 litters. Each litter produced by a founder animal consisted of pups that were all GFP-negative, indicating that the germ cells from their founder parent were 100% from R1 stem cell origin (Table 2.5; Figure 2.5).

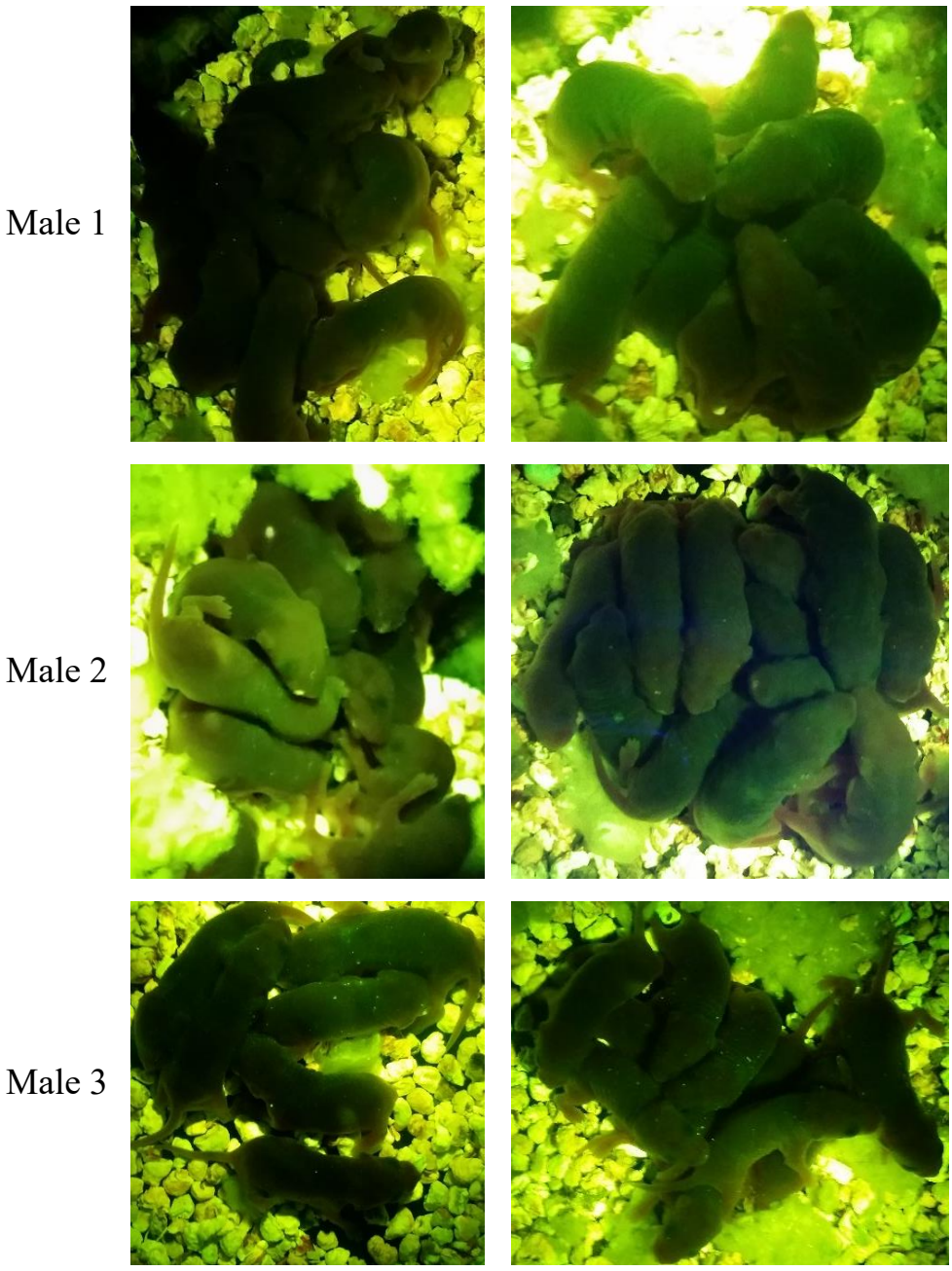


Figure 2.5 Two representative F_1 litters generated from each of the R1 chimera founder males. Lack of GFP expression indicates germline occupied solely by ESC background.

Table 2.5 Summary of mating results of founder R1 chimeras with WT females.

Mouse ID	Sex	Mate	Litter Birthdate	Litter Size	Germline Origin
1R1	M	C57BL/6J	7/11/2017	10	R1 ESC
		C57BL/6J	8/4/2017	9	
		CD1	8/9/2017	8	
		CD1	9/24/2017	7	
2R1	M	C57BL/6J	7/10/2017	11	R1 ESC
		C57BL/6J	8/3/2017	5	
		CD1	8/8/2017	3	
		C57BL/6J	8/28/2017	11	
3R1	M	C57BL/6J	9/19/2017	10	R1 ESC
		C57BL/6J	9/26/2017	6	

$Cwc15^{+/-}$ founder animals show lack of chimerism and $Cwc15^{+/-}$ ESCs do not contribute to the germline

Blastocyst complementation was also performed using ESCs that were mutant for *Cwc15* (*$Cwc15^{+/-}$*). *Cwc15* is a gene that is responsible for activation of pre-mRNA splicing and this stem cell line had been in use for a different experiment in our laboratory. This stem cell line was chosen because previous experience in our lab with this line generated chimeras quite easily but none of these were germline chimeras. It was therefore decided that the *$Cwc15^{+/-}$* line would be an ideal candidate to determine if previously germline-incompetent ESCs could be pushed toward occupation of the germline once endogenous PGC competition was removed. Using *$Cwc15^{+/-}$* ESCs as donor cells, 16 embryo transfers were performed, resulting in 11 pregnancies. From these 11 pregnancies, only 5 pups survived past weaning age. Unfortunately, the blastocyst complementation approach yielded only 1 visible chimera, which died at 12 weeks of age prior to siring a litter. The other founder animals showed low contribution by ESCs, and did not yield any visible chimeras. Instead, all pups produced from this experiment were almost entirely GFP positive, indicating that the offspring were derived solely from the host embryo, with little to no contribution by the *$Cwc15^{+/-}$* ESCs. A total of 5 founder animals were produced, with only 2 showing any sign of contribution by the stem cells. Two of the limited chimeric animals however were phenotypically female, indicating that the ESCs did not contribute to their reproductive systems, as the ESCs were karyotypically XY, and therefore should only give rise to male offspring if incorporated into the reproductive tract during development. Images of the founder animals and breeding data can be found in Figure 2.6 and Table 2.6, respectively.

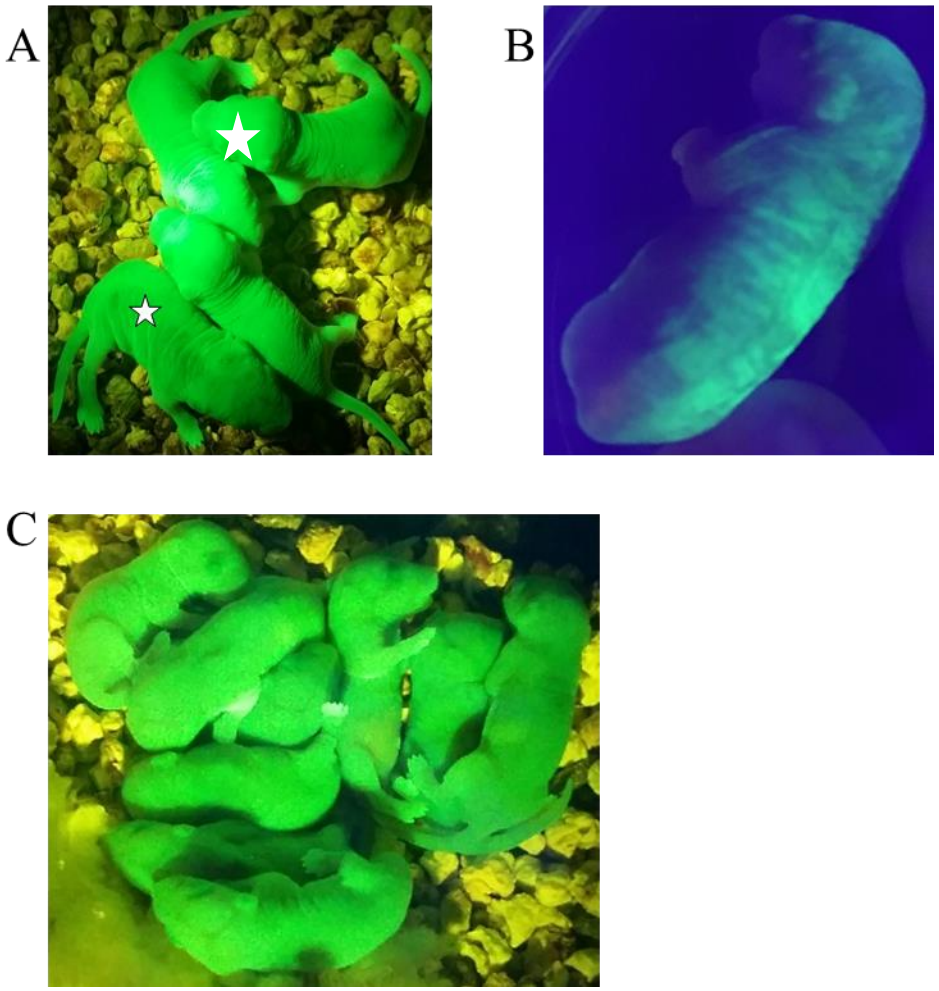


Figure 2.6 Founder (F_0) pups born from blastocyst complementation with *Cwc15*$^{+/-}$ cells. A) Pups from replicate 1; B) Pup from replicate 2. C) Representative F_1 pups from mating of F_0 to wild-type partners. A star indicates the founder chimera that had low levels of chimerism (<5%).

Table 2.6 Summary of mating results of founder *Cwc15*$^{+/-}$ chimeras with WT mates. An asterisk indicates the one visible chimera that died at 12 weeks of age.

Mouse ID	Sex	Mate	Litter Birthdate	Litter Size	Germline Origin
1CWC	F	C57BL/6J	10/6/17	3	Host Embryo
			11/16/17	4	Host Embryo
2CWC	M	C57BL/6J	12/2/17	7	Host Embryo
3CWC	M	C57BL/6J	10/17/17	8	Host Embryo
			11/19/17	8	Host Embryo
4CWC	F	C57BL/6J	10/2/2017	9	Host Embryo
			11/19/17	4	Host Embryo
5CWC*	M	C57BL/6J	None sired	None sired	N/A

Discussion

In this study, we sought to determine if ESCs could be preferentially biased toward a germline fate by eliminating endogenous PGCs in the host embryo. To accomplish this, *Prdm14* was knocked out in a host embryo that was then aggregated with either another WT embryo or with ESCs. Previously, our lab obtained results from a chimera experiment in which it was possible to generate chimeras from the *Cwc15*$^{+/-}$ ESC line (personal data, unpublished). However, none of the chimeras produced showed germline transmission, as offspring from these chimeras were all wild-type. This led to an important experimental question: were the stem cells simply unable to contribute to the germline and therefore not fully competent, or were the stem cells being outcompeted by the endogenous germ cell program? As evidenced by this study, the answer may comprise a bit of both explanations.

It is already known that stem cells are able to rescue a knockout phenotype when introduced into a mutant blastocyst. In fact, this has been reported numerous times with researchers targeting genes important for whole organ generation, such as *Pdx1* for the pancreas and *Nkx2.5* for the heart. So far, blastocyst complementation (the process of injecting stem cells into a genetically modified embryo) has been used in the mouse to rescue the function of *Runx2* (Chubb, Oh et al. 2017), *Nkx2.5* (Sturzu, Rajarajan et al. 2015), *Pou5f1* (Le Bin, Munoz-Descalzo et al. 2014), *Sall1* (Usui, Kobayashi et al. 2012), *Rag2* (Chen, Lansford et al. 1993), *Pdx1* (Kobayashi, Yamaguchi et al. 2010, Kobayashi, Kato-Itoh et al. 2015), and *Id1/2/3* (Fraidenraich, Stillwell et al. 2004), among countless others. However, blastocyst complementation and the rescue of the phenotype has not been shown in the reproductive system to

date. This study is the first step in generating a rescue phenotype for primordial germ cells via blastocyst complementation.

In addition to providing evidence that ESCs can rescue a knockout PGC phenotype, this study provides a foundation for generating chimeras from ESCs that were previously unable to show germline transmission. While the robust R1 ESC line generated chimeras easily, the *Cwc15*$^{+/-}$ line did not, which was unexpected as chimeras had been previously produced by our group. This may be explained by a change in experimental methods between the two attempts. Previously, our chimeras were generated by collection of embryos at the blastocyst stage, injection of 10-12 stem cells into the blastocoele, and immediate transfer back into the surrogate mother. Due to the nature of this experiment and the need for CRISPR/Cas9 injection, embryos were cultured from zygote stage to blastocyst stage, a time spanning 4 days. After injection of ESCs into the blastocyst, embryos were allowed to recover for 1-2 hours. This combination of experimental conditions could explain the decrease in developmental potential of the whole embryos. The use of blastocyst complementation may also explain the low derivation of pups, as the embryos were manipulated twice.

In addition to not generating chimeras efficiently, of those that were produced, the degree of chimerism was quite low and there was little to no contribution by the *Cwc15*$^{+/-}$ ESCs to the whole animal. Despite the low degree of chimerism seen externally, it was still possible however unlikely that the germ cell population was generated by the *Cwc15*$^{+/-}$ cells so the founder animals were mated to determine the status of the germ cell lineage. As expected, offspring generated from these matings

were entirely GFP positive, indicating a lack of contribution to the germline by the *Cwc15*$^{+/-}$ ESCs, as in this chimera experiment the host embryo had GFP expression while the donor ESCs did not.

The ability for these non-chimeric founder animals to produce litters is unexpected, because the germ cells would need to come from cells that still retained a functional *Prdm14* allele. The lack of contribution to the offspring by the *Cwc15*$^{+/-}$ ESCs suggests that the origin of the germ cells was from the host embryo that was CRISPR-injected. However, if the CRISPR was able to target both alleles of the zygote, the animals should have been sterile because the function of *Prdm14* would have been interrupted. The surprising result that these animals were able to produce litters suggests that the guide RNA and Cas9 complex were not able to completely target and interrupt the function of the endogenous *Prdm14* locus in these animals.

Despite the lack of contribution to the germline by the *Cwc15*$^{+/-}$ESCs, it may still be possible to use this technique to generate germline competent chimeras. Additional ESC lines should be tested to generate animals with a higher degree of chimerism than were produced in this study. Producing founders with a high degree of chimerism is a critical first step in determining if ESCs can contribute to the germline. Without that criterion met, this study did not have a good baseline from which to determine germline competence of the *Cwc15*$^{+/-}$ ESCs.

The inability to produce appropriate numbers of chimeras may not entirely be explained by the cell line chosen for use. Because the C-section technique was a new technique to the laboratory used for the first time in this experiment, the number of surviving pups was low. Foster mothers were often unaccepting of the cross-fostered

pups, and further practice and improvements to this technique would likely result in an increased number of founder animals produced by blastocyst complementation. Additionally, improved embryo transfer technique would also be expected to increase the number of founders produced (and perhaps the decrease the mortality rate), as no more than 4 pups were ever produced from 1 transfer, and it is known that small litter sizes encourage cannibalism.

The present chimeric mouse study in which embryonic stem cells were directed toward the germ cell lineage provides another approach to generating germline chimeras. The mouse ESC field has been plagued with cells that are either developmentally incompetent for PGCs or are outcompeted by the endogenous PGCs. This study provides a useful mechanism to overcome the germline transmission barrier so that the field can continue to move forward and characterize the function of genes, the modeling of human disease, and the biology of reproduction.

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Chapter 3: Expression of Germline-specific Genes in the Early Porcine Embryo

Abstract

Cells of the mammalian germline share some characteristics with cells of the early totipotent embryo. Mammalian germ cells originate *de novo* from mesodermal progenitors in the primitive streak of the gastrulation stage embryo. The primordial germ cells (PGCs) upregulate key pluripotency genes that are also expressed in the early embryo. In the mouse model, *Prdm14* and *Tfap2c*, which are critical for PGC development, are also expressed in the preimplantation embryo. However, little is known about the expression patterns of these germ cell-related genes in the early porcine embryo. In this study, temporal expression of select pluripotency, epigenetic regulators, and PGC genes: *SOX2*, *POU5F1*, *NANOG*, *ESRRB*, *CARM1*, *TET1*, *TET2*, *PRDM14*, *PRDM1*, *TFAP2C*, *DAZL*, *VASA*, and *STRA8* were investigated. Embryos were collected, from artificially inseminated gilts, at various stages of development between zygote and blastocyst, at embryonic day (E) 26 when PGCs have migrated to embryonic gonads. Unlike mouse embryos, pig embryos show little expression of germline-specific genes during preimplantation development. This study provided evidence that porcine and potentially other non-rodent models may not mirror the developmental signaling processes of the mouse model and warrants further investigation.

Introduction

Germ cells are lineage committed stem cells restricted in their fate to become a spermatozoon or oocyte, depending on the biological sex of the organism. The germ cells become totipotent following fertilization, and are capable of giving rise to every cell type in the body, and for mammals the placenta as well. While germ cells are not totipotent themselves, the ability for them to give rise to a zygote upon fertilization engenders some shared characteristics. Primordial germ cells, the precursors to mature sperm or oocytes, and embryonic cells have many similarities, including expression of canonical pluripotency genes, DNA methylation status, and chromatin state (Mise, Fuchikami et al. 2008).

PGCs are often characterized by their transcript profile, which has many overlaps with that of the early preimplantation embryo. It is known in mice and many other species that PGCs express pluripotency factors *Pou5f1*, *Sox2*, *Klf4*, *Dppa4*, *Ssea1*, and *Nanog* which are also upregulated in the early embryo as well as in embryonic stem cells and induced pluripotent stem cells (Surani 2001, Sorrentino, Nazzicone et al. 2007, Surani 2007, Mise, Fuchikami et al. 2008). In addition to these pluripotency factors, there is evidence that in the mouse a key PGC gene, *Prdm14*, is also involved in the maintenance of naïve pluripotency, suggesting that there may be some overlap between the signaling pathways that are active in PGCs and pluripotent cells (Grabole, Tischler et al. 2013).

In the mouse embryo, *Prdm14* is expressed as early as the 2-cell stage and another family member, *Prdm1* is expressed at the early epiblast stage (Saitou 2009, Burton, Muller et al. 2013). Additionally, *Prdm14* expression is asymmetrical in the

late four cell stage embryo, with two blastomeres showing expression at the mRNA and protein levels while the other two blastomeres show little to no expression at all (Burton, Muller et al. 2013). At this stage, PRDM14 is spatially correlated with POU5F1 and NANOG expression (Burton, Muller et al. 2013). PRDM14 is also weakly and transiently expressed in embryonic day 3.5 mouse blastocysts, whereas POU5F1 and NANOG show strong expression (Burton, Muller et al. 2013). At the molecular level, PRDM14 has been shown to co-occupy target genes along with POU5F1, SOX2, and NANOG (Yamaji, Seki et al. 2008). *PRDM14* is among the genes reported to be bound by POU5F1, SOX2, and NANOG, and PRDM14 itself is also capable of binding to the proximal enhancer of *Pou5f1* to activate expression (Boyer, Lee et al. 2005, Nakaki and Saitou 2014).

In addition to the expression of pluripotency genes, there are other essential genes that are also upregulated in both cell populations, which are related to epigenetic changes that occur during germ cell and embryo development. Embryos undergo two main stages of demethylation: one at the zygote stage in which existing marks on the gamete are erased, and another at the PGC stage in which future germ cells silence the somatic epigenetic pattern inherited from the cells of the younger embryo (Kar, Parbin et al. 2014). PGCs undergo demethylation in order to prepare for the pluripotent state needed for eventual gamete identity. Demethylation in PGCs allows for aberrant epigenetic marks to be reset in order to prevent their transmission to the next generation.

Among the genes involved in epigenetic modifications are the TET proteins (ten-eleven translocation enzymes), which play a role in active DNA demethylation.

This family of proteins is responsible for the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), which has been described as the initial step of active demethylation in mammals (Okashita, Sakashita et al. 2015). Due to their role in demethylation, TET proteins have been shown to be expressed in PGCs, cells of the early embryo, and embryonic stem cells (Seisenberger, Andrews et al. 2012). TET proteins have also been shown to physically interact with *Prdm14*, as *Prdm14* enhances recruitment of TET1 and TET2 at target loci to aid in DNA demethylation (Okashita, Kumaki et al. 2014). Another epigenetic modifier, *Carm1*, an arginine methyltransferase, has also been reported to be expressed in early embryos and PGCs in the mouse (Burton, Muller et al. 2013). *Carm1* is expressed as early as the 2-4 cell stage in the mouse and even associates with PRDM14 during this time period, making it an interesting candidate for study in the pig system.

Because of its role in both PGC specification and maintenance of pluripotency in the mouse, and its expression in human ESCs, investigation of the role of *PRDM14* in a non-rodent "bridge model" is warranted. While the pig genome has been sequenced, not all genes have been annotated and many annotated genes have yet to have their functions described. In order to determine the function of *PRDM14* in the pig, it was necessary to first determine its normal expression profile at similar developmental time points and locations as are found in the mouse.

Along with determining the expression of *PRDM14* at varying time points in early pig development, several other genes of interest were also chosen for analysis. There were 4 groupings of genes to consider: PGC-related genes (*PRDM1* and *TFAP2C*), genes involved in epigenetic modifications (*CARM1*, *TET1*, and *TET2*),

pluripotency genes (*POU5F1*, *SOX2*, *NANOG*, and *ESRRB*), and germ cell markers (*DDX4*, *DAZL*, and *STRA8*). These groups of genes were chosen based on known information about their function in the mouse system, as well as interest in how genes that are associated with *PRDM14* may be functioning at these developmental time points.

Materials and Methods

Collection of in vivo embryos

A cohort of gilts were synchronized by oral administration of progesterone analog Regu-Mate® (0.22% altrenogest solution, 2.2 mg/mL) starting from day 15 after a gilt showed behavioral heat. Animals were given 22 mg (10 mL) Regu-Mate® once daily via a drench gun for a minimum of 6 days prior to withdrawal. Approximately 5-7 days after Regu-Mate® withdrawal, gilts in standing estrus were bred 2-3 times via artificial insemination (AI). Semen for AI was provided by Genus PIC in individual doses. Animals were sacrificed based on the stage of embryo desired, as shown in the table below.

Table 3.1 Euthanasia schedule based upon breeding date

Developmental Stage	**Day euthanized after breeding**
Zygote	1
2-cell	2
4-cell	3
Morula	4
Blastocyst	6
E26	26

Reproductive tracts were removed from the females and flushed via 18 gauge needle and syringe using 30-35 mL warmed Dulbecco's Modified Eagle Medium (DMEM, Gibco; Grand Island, NY). Depending upon the stage of development, either the oviduct (zygote and 2-cell), uterine horns (blastocyst), or both (4-cell and morula) were flushed. For embryonic day (E) 26 samples, fetuses were carefully removed from the reproductive tract inside a laminar flow hood and the gonads were dissected for collection. All embryos were then immediately processed for RNA. Gonads from E26 fetuses were snap frozen in liquid nitrogen prior to RNA extraction.

RNA collection and cDNA synthesis

RNA from embryos was collected using the Dynabeads™ mRNA Purification kit according to manufacturer's instructions (ThermoFisher; Waltham, MA), eluting into a final volume of 10 µL. First, the zona pellucida of each embryo was removed by incubation in acidic Tyrode's solution (Sigma; St. Louis, MO) for 2-3 minutes until the zona was completely dissolved. For embryos from developmental stages zygote through morula, 2-3 embryos were pooled for RNA collection to ensure enough RNA to process for PCR. Blastocysts were harvested individually based on evidence from previous publications (Park, Jeong et al. 2012). For E26 gonad samples, RNA was extracted using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen; Hilden, Germany). Briefly, the sample was ground using a tenbroeck homogenizer that was pre-chilled in liquid nitrogen. The sample was then passed through a 20-gauge needle before proceeding with the RNeasy kit, and eluted in a final volume of 30 µL.

cDNA was then synthesized via the oligo(dT) method using the SuperScript™ IV First-Strand Synthesis System according to manufacturer's instructions (ThermoFisher, Waltham, MA). Using a final volume of 20 µL, synthesis was carried out at 50°C for 15 minutes and the reverse transcription reaction was terminated by incubating at 80°C for 10 minutes.

Droplet Digital™ PCR

PCR to identify expression of genes of interest was performed using the Bio-Rad QX200 Droplet Digital™ PCR system (ddPCR™) according to manufacturer's recommendations (Hercules, CA). In this system, a single PCR reaction is partitioned into thousands of reactions by placement inside of oil droplets which are amplified and quantitated individually. This allows for quantitative analysis of samples with low starting material or copy number, while giving thousands of data points for a single sample. This system also provides absolute measurement of copy number without the need for running standard curves.

For each sample, a 22 µL reaction containing ddPCR™ EvaGreen Supermix was loaded into a 96-well plate with the specific primers listed below used for amplification. The plate was loaded into the QX200™ Automated Droplet Generator (Bio-Rad; Hercules, CA) where the sample was fractionated into 12,000-20,000 individual droplets. After droplet generation, samples were amplified using the C1000 Touch™ Thermal Cycler (Bio-Rad; Hercules, CA) using the following conditions: 95°C for 10 minutes followed by 40 cycles of 94°C for 30 sec and 58°C for 1 min, and a final signal stabilization step of 98°C for 10 minutes. After amplification, the

plate was transferred to the QX200™ Droplet Reader for reading and analysis of the droplets using the absolute quantification setting on the machine.

Table 3.2 Primer sequences used for ddPCR™.

Gene	Primer
RPS18	F: 5'-GGCTACCACATCCAAGGAAG-3'
	R: 5'-TCCAATGGATCCTCGCGGAA-3'
PRDM14	F: 5'-GAAGTCAAGACCCACGGAGA-3'
	R: 5'-AGTTCCCAGCACCTCCTTTT-3'
PRDM1	F: 5'-TGTGGGTACGACCTTGGCTG-3'
	R: 5'-CATATCCGCGTCCTCCATGT-3'
CARM1	F: 5'-ACCACACGGACTTCAAGGAC-3'
	R: 5'-CGTAGATCTTCCTCGCTCCA-3'
TFAP2C	F: 5'-TCGTGCTCTGACCTTGAAGT-3'
	R: 5'-CTCTTGCCATCTCCTTGTGC-3'
TET1	F: 5'-AGGCGGCCGACCAAAAC-3'
	R: 5'-GGCACGAGGAACAGAGTCAT-3'
TET2	F: 5'-AGCTCACCGAAACACTGAGG-3'
	R: 5'-CAGGCACAGGTTCTCTCTTCA-3'
ESRRB	F: 5'-GCCCGTACCTGAGCTTACAG-3'
	R: 5'-AGGCATGGCGTAGAGTTTGT-3'
STRA8	F: 5'-ATGTGGCAAGTTTCCTGGAC-3'
	R: 5'-GAAACTTCTCCTCGGGCTTT-3'
DAZL	F: 5'-CCTCCAACCATGATGAATCC-3'
	R: 5'-ACACAGGCAGCTGATAACCA-3'
DDX4	F: 5'-GAGAGGCGGCTTTCAAGATG-3'
	R: 5'-TAACCACCTCGTCCACTTCC-3'
SOX2	F: 5'-CCTACGGGACATGATCAGCA-3'
	R: 5'-CTCCAGTTCACTGTCCGGC-3'
NANOG	F: 5'-GGTTTATGGGCCTGAAGAAA-3'
	R: 5'-GATCCATGGAGGAAGGAAGA-3'
POU5F1	F: 5'-GGGTTCTCTTTGGGAAGGTGT-3'
	R: 5'-TGCCTTGCATATCTCCTGCA-3'

Statistical analysis

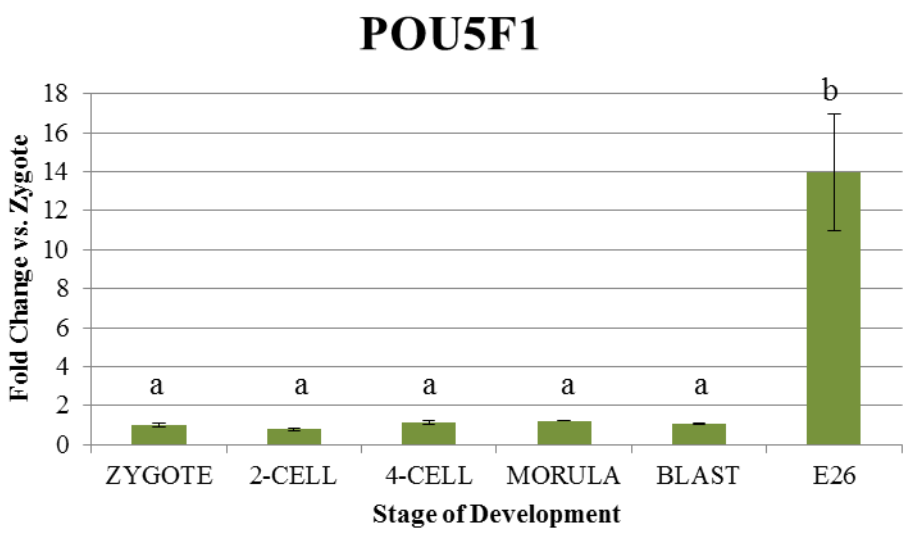
Transcript copy number for each target gene at each developmental stage was normalized to an internal reference (40S Ribosomal protein S18; *RPS18*) corresponding to the appropriate developmental stage to correct for differing amounts of starting RNA. *RPS18* was chosen for use from among 7 candidate housekeeping genes because it demonstrated the most consistent expression throughout each of the developmental time points considered. The following equation was used for normalization of each target gene: mRNA level = $(\text{Transcript copy number})_{\text{target}}/(\text{Transcript copy number})_{\text{RPS18}}$. The data were log2-transformed prior to analysis by ANOVA using the MIXED models procedure of SAS (SAS Institute; Cary, NC) and differences between the developmental stages were examined using the test of least significant difference (PDIFF). A significance level of $p<0.05$ was used to determine significance. The data are presented relative to the earliest embryonic stage examined for each gene, which was expected to have the lowest level of expression among developmental stages.

Results

Pluripotency genes are upregulated in the early embryo and in the E26 fetal gonad

Pluripotency genes *POU5F1*, *SOX2*, *NANOG*, and *ESRRB* were chosen for inclusion in this study to serve as positive controls for early embryo expression, and to determine if they were also characteristic markers of the PGC population at E26. Until recently, *POU5F1* and *NANOG* were some of the only markers used to identify PGCs during porcine fetal development due to the lack of knowledge regarding PGC

signaling and specification pathways. These data show a similar pattern of *POU5F1* and *NANOG*: expression at all stages of development analyzed with higher expression in the E26 fetal gonad than in the preimplantation embryo (Figure 3.1). As expected, *ESRRB* is also upregulated in PGCs at E26, as well as in the 4-cell to blastocyst stages of the preimplantation embryo. *SOX2* however showed diminished expression throughout development when compared to expression levels at the zygote stage (Figure 3.2).



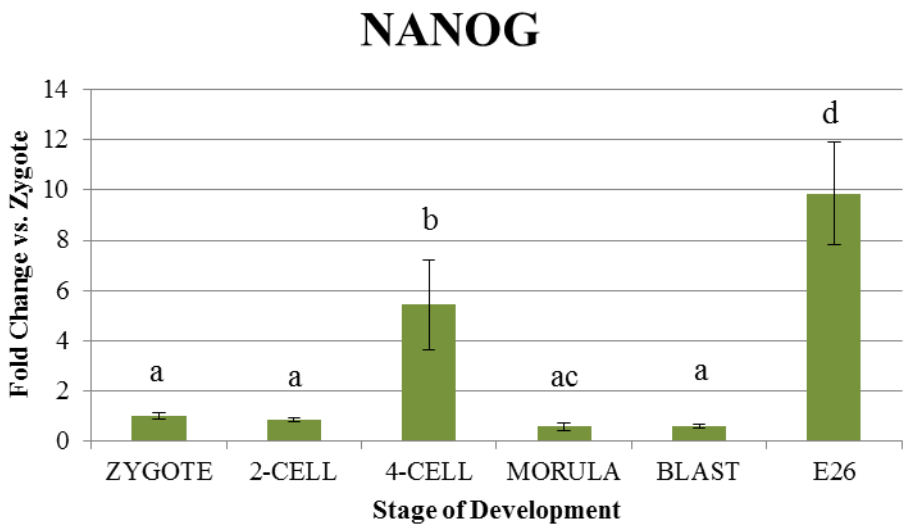


Figure 3.1 Gene expression of *POU5F1* and *NANOG* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

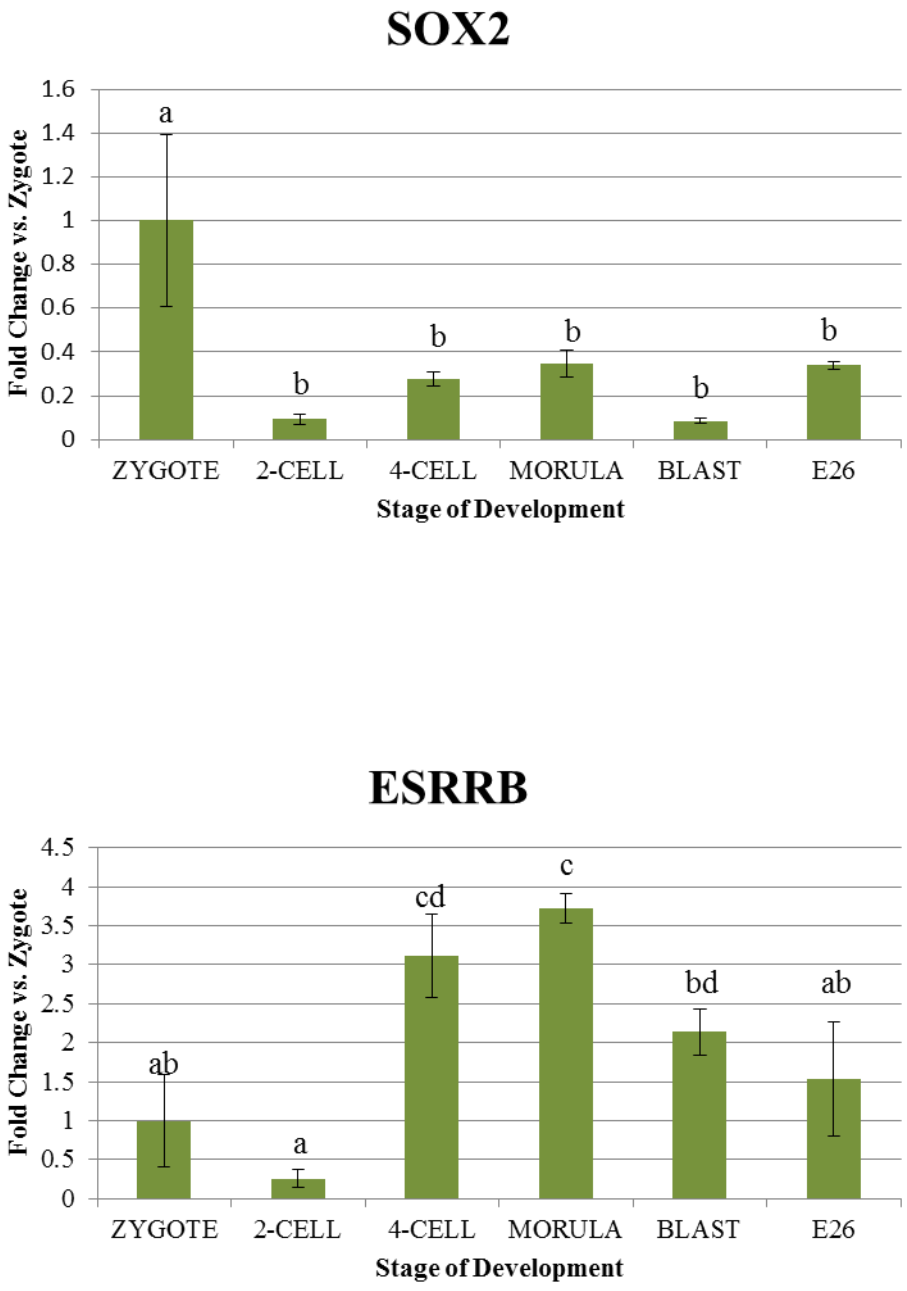


Figure 3.2 Gene expression of *SOX2* and *ESRRB* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

Primordial germ cell-related genes show little expression in the early embryo

In order to determine if the genes important for germ cell specification in the mouse are also expressed at key time points in the pig, we chose for analysis the three PGC-related genes that are necessary and sufficient for mouse PGC specification: *PRDM1*, *PRDM14*, and *TFAP2C*. *PRDM1* and *PRDM14* showed differential expression across the 6 stages of development analyzed, with reduced expression as development progresses (Figure 3.3). *TFAP2C* showed no significant pattern of expression across developmental time points (Figure 3.4).

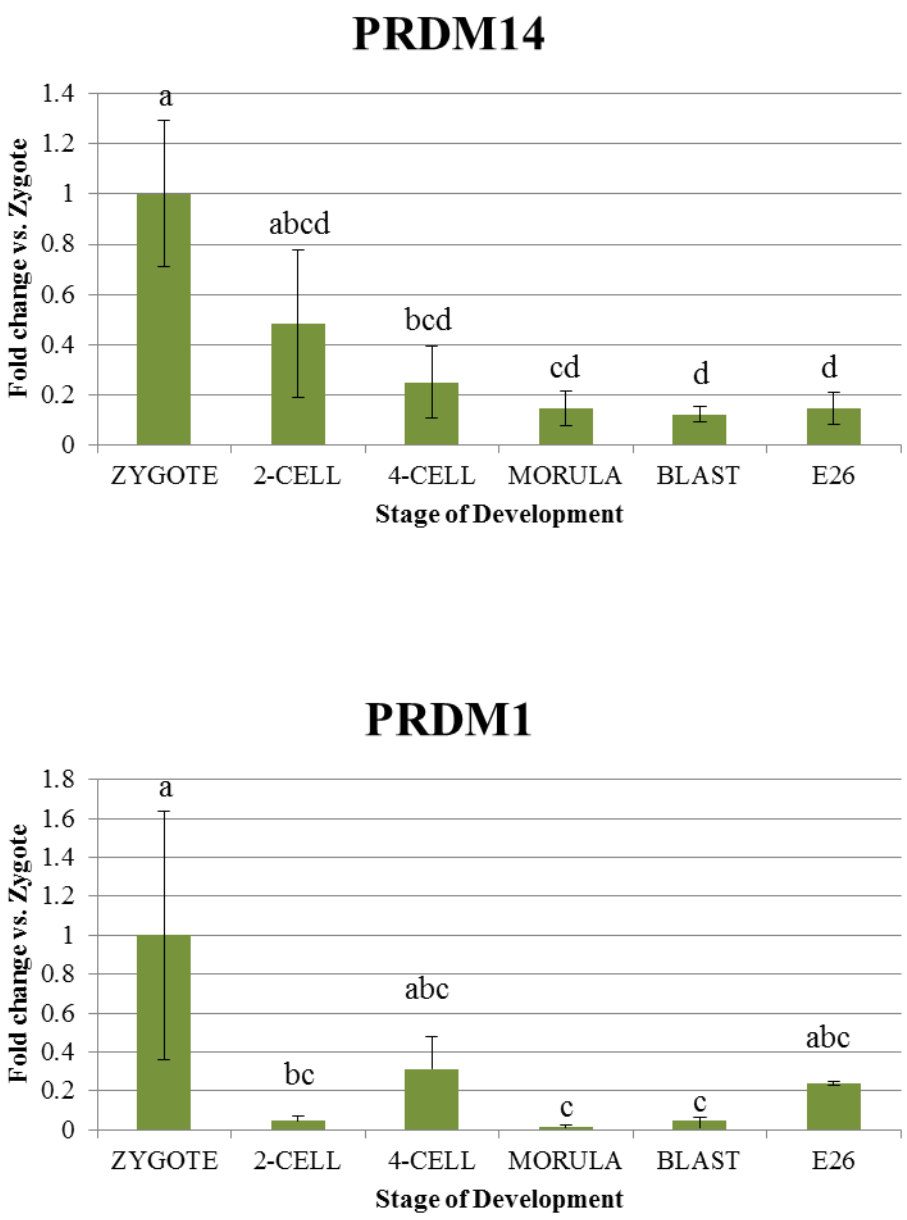


Figure 3.3 Gene expression of *PRDM14* and *PRDM1* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

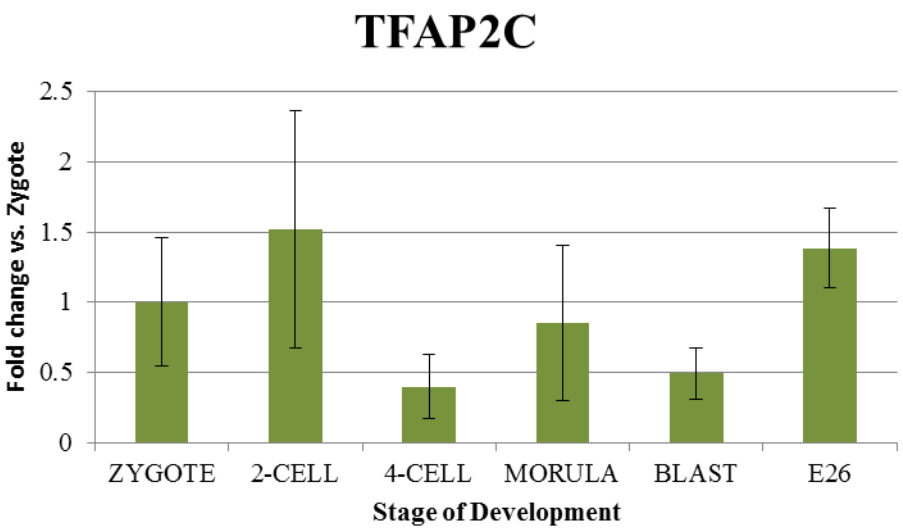


Figure 3.4 Gene expression of *TFAP2C* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

Germ cell markers are not expressed in the early embryo

DAZL, *VASA*, and *STRA8* are all markers of the germ cell population. Unlike the other two genes, *STRA8* is restricted to the post-natal male lineage. In this experiment, we included germ cell markers in the study to determine if their expression was limited to the germ cell population, or if there was some earlier expression in pluripotent cells of the preimplantation embryo. *DAZL* and *VASA* both showed high expression at the zygote stage, with tapering levels as development continued (Figure 3.5). *DAZL* in particular showed an increased level of expression at the E26 time point, indicative of its role in the pre-natal germ cell population. *STRA8* transcript levels were low across all time points and this gene did not exhibit any significant trends in expression across time points (Figure 3.6).

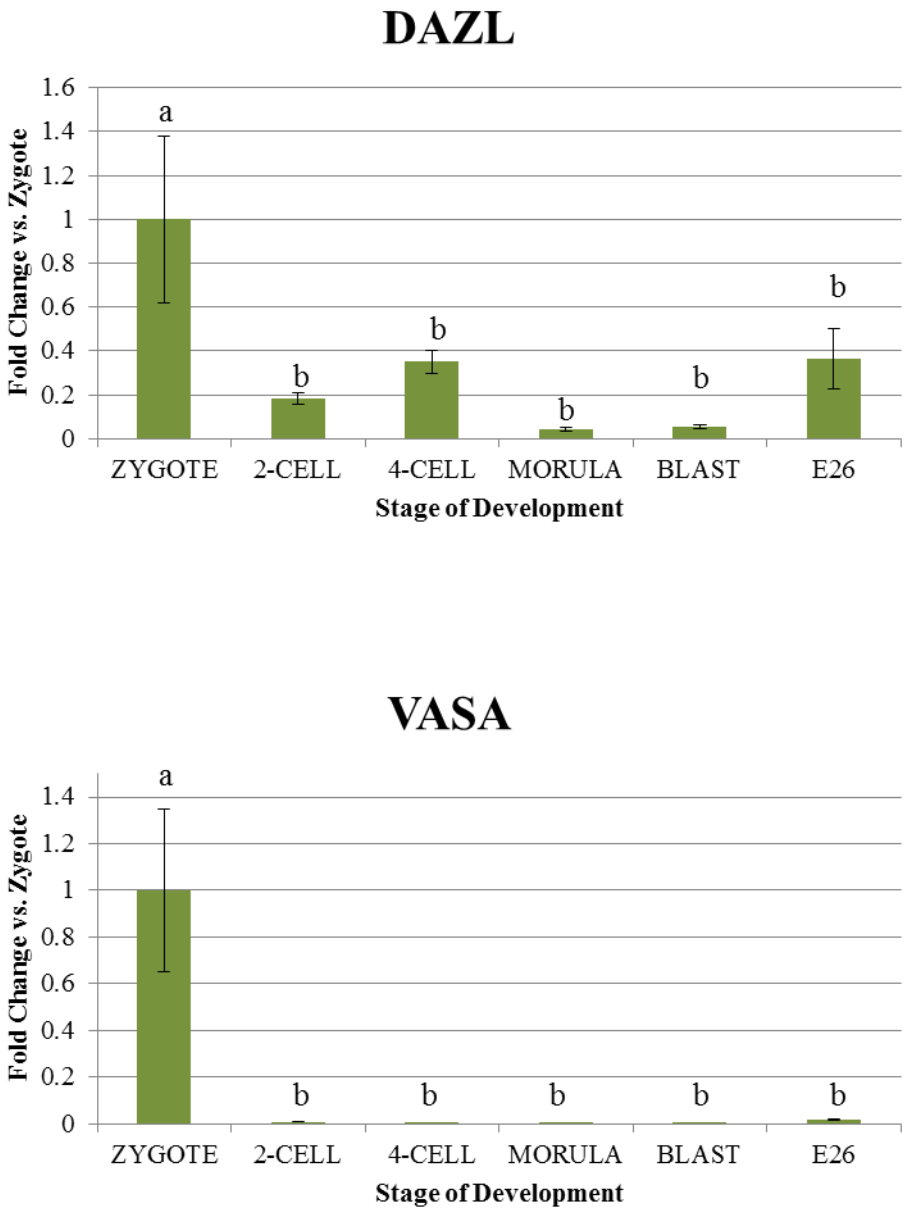


Figure 3.5 Gene expression of *DAZL* and *VASA* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

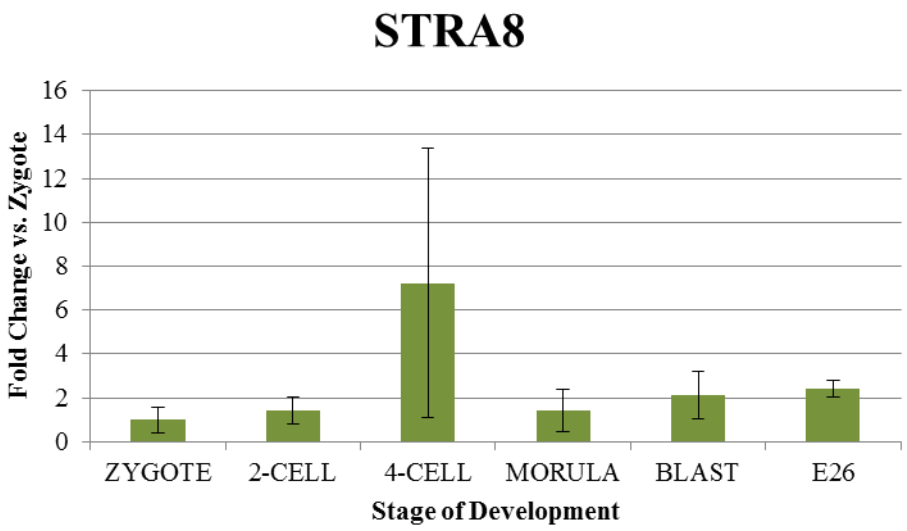


Figure 3.6 Gene expression of *STRA8* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

Genes involved in epigenetic reprogramming are upregulated during periods of genome-wide demethylation

The mammalian embryo undergoes two main rounds of genome-wide DNA methylation reprogramming: in the early preimplantation embryo and in PGCs. Therefore, we chose to investigate three factors involved in DNA methylation reprogramming that have also been shown to interact with PRDM14 in other species. Of the genes chosen for analysis, *CARM1* and *TET1* were both upregulated in E26 gonads (Figure 3.7). In contrast, *TET2* showed highest expression at the zygote stage (Figure 3.8). All three of these genes showed the highest level of expression at one of the two developmental time points where epigenetic marks are being erased genome-wide, indicating a positive correlation with this process.

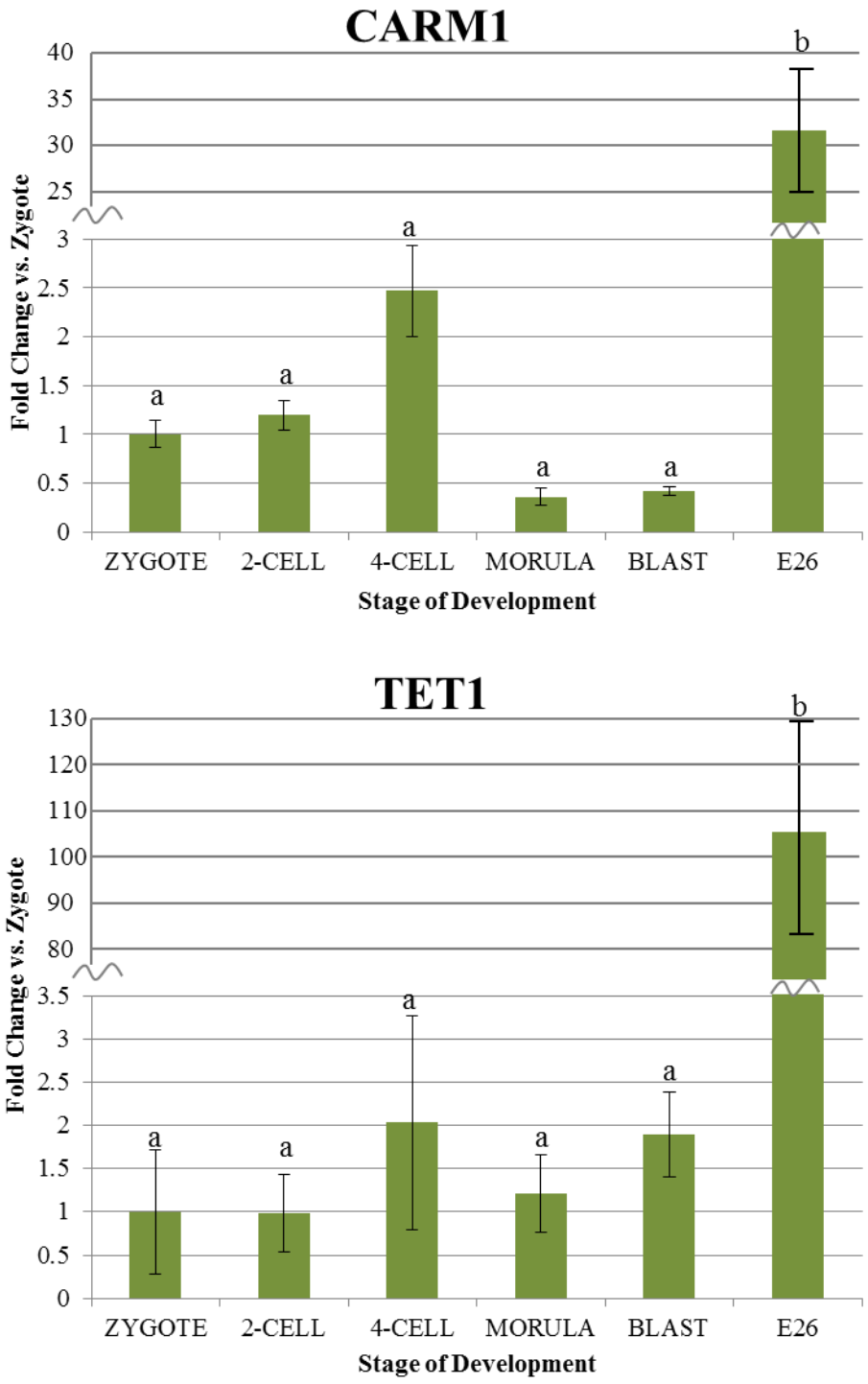


Figure 3.7 Gene expression of *CARM1* and *TET1* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

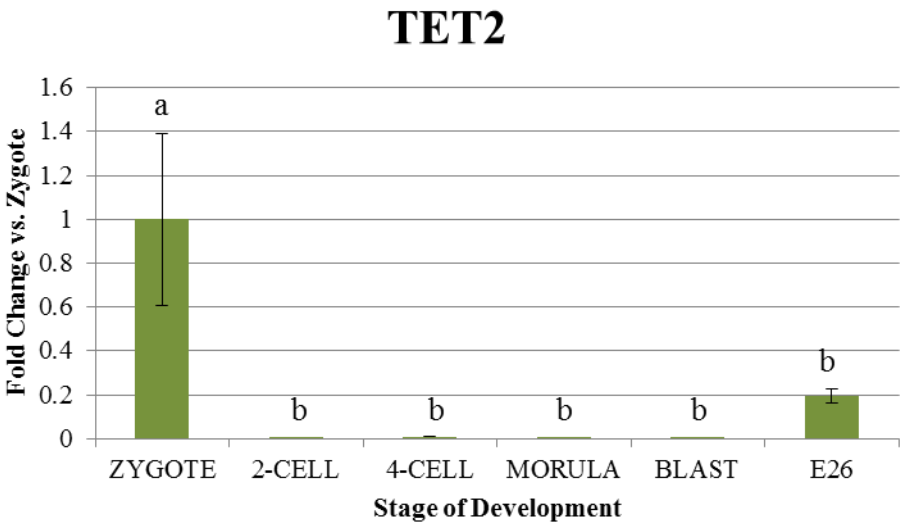


Figure 3.8 Gene expression of *TET2* at varying stages of embryo development. Least-square means of the natural log of gene copy number ± SE are presented. $p<0.05$

Discussion

The above study describes for the first time in the porcine system the expression pattern of several PGC, germ cell, and epigenetic markers in the preimplantation embryo. The genes in this experiment were chosen based on their proposed role within the germ cell program or their association with the major gene of interest, *PRDM14*.

The pluripotency markers *POU5F1* and *NANOG* showed stable expression as the embryo continued to develop, with highest expression levels at E26. *NANOG* also showed a small increase in expression at the 4-cell stage. The high expression at E26 is likely due to an increase in the number of cells of the embryo through blastocyst stage, as well as very high expression in the pluripotent PGCs that reached the genital ridge by E26. *SOX2* and *ESRRB* did not have this same trend for increased expression at the E26 stage. It is possible that the lower expression at E26 of these factors is due to lower overall expression as compared to *POU5F1* and *NANOG*. This same effect could be true for several other factors that were expected to be highly expressed in the genital ridge (*PRDM14*, *PRDM1*, *DAZL*, *VASA*).

The PGC marker *PRDM1* showed highest expression at the zygote stage. This indicates that there was little to no expression as development occurred, at least through blastocyst stage. In the pig, *PRDM1* has been shown to be expressed in the PGCs and prespermatogenic population of cells (Petkov, Marks et al. 2011, Kakiuchi, Tsuda et al. 2014, Kobayashi, Zhang et al. 2017). Kobayashi et al found that it had higher expression than *PRDM14* in the PGC population of both pigs and humans, so it is expected that there would be some expression at E26 in this study. Dilution of the

effect due to large number of non-PGC cells in the gonad at this stage may account for the reduced effect seen graphically. In the pig, *PRDM1* has 7 transcript variants to examine. The primers used here detected variants X2 and X7, which are two of the three longest transcript variants produced. It is therefore possible that amplification of the other 5 variants would result in some expression in the early porcine embryo. Currently it is unknown which variants are expressed in the germ cell lineage in pigs, because *PRDM1* is also expressed in other tissues of the body, as evidenced in the mouse (Mould, Morgan et al. 2015, Ahmed, Elias et al. 2016, Bankoti, Ogawa et al. 2017). However, this same primer set showed amplification of message within the whole fetal gonad in a later study, indicating it was appropriate for use in this study (Figures 4.3 and 4.5).

PRDM14 also had low expression throughout early embryo development and even at E26, which has been indicated by other studies and the recent suggestion that *PRDM14* does not have a major role in the porcine PGC specification process (Kobayashi, Zhang et al. 2017). While *TFAP2C* expression is low in this experiment, TFAP2C may be constitutively expressed and may not be upregulated at any specific time point examined. In the mouse system, *Tfap2c* is important for specification of the trophectoderm lineage during the morula to blastocyst transition, as well as placental development (Auman, Nottoli et al. 2002, Winger, Huang et al. 2006, Choi, Carey et al. 2012, Cao, Carey et al. 2015). However, this expression pattern in the preimplantation embryo was not seen in this pig study.

Three germ cell markers that identify pre- and post-natal germ cells were included for analysis – *DAZL*, *VASA*, and *STRA8*. Similar to PGC markers, these were

chosen because they are indicative of cells in a pluripotent pathway and may therefore have shown expression in the early embryo. Both *DAZL* and *VASA* showed highest expression at the zygote stage with little to no expression after that time period. *DAZL* in particular showed another increase of expression at E26, although this change was not significant. Because these two are markers of post-natal germ cells, it is likely that the high expression at zygote stage is holdover of transcript deposited prior to fertilization, especially since levels drastically decreased following the first cleavage event. The third germ cell marker, *STRA8*, is considered a meiotic gatekeeper gene as it regulates a germ cell's entry into meiosis (Anderson, Baltus et al. 2008, Feng, Bowles et al. 2014, Wang, Chen et al. 2014). In the early pig embryo, it has very little expression, indicating it is not required for early development.

The last suite of genes chosen for analysis were genes involved in epigenetic reprogramming – *CARM1*, *TET1*, and *TET2*. Each of these genes has been shown to interact with PRDM14 in the mouse system, as described previously. The ddPCR™ data shown here indicate that they are active in the pig system, with highest expression at one of the two periods of genome-wide epigenetic reprogramming. *CARM1* and *TET1* both showed exceptionally high expression during the PGC stage of development while *TET2* showed higher expression at the zygote stage.

The increase in expression of *STRA8* at the 4-cell stage along with three other genes that show similar anomalies at that stage (*NANOG*, *PRDM1*, *DAZL*) is of interest. This rise in expression at that single time point could be due to a reduction in overall transcription that would result in a skewed ratio of the gene of interest to the housekeeping gene. In pigs, the maternal to zygotic transition occurs during the 4-8

cell stage (Prather 1993, Lee, Hamm et al. 2014). During this time, the zygotic genome is activated resulting in new transcription from the embryonic genome, and degradation of maternally inherited mRNA transcripts. The 4-cell stage embryos used for analysis in this experiment could have been collected during this critical transition phase, resulting in the differences in expression for these four genes.

This study provides valuable information regarding the expression of key germline-related factors during early pig development. These results clearly show low levels expression for the germ cell-related genes (*PRDM14*, *PRDM1*, *TFAP2C*, *DAZL*, *VASA*, *STRA8*, indicating their lack of importance in early embryonic development of the pig. For *PRDM14* and *TFAP2C*, this is contrary to what is known in the mouse system. However, confirmation of their diminished role via knockout experiments would be required to confirm the present data. The data presented in this experiment represent a key first step in describing the normal expression profile of the porcine embryo, as well as delineating the role of *PRDM14* at these early developmental stages.

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Chapter 4: Determination of the Role of *PRDM14* in the Male Porcine Germ Cell Niche

Abstract

Primordial germ cells (PGCs), the precursor cells that give rise to mature sperm or oocytes, are induced from a subset of mesoderm cells via BMP signaling during the epiblast stage of the early mammalian embryo. In the mouse, PGCs are specified via an initial tripartite signaling network. These factors are responsible for genome-wide epigenetic reprogramming, re-acquisition of pluripotency, and repression of the somatic program in this subset of cells. Among these genes, in mice *Prdm14* has been shown to be an essential transcription factor for PGC specification. Ablation of *Prdm14* results in the loss of PGCs in mouse models, while still maintaining an otherwise wild-type phenotype. Recently, the domestic pig has emerged as a bridge between mouse models and human research to explore and understand effects of diseases and their treatments, due to differing translational results between mouse and human. In this study, the role of *PRDM14*, if any, in PGC specification was investigated in the domestic pig model. Using CRISPR/Cas9 technology, a region of exon 1 was targeted for disruption in the open reading frame and generation of a functional knockout. Porcine embryos microinjected with CRISPR/Cas9 guide RNA and protein were then transferred into surrogate females and the animals were sacrificed at gestation day 60 or at sexual maturity. All animals were genotyped using ear and tail snips and were assessed for germ cell status. Two knockout males were produced, but these animals still had germ cells indicating

PRDM14 may not be essential for gametogenesis in the male pig. The role of *PRDM14* in the female reproductive system has yet to be determined.

Introduction

The role of *PRDM14* in the pig is currently unknown. It is often the case that gene function is conserved among species, but not always. To understand the function of *PRDM14* in the pig we first took some cues from what is known in the mouse. In the mouse model, specification of PGCs is reliant on three factors that together are responsible for repression of the somatic program, genome-wide epigenetic reprogramming, and re-acquisition of pluripotency (Yamaji, Seki et al. 2008). These three genes form a tripartite network in which *Prdm1* is the first to be expressed at E6.25. *Prdm1* in turn can activate both *Prdm14* and *Tfap2c* to initiate the specification of PGCs (Yamaji, Seki et al. 2008, Magnusdottir, Dietmann et al. 2013). In the mouse, *Prdm14* in particular is responsible for two important functions: specification of PGCs and maintenance of pluripotency in the early embryo. This role has been evidenced through knockout studies of *Prdm14* that show PGC specification fails at an early stage and the animals are sterile (Yamaji, Seki et al. 2008). Expression profiling studies in both the early embryo and in ESCs show that *Prdm14* is expressed as early as the two-cell stage and that it is also expressed in ESCs (Assou, Le Carrour et al. 2007, Burton, Muller et al. 2013). Its role in pluripotency in the mouse has been shown via its interactions with *Pou5f1* as well as its ability to convert epiblast-like cells to ESC-like cells when overexpressed in culture (Iseki, Nakachi et al. 2016).

The role of PRDM14 in PGC specification remains controversial in humans and non-rodent models. Recent studies that were published near the completion of this research have highlighted a reduced role for *PRDM14* in pig PGC specification. Instead, researchers have proposed a larger role for *SOX17* and *PRDM1* in specification of PGCs, which is more similar to what appears to occur in humans (Irie, Weinberger et al. 2015, Kobayashi, Zhang et al. 2017). Indeed, both of these factors show high expression at the time of PGC specification in the pig, around embryonic day 9.5-12. Very weak expression of PRDM14 is exhibited during this same time period, although PRDM14 is expressed once the PGCs reach the genital ridge and settle in the gonad (Kobayashi, Zhang et al. 2017). Nevertheless, this project sought to corroborate these findings by generating a functional knockout of *PRDM14* to determine whether it is dispensable for PGC development in the pig.

Materials and Methods

Generation of CRISPR guide RNA

The beginning of exon 1 was targeted for knockout (Figure 4.1). This site was chosen so that any edits that may generate frameshifts or large deletions would interfere with the downstream structure and/or function of the protein. A 200 bp sequence was screened for guide RNA targets using the MIT CRISPR Design web tool run by the Zhang Lab (crispr.mit.edu). The website populates a list of all possible guide sequences and then compares them to the entirety of the pig genome to generate unique sites that are unlikely to be found in coding exons elsewhere in the genome. Three to four guides were chosen from this list to test their efficacy *in vitro* before

using them for embryo transfer. To start, this experiment used one guide only. However, the second and third attempts to generate a knockout animal used two guides spaced 223 base pairs apart to attempt to generate larger deletions. Chosen guides were transcribed *in vitro* using the MEGAshortscript T7 kit (Ambion; Austin, TX) and stored at -80°C until their use in microinjection.

Figure 4.1 Diagram of the structure of porcine *PRDM14* and guide RNA target sites. The top guide site was used for experiments 1-3. The bottom guide site was used for experiments 2-3. Gray boxes: non-coding regions; Green boxes: open reading frame.

Preparation of gene edited embryos for transfer

Sows chosen for use as embryo donors and surrogate mothers were synchronized using Regu-Mate® (0.22% altrenogest solution, 2.2 mg/mL), which was started 15 days after a sow showed heat. Animals were given 22 mg (10 mL) Regu-Mate® once daily via a drench gun for a minimum of 6 days prior to withdrawal. Twenty-eight hours after Regu-Mate® withdrawal, sows were given an intramuscular injection of 1500 IU PMSG (Prospec Bio; Rehovot, Israel). This injection was followed by an intramuscular injection of 750 IU hCG (Prospec Bio; Rehovot, Israel) 82.5 hours after PMSG injection. Two rounds of artificial

insemination (AI) were performed the day after hCG injection, approximately 6 hours apart, and surgical collection of embryos was performed the day after AI.

Prior to surgical collection of embryos, sows were restrained in a chute and were administered an intramuscular shot of TKX (a combination of Telazol 50 mg/ml [Zoetis, Inc.; Kalamazoo, MI], Ketaset 50 mg/ml [Zoetis, Inc.; Kalamazoo, MI] , and Rompun 50 mg/ml [Xylazine –Bayer; Shawnee Mission, KS]) dosed at 50 mg/100 pounds of body weight. When the animal was sedate enough to manipulate the ear for intravenous injection, a 25 mg/100 pound body weight dose was administered via IV injection into an ear vein. After a few minutes when the animal was fully sedated, she was rolled onto a stretcher.

Once on the stretcher, all four feet were tied to the stretcher to keep them in place during surgery. The abdomen was shaved and a three step surgical scrub procedure was used to prepare the area for surgery. First, a mild soap and water were used to scrub off any visible dirt. Next, a 0.05% chlorhexidine solution was used to scrub in a circular motion from the center of the surgical area toward the outer edge. The solution was wiped off from the inner area toward the outer area using a clean paper towel. Lastly, the area was thoroughly sprayed with a 70% ethanol solution. The pig was then moved into the surgical suite for surgery.

Once in the surgical suite, a modified nose cone was placed over the nose. This nose cone was fitted with a latex punch balloon instead of a hard cone to allow for a better seal between the equipment and the animal and to prevent leaks of isoflurane into the operating room. The animal was also connected to a monitoring machine that was able to read heart rate, blood pressure, and oxygen saturation level.

The animal was initially placed on a 2.0% oxygen flow and 5% isoflurane (MWI Animal Health; Boise, ID) flow using a rebreathing anesthesia machine (Matrx VMS VIP300; Midmark Corporation; Dayton, OH). As surgery progressed, the percentage of isoflurane was reduced by 1% every 5-10 minutes so that by the end, she was breathing 100% oxygen. Vital signs were recorded every 5-10 minutes until the last suture was in place.

To begin the surgery, a midline incision was made through the skin at the posterior three rows of nipples. Another incision was made through the fat and muscle layers and any nicked veins were clamped with hemostats to prevent excessive bleeding. A final incision was made through the peritoneal wall to visualize the reproductive tract. One uterine horn was located and followed anteriorly to locate the ovary. A small funnel was placed inside the infundibulum and firmly held in place. At the same time, an 18-gauge blunt needle attached to a 35 mL syringe containing 35 mL of pre-warmed DMEM (Dulbecco's Modified Eagle Medium; Gibco; Grand Island, NY) was inserted into the uterus just posterior to the uterotubal junction and firmly held in place. Once in place, the syringe was depressed and the DMEM was flushed through the oviduct and embryos that passed through the infundibulum were collected in a 50 mL conical tube. This conical tube was immediately placed into a warmed traveling case and taken directly to the laboratory. The same procedure was repeated on the contralateral oviduct.

After the oviducts were flushed, the reproductive tract was placed back into the abdominal cavity and 500 mL of warmed saline was poured into the cavity. The peritoneum was sutured using a continuous locked suture (#2, Ethicon; Cincinnati,

OH), followed by the muscle and fat layer and finally the skin (2-0, Ethicon; Cincinnati, OH). A final layer of aluminum spray (AluSpray, Neogen; Lexington, KY) was applied over the outer sutures. The modified nose cone was removed and the sow was moved into a recovery pen. While in the recovery pen, she was given an intramuscular injection of 2 ml/100 pounds Excede (Zoetis, Inc.; Kalamazoo, MI), and 2 ml/100 pounds Banamine (Merck Animal Health; Madison, NJ). After surgery, animals were monitored every half hour until they were fully awake, able to stand and walk, and responded to outside stimuli. At each post-surgical check, breathing rate, color, and response to stimuli were monitored and recorded.

Microinjection of surgically collected zygotes

After collection, the zygotes were immediately taken back to the laboratory for culture and microinjection. Media containing the flushed zygotes was poured into a 10 cm square dish and searched for embryos. Embryos were then placed into PZM3 culture media in a 38.5°C incubator under 5% oxygen and 5% CO_2 until microinjection. Prior to microinjection, the *in vitro* transcribed guide RNA was complexed with Cas9 protein (PNA Bio, Thousand Oaks, CA) at room temperature for 10 minutes. The complex consisted of 25 ng/µL Cas9 protein and 12.5 ng/µL guide RNA. After microinjection, embryos were cultured overnight at 38.5°C under 5% oxygen and 5% CO_2.

Embryo transfer of microinjected embryos

Embryo transfer was performed in much the same way as embryo collection, and was done the day after collection so that only embryos that had cleaved were used for transfer. The transfer surgeries were performed as previously described for embryo collection. In transfer surgery, once the oviduct was located, an 18 gauge blunt needle was used to create a small hole in the oviduct. Then, the embryos (30-40 per animal, split between both uterine horns) were loaded into a positive displacement pipette and the pipette was guided into the hole that the needle had made. The embryos were then transferred into the oviduct. The same procedure was done for the remaining embryos on the contralateral oviduct. After transfer, the animals were closed in the same manner as previously described, given postoperative medications, and monitored after surgery as previously described.

Somatic cell nuclear transfer (SCNT)

Cumulus-oocyte complexes (COCs) were purchased from a commercial supplier (DeSoto Biosciences). Briefly, matured oocytes were enucleated by aspirating the polar body and MII chromosomes with an enucleation pipette (Humagen; Charlottesville, VA). Enucleation was carried out in TL-HEPES media supplemented with 0.4% BSA and 5 mg/ml cytochalasin B (Sigma-Aldrich; St. Louis, MO). After enucleation, a donor cell was introduced into the perivitelline space of an enucleated oocyte. Fusion of injected oocytes was induced in a fusion medium (280 mM mannitol, 0.001 mM CaCl2, and 0.05 mM MgCl2) by two DC pulses (1 sec interval) of 2.0 kV/cm for 30 μs by using a BTX Cell Manipulator 200 (BTX;

Holliston, MA). After fusion, oocytes were incubated for 1 hour in TL-HEPES. The reconstructed oocytes were activated by an electric pulse (1.0 kV/cm for 60 μs) followed by 4 hour of incubation in PZM3 medium containing 2 mM 6-dimethylaminopurine (6-DMAP). Cleaved embryos were transferred into the recipient the following day.

Birth of embryo transfer animals

At around day 110 of gestation, pregnant females were brought to the farrowing barn and loaded into a gestational crate in preparation for farrowing at day 114. Once the piglets were born, they were monitored closely for the first 4-6 hours to ensure they were moving, nursing, and in perceived good health. The next morning after birth, animals were given an iron supplement (1 ml Ferrodex; AgriLabs; St. Joseph, MO), needle teeth clipped, tails docked, and ears notched for identification. The tissue from tail docking and ear notching was collected for genotyping purposes. These piglets were allowed to grow until puberty, when their reproductive capacity was assessed.

Collection of tissue from fetuses

Two experiments required the surrogate sows to be sacrificed at embryonic day 60 to determine the effect of *PRDM14* knockout on fetal germ cell development. Sows were sacrificed and the reproductive tracts removed and taken back to the laboratory. Once there, they were put into a laminar flow hood and the fetuses were carefully dissected out of the tract. The gonads were removed and collected for either

RNA or immunohistochemistry. From each fetus, one gonad was snap frozen in liquid nitrogen for RNA collection, and the other gonad was placed into a solution of 10% neutral buffered formalin for fixation.

Genotyping

Tissue samples collected from piglets and fetuses were subjected to genotyping to determine edits by the CRISPR/Cas9 system. Ear and tail samples were incubated in lysis buffer containing 10 µL/ml proteinase K (Sigma-Aldrich; St. Louis, MO) overnight at 50°C with agitation. The next day, DNA was purified using phenol-chloroform extraction followed by ethanol precipitation. DNA was amplified using primers surrounding the two CRISPR targeting sites F: GTCAGGCGGAATCTCGGTAG, R: GAGCCAGGTAACAACTGGGA. The following PCR cycling conditions were used: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 60°C for 20 sec, and 68°C for 45 sec, followed by a single elongation step at 68°C for 5 min. PCR products were cloned into pJET1.2/blunt entry vector to separate out individual alleles prior to sequencing (ThermoFisher; Waltham, MA). DNA was sent for sequencing to Macrogen USA (Rockville, MD) and the results were aligned with wild-type sequences to determine specific edits around the guide RNA site(s).

Genotyping of ear and tail samples was confirmed by also genotyping the gonadal samples after immunohistochemistry was performed. The formalin-fixed, paraffin embedded sections were extracted for genomic DNA via the QIAamp DNA FFPE Tissue Kit (Qiagen; Hilden, Germany), amplified using the same primers as

above, and again cloned into the pJET1.2/blunt vector (ThermoFisher; Waltham, MA) prior to sequencing by Macrogen USA (Rockville, MD).

RNA extraction and cDNA synthesis

RNA was collected using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen; Hilden, Germany). Briefly, the sample was ground using a tenbroeck homogenizer that was pre-chilled in liquid nitrogen. The sample was then passed through a 20-gauge needle before proceeding with the RNeasy kit, and eluted in a final volume of 30 µL nuclease-free water.

cDNA was then synthesized via the oligo(dT) method using the SuperScript™ IV First-Strand Synthesis System according to manufacturer's instructions (ThermoFisher; Waltham, MA). Using a final volume of 20 µL, synthesis was carried out at 50°C for 15 minutes and the reverse transcription reaction was terminated by incubating at 80°C for 10 minutes.

Immunohistochemistry for paraffin-embedded samples

After fixation for 24 hours at room temperature, samples were moved through a series of ethanol washes. Samples were washed twice with each concentration of 20%, 40%, 60%, and 70% ethanol for 30 minutes per wash. After the final wash, samples were stored in 70% ethanol and sent to American Histolabs (Gaithersburg, MD) for paraffin embedding and sectioning.

Tissue sections were subjected to a deparaffinization and rehydration procedure prior to immunostaining. Each wash was performed for 2 minutes each.

First were 2 xylene washes, followed by: 1x xylene/100% ethanol, 2x 100% ethanol, 1x 95% ethanol, 1x 70% ethanol, 1x 50% ethanol, 1x tap water. Slides were blocked for endogenous peroxidase activity using 3% hydrogen peroxide in PBS for 1 hour. Antigen retrieval was performed using 95°C Tris-EDTA buffer for 20 minutes. Following antigen retrieval, slides were washed twice for 5 minutes each in TBS containing 0.025% Triton X-100 (TBS-T). Tissue sections were then blocked for 1 hour using SuperBlock Protein Block (Thermo Fisher Scientific; Waltham, MA). Primary antibody was diluted in blocking buffer (PRDM14 1:50; Santa Cruz Biotechnology; Dallas, TX), applied to the slide, and slides were incubated overnight at 4°C.

The next day, slides were rinsed three times in TBS-T for 5 minutes each rinse with agitation. Then, they were incubated with secondary antibody (Donkey Anti-Rabbit IgG; Jackson ImmunoResearch Laboratories; West Grove, PA) diluted 1:500 in TBS-T for one hour. After, they were rinsed 3 more times in TBS-T for 5 minutes each rinse. Color development was performed using SignalStain® DAB Substrate kit according to manufacturer's instructions (Cell Signaling Technology; Danvers, MA). Following color development, tissue sections were counterstained with hematoxylin (BBC Biochemical; Mount Vernon, WA) and dehydrated through a series of ethanol and xylene washes. These washes were in the reverse order of the rehydration step described above. After dehydration, slides were sealed with Permount, cured overnight, and sealed with nail polish.

Immunohistochemistry for OCT-embedded samples

Samples that were prepared for OCT (optimal cutting temperature) embedding were placed into a cryomold containing OCT compound (Sakura® Finetek; Torrance, CA). They were then placed over a bed of dry ice and methanol until completely cooled and hardened, when they were placed into liquid nitrogen for transport and eventually stored in a -80°C freezer until processing.

OCT embedded tissues were cryosectioned and placed onto room temperature slides. The tissue sections were then fixed using 4% PFA (Electron Microscopy Sciences; Hatfield, PA) for 5 minutes. After fixation, they were blocked with a 50mM glycine solution diluted in PBS for 5 minutes. Sections were permeabilized using a 0.1% Triton X-100 solution for 15 minutes. Next, sections were blocked using Dako serum-free protein block (Agilent; Santa Clara, CA) for 30 minutes. Primary antibody (PRDM14 1:50; Santa Cruz Biotechnology; Dallas, TX) was diluted in blocking buffer, applied to the slide, and slides were incubated overnight at 4°C.

The next day, slides were rinsed thrice using a 0.01% Triton X-100 solution for 5 minutes each. Then, slides were incubated in a 1:2000 dilution of secondary antibody (Donkey anti-Rabbit IgG (H+L) Alexa Fluor®488; ThermoFisher; Waltham, MA) for 30 minutes at room temperature. Slides were then rinsed three times in 0.01% Triton X-100 for 5 minutes each. Finally, slides were dried and mounted using ProLong™ Gold Antifade containing DAPI (ThermoFisher; Waltham, MA), and sealed with nail polish the next day.

Results

This study was a combination of three smaller experiments. In Experiment 1, one CRISPR guide was used to target *PRDM14* and pregnancies were allowed to go to term. Piglets were grown until sexual maturity to assess their germ cell status. In Experiment 2, a combination of two CRISPR guides was used to induce a larger deletion in *PRDM14*. Pregnancies were terminated at embryonic day 60 and the gonads of each fetus were assessed for germ cell status. In Experiment 3, somatic cell nuclear transfer (SCNT) was performed using fibroblast cells from fetuses from the second experiment. This was done due to the high incidence of mosaicism in animals from the second experiment. These pregnancies were also sacrificed at E60 and gonads analyzed for germ cell status.

Experiment one: knockout of PRDM14 using a single CRISPR guide RNA

All piglets born were gene edited

Piglets born from embryos microinjected with a single guide RNA were subjected to genotyping from tissue samples at two locations: the ear and the tail. This was done to determine if there was any mosaicism due to the Cas9 nuclease inducing a double strand break after the zygote cleaved. All 11 piglets showed some degree of editing, as there were no wild-type alleles found during genotyping. We then predicted the genotype (bi-allelic or mono-allelic/heterozygous) based on the edits discovered (Table 4.1).

Gene edited animals still produce germ cells

Animals generated from using a single guide RNA to induce random insertions or deletions around the guide site were allowed to mature until puberty, when their germ cell status was assessed. Heterozygous males were sacrificed and in all cases, motile sperm was collected from their cauda epididymides. Semen from bi-allelic males was collected and contained motile sperm. As expected, heterozygous males also showed germ cell development. This is evident in H&E morphological staining and immunohistochemical staining for PRDM14, a PGC marker (Figure 4.2). Only one bi-allelic female was generated in this experiment and at puberty, she was monitored for estrus and artificially inseminated with either wild-type sperm or sperm from a bi-allelic KO male at least 4 times. She was never able to establish a pregnancy, but her ovary did contain mature follicles as noted upon euthanasia. Hematoxylin and eosin stained histology sections of this female's ovary also showed production of oocytes (Supplementary Figure 1). A summary of these results can be found in Table 4.2.

Table 4.1 Genomic edits of newborn piglets. Animals 50-52 were stillborn or died shortly after birth. bp: base pair

Piglet ID	Sex	Genotype	Edits (nucleotide)
30	M	Bi-allelic Heterozygous	2bp insertion, 17bp insertion
31	M	Wild-type Heterozygous	3bp deletion, 1bp mutation
32	M	Wild-type Heterozygous	3bp deletion, 2bp mutation, many single nucleotide mutations, 3bp deletion + 2bp mutation
33	M	Bi-allelic Heterozygous	6bp deletion + 1bp mutation, 9bp deletion + 2bp mutation, 10bp insertion
34	M	Bi-allelic Heterozygous	2bp insertion, 21bp deletion
35	M	Bi-allelic Heterozygous	3bp insertion, 5bp deletion + 5bp mutation, 2bp mutation, 21bp deletion, 6bp deletion + 2bp mutation
36	F	Wild-type Heterozygous	3bp deletion
37	F	Wild-type Heterozygous	9bp deletion, 8bp deletion
50	F	Wild-type Heterozygous	10bp insertion, 3bp deletion + 1bp mutation
51	M	Wild-type Heterozygous	3bp mutation, 14bp deletion
52	M	Wild-type Heterozygous	3bp insertion w/ mutation, 9bp deletion

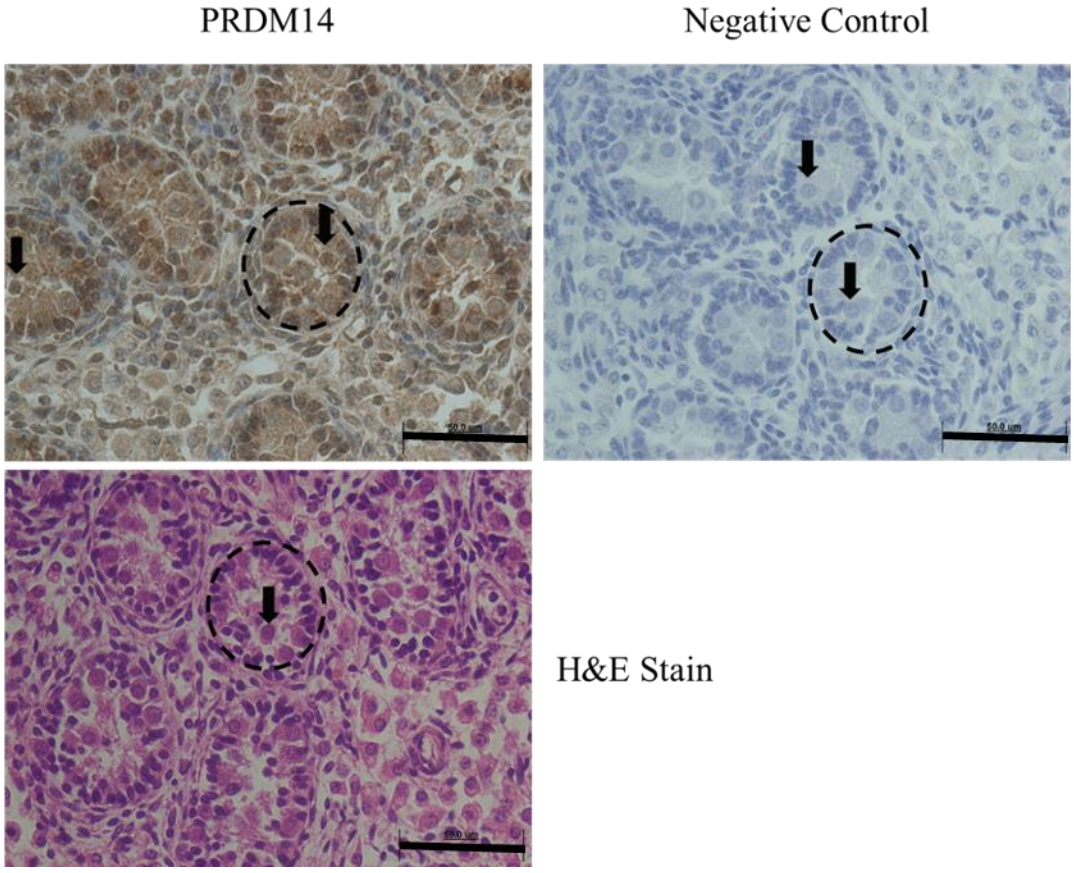

Figure 4.2 Representative images of heterozygous male gonads (Piglets 51 and 52). Immunohistochemical staining for PRDM14, Negative control which included no primary antibody, and H&E stained section. Dashed circles indicate seminiferous tubules; arrows indicate germ cells. Scale bar: 50 µm.

Table 4.2 Germ cell status of all piglets in experiment one. All animals regardless of genotype had germ cells present. Female 36 was not analyzed because her phenotype was predicted to be WT.

Piglet ID	Sex	Genotype	Germ Cell Status
30	M	Bi-allelic Heterozygous	Motile sperm
31	M	Wild-type Heterozygous	Motile sperm
32	M	Wild-type Heterozygous	Motile sperm
33	M	Bi-allelic Heterozygous	Motile sperm
34	M	Bi-allelic Heterozygous	Motile sperm
35	M	Bi-allelic Heterozygous	Motile sperm
36	F	Wild-type Heterozygous	N/A
37	F	Wild-type Heterozygous	Follicles and oocytes present
50	F	Wild-type Heterozygous	Primordial germ cells present
51	M	Wild-type Heterozygous	Germ cells present
52	M	Wild-type Heterozygous	Germ cells present

Experiment two: knockout of PRDM14 using two CRISPR guide RNAs

Fetuses produced from double guide RNA microinjection show germ cell production

In order to determine if the results from the first embryo transfer experiment were due to an incomplete knockout or if ablation of endogenous *PRDM14* truly does not result in sterility in the pig, we used a dual guide approach to generate larger deletions to remove a larger portion of the genomic sequence. The additional target site was 246 base pairs downstream from the original target site. Unfortunately, due to the scheduling of the embryo collection surgeries, the embryos had already advanced to the 2-cell stage. So as not to waste the embryos, they were microinjected at the 2-cell stage, generating a situation in which mosaicism was much more likely. These pregnancies were terminated at embryonic day 60 to observe possible changes at the time of fetal gonadal development. As expected, genotyping results from ear and tail tissue showed these fetuses to have a variety of edits at both target sites (Table 4.3). This combination of edits in all 5 fetuses compounded the interpretation of the results.

Nevertheless, transcript data collected from the fetal gonads showed expression of *PRDM14* in female fetuses 1-2 and male 1 but transcript was undetectable in female 3 or male 2. The same expression pattern was true for *DAZL*, a marker of pre- and post-natal germ cells. *PRDM1* and *VASA* however, were both detected in all fetuses (Figure 4.3). Immunohistochemical staining of gonads from male fetuses showed expression of PRDM14 protein (Figure 4.4), as did female fetuses 2 and 3 (Supplementary Figure 2). The morphology of the germ cells appeared normal.

Table 4.3 Genomic edits of fetuses microinjected with two CRISPR guide RNAs. bp: base pair; mutation: single base pair change.

Fetus ID	Sex	Genotype	Site 1 Edits	Site 2 Edits
1	F	Heterozygous	1bp deletion, mutation, 246bp deletion	3bp deletion, 246bp deletion, 2bp deletion, mutation
2	F	Heterozygous	1bp deletion, 246bp deletion	3bp deletion, 246bp deletion
3	F	Heterozygous	1+1bp deletion, 186bp deletion, 246bp deletion, 4bp deletion, mutation	186bp deletion, 246bp deletion, 3bp deletion
1	M	Heterozygous	3bp deletion, 1+3bp deletion, 171bp deletion, 246 bp deletion	WT, 71bp deletion, 246bp deletion
2	M	Heterozygous	186bp deletion, 246bp deletion, 1bp deletion, mutation	186bp deletion, 246bp deletion, 3bp deletion, mutations

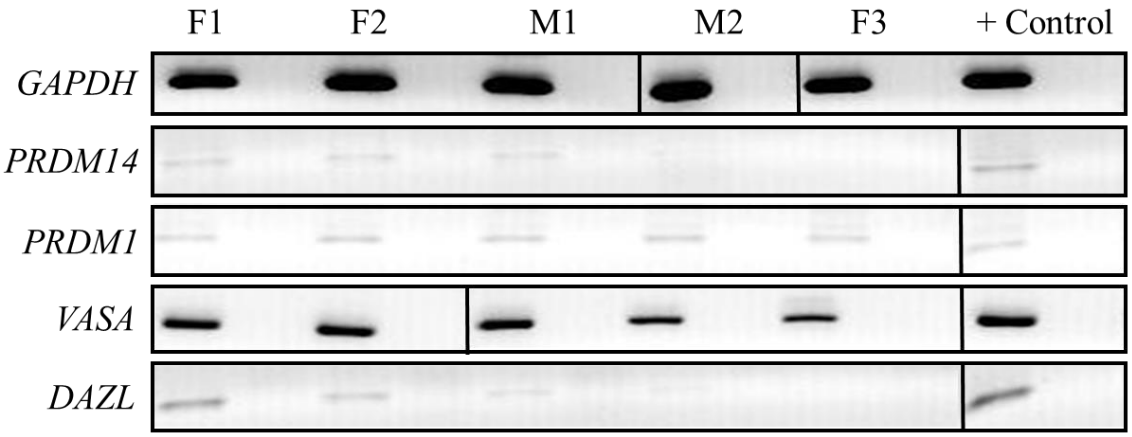

Figure 4.3 RT-PCR of PGC and germ cell markers expressed in fetal gonads from experiment 2 (two guide RNA injection). All PCR was performed in the same plate. Black lines indicate the PCR reaction was run on different gels at the same time. Each sample is accompanied by a negative RT reaction in the adjacent well.

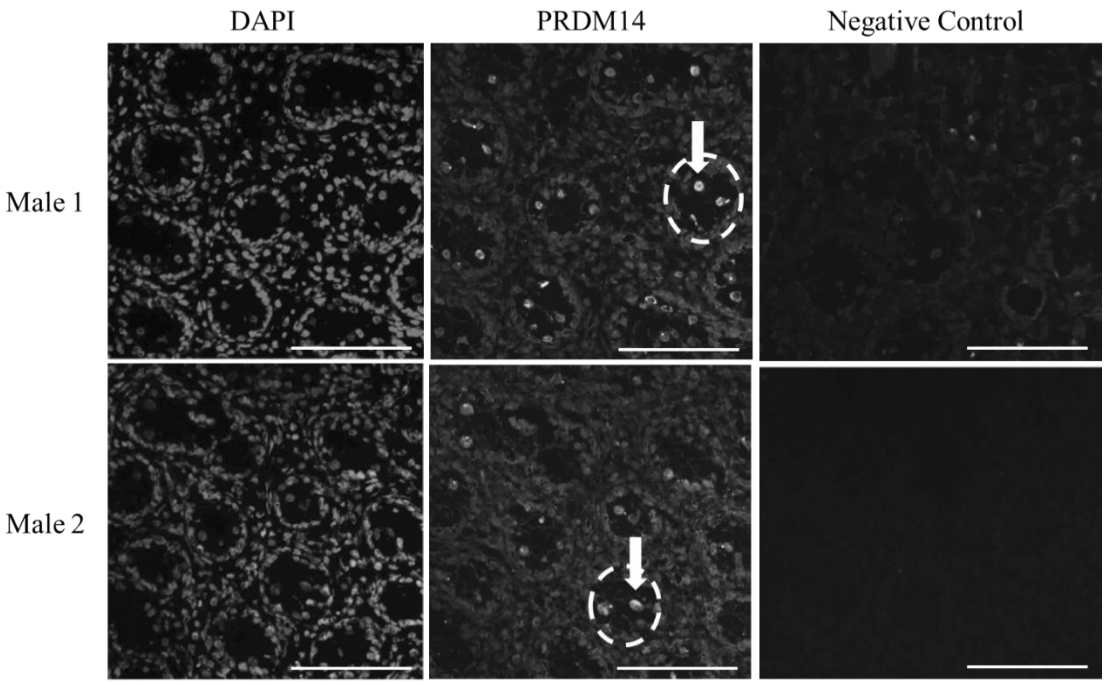

Figure 4.4 Fluorescent immunohistochemistry images of PRDM14 from male fetuses in experiment two. Dashed circles indicate seminiferous tubules; arrows indicate germ cells. Scale bar: 500 μm.

Experiment three: cloning of PRDM14 knockout fetuses from experiment one

Knockout fetuses still have germ cells

In order to generate knockout animals without mosaicism and only 2 alleles with large deletions, we performed somatic cell nuclear transfer (SCNT) with cells from female 2 and male 2 from the previous experiment. These 2 fetuses were chosen because they had some alleles with large deletions and they also showed a lack of *PRDM14* transcript as detected by reverse transcriptase PCR. The pregnancies established from SCNT were also terminated at embryonic day 60. Genotyping of these 6 fetuses showed the presence of a large 246 base pair deletion in all fetuses. This represents a removal of the entire sequence between the two CRISPR target sites. Multiple sequencing runs for each fetus showed that for all male fetuses this was the only allele found (Table 4.4). However, female fetuses 1 and 2 had a second allele consisting of a 186 base pair deletion, occurring between the two CRISPR target sites (Supplementary Table 1).

RNA taken from the gonads from these fetuses indicated reduction of *PRDM14* transcript from fetuses 1 and 2 at several regions of the message. However, other germ cell markers *PRDM1* and *VASA* were still present at the transcript level in these fetuses. Surprisingly, *DAZL* was not expressed in fetus 2 but was expressed in all other fetuses (Figure 4.5). Gonads from male fetuses lacking *PRDM14* transcript also showed a lack of PRDM14 protein, as compared to a negative control using only the secondary antibody (Figure 4.6). Morphologically, H&E staining clearly showed that despite the absence of *PRDM14* message and protein, there were still cells present within the seminiferous tubules of the fetal gonad (Figure 4.7). Based on their

location within the seminiferous tubules, these cells are likely germ cells. Immunohistochemical staining and H&E staining of the female gonadal tissue indicates the presence of germ cells (Supplementary Figure 3).

Table 4.4 Genomic edits of cloned fetuses microinjected with two CRISPR guide RNAs. bp: base pair; aa: amino acid.

Fetus ID	Sex	Genotype	Edits (nucleotide)	Edits (amino acid)
1	M	Homozygous KO	246 bp deletion	82 aa deletion
2	M	Homozygous KO	246 bp deletion	82 aa deletion
3	M	Homozygous KO	246 bp deletion	82 aa deletion
4	M	Homozygous KO	246 bp deletion	82 aa deletion

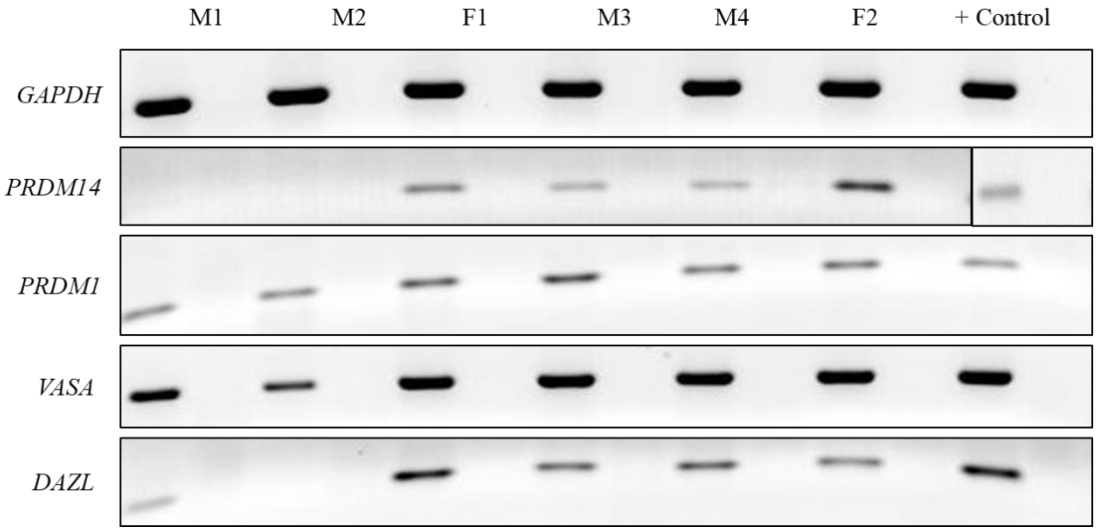

Figure 4.5 RT-PCR of PGC and germ cell markers expressed in fetal gonads from experiment 3 (cloned fetuses). All PCR was performed in the same plate. Black lines indicate the PCR reaction was run on different gels at the same time. Each sample is accompanied by a negative RT reaction in the adjacent well.

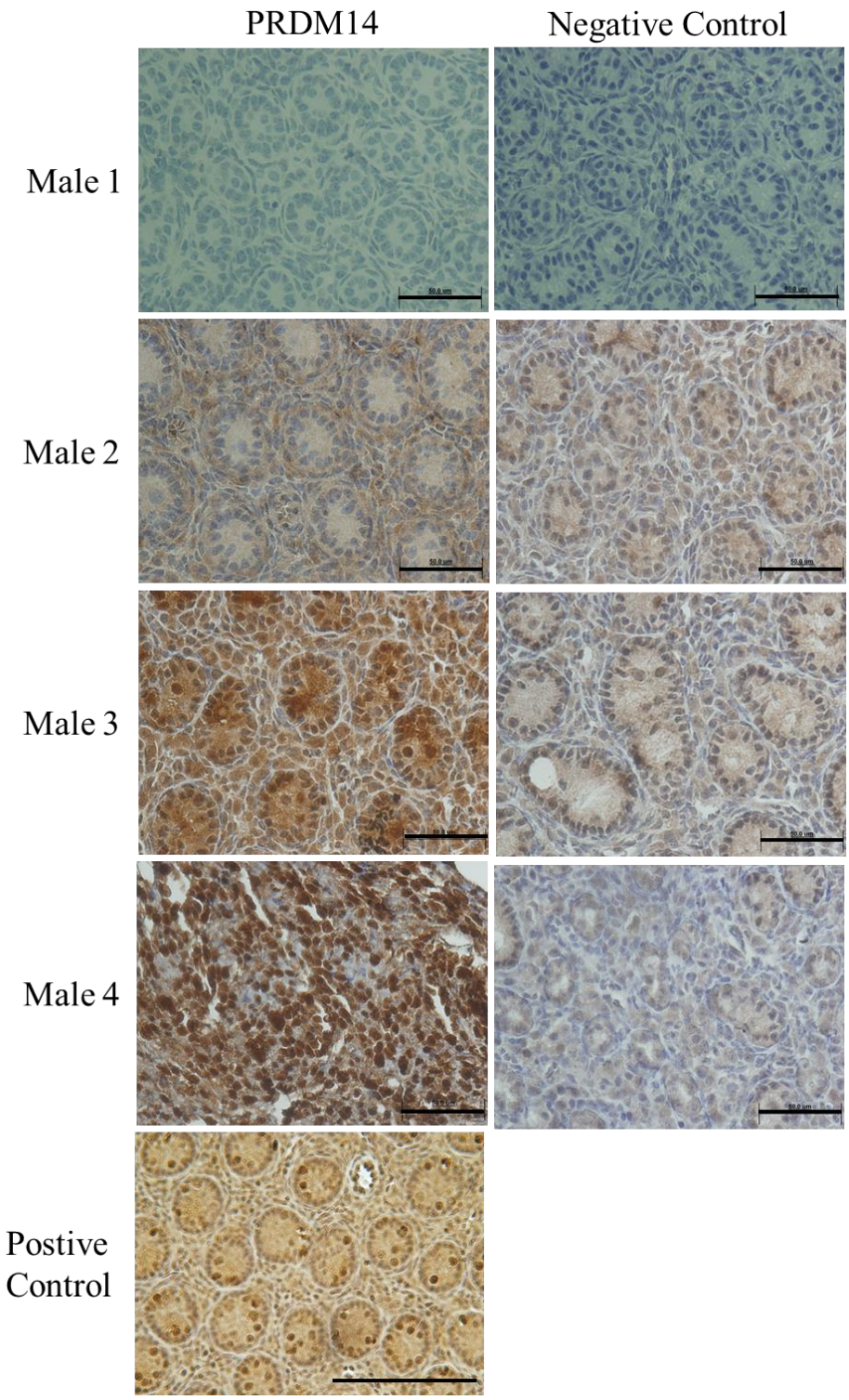

Figure 4.6 Immunohistochemical images from cloned male fetuses. Fetus 1 and 2 are presumptive knockouts while fetuses 3 and 4 are not. Scale bar: 200 µM

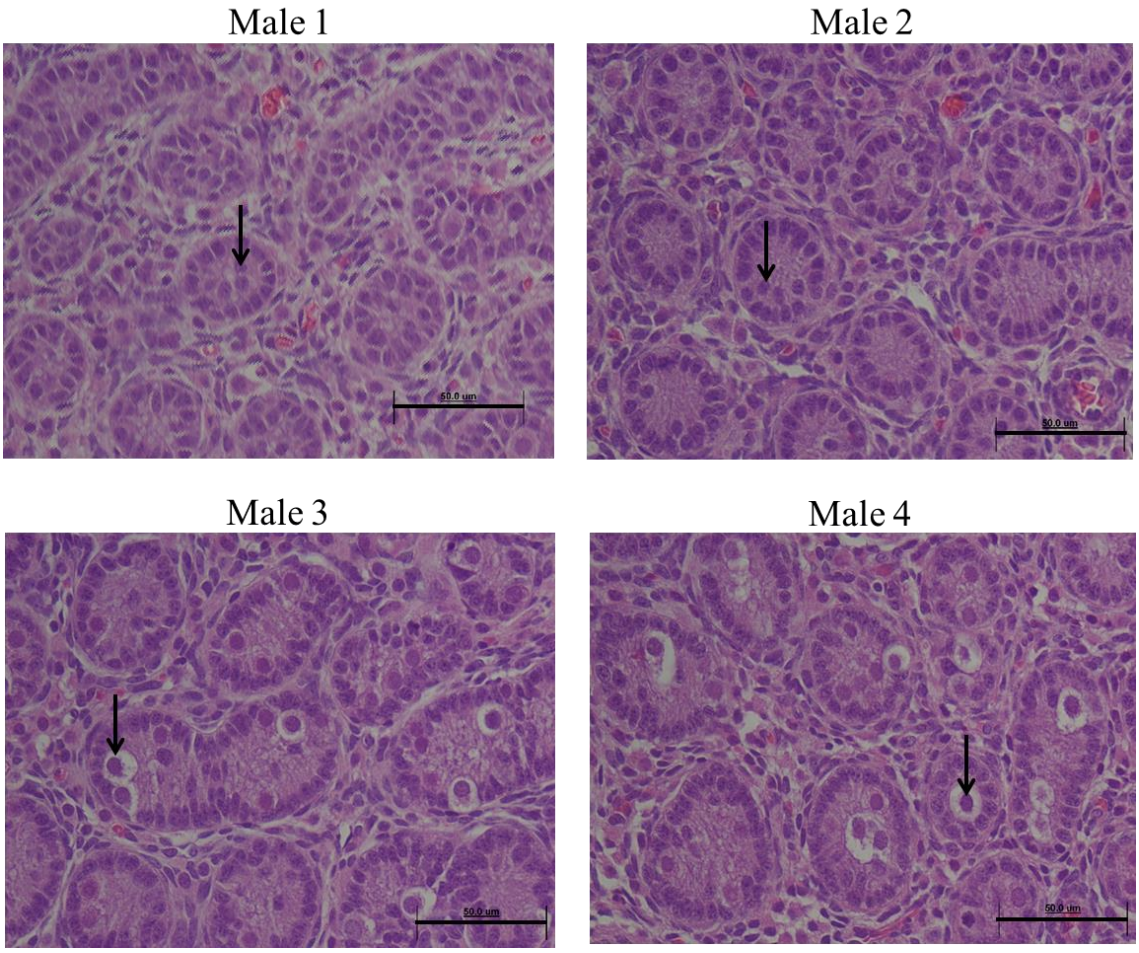

Figure 4.7 H&E staining of male gonads from cloned fetuses. Scale bar: 50 μm

Discussion

We used a targeted gene editing approach to disrupt the open reading frame, and consequently disruption of protein expression and function of *PRDM14* in the pig. In the absence of pluripotent stem cells, the simplest way to generate a knockout pig is through the use of the CRISPR/Cas9 gene editing system. This approach allows for random insertions, deletions, or mutations to be introduced to the genomic sequence at the zygote stage (Youds and Boulton 2011, Jinek, Chylinski et al. 2012, Gaj, Gersbach et al. 2013). These changes to the genetic sequence also known as indels can result in interruption of the gene's endogenous function due to imperfect DNA repair within the cell.

We took advantage of this system in order to determine the function of *PRDM14* in the pig by creating a knockout animal. The data presented here demonstrate for the first time an attempt to knockout *PRDM14* in the domestic pig. Due to the nature of the technique and how variable editing outcomes can be, this experiment required several iterations to generate an animal that could be considered a knockout. This attempt to knockout *PRDM14* in the pig required several refinements, including the use of a two CRISPR guide system in order to generate a large deletion, especially because *PRDM14* is a large multi-exonic gene. This technique of using multiple CRISPR guides to target one gene for a large deletion has been successful in other species, including cattle (Bevacqua, Fernandez-Martin et al. 2016).

Experiment one: knockout of PRDM14 using a single CRISPR guide RNA

In experiment one, a single CRISPR guide RNA was used to target early in the first exon of the open reading frame of *PRDM14*. In this first experiment, the use of a single guide RNA resulted in animals with limited edits – mostly small deletions or point mutations that were not expected to have an effect on the resulting phenotype (Table 4.1). After these animals were grown to maturity, sperm samples taken from the testes indicated the presence of motile sperm. Semen from two edited males from this experiment was used to inseminate an edited female (#37), but no pregnancy occurred after several attempts. Even though the edited male and female both had germ cells, the female was not able to establish a pregnancy, indicating that *PRDM14* may have another role affecting fertility in the pig outside of its predicted role in initial PGC specification. Further experiments with edited males would be required to determine if this is true. These data combined suggested one of two conclusions: that the CRISPR guide RNA was not completely effective and a true knockout was not produced, or that *PRDM14* is not required for PGC development in the pig. In order to rule out the possibility that the presence of germ cells was not due to the ineffectiveness of the CRISPR system, a second experiment using two CRISPR guides was attempted.

Experiment two: knockout of PRDM14 using two CRISPR guide RNAs

The second experiment in this study used a two CRISPR guide RNA approach to generate a knockout. The second guide was located 223 base pairs downstream of the first guide, and also within exon 1. While animals in the first experiment were

grown to sexual maturity, that time point was not required to answer the initial research question of whether *PRDM14* is required for PGC formation. Therefore, in this second experiment animals were sacrificed at an earlier time point to expedite the characterization process.

The results from experiment 2 show a reduction in *PRDM14* message in both males, and for male 2, a complete inability to detect transcript (Figure 4.3). The primer annealing site for detection of *PRDM14* transcript was located at the boundary of exon 1 and exon 2 in order to avoid amplification of genomic DNA. Based upon the primer design, it would still be possible to detect transcript in edited animals because the annealing site was downstream from the CRISPR guide RNA target site. Reductions in transcript detection could therefore be explained by a difference in overall expression level of *PRDM14*.

At the protein level, gonads from both male fetuses still show expression of PRDM14 (Figure 4.4). Even in the absence of detectable *PRDM14* message from male 2, expression of protein is still seen. This could be due to the procedures used – since the RT-PCR performed was qualitative, there could be an undetectable level of transcript that was still translated to protein, resulting in the positive expression seen in the immunohistochemistry results. The antibody chosen for use in this study was raised against an epitope at the C terminus of the protein, due to its high amino acid sequence conservation among species. The epitope is relatively far from the CRISPR guide RNA target site. However, interruptions to the reading frame earlier in the sequence could result in misfolding of the protein and therefore interfere with the antibody's ability to access the epitope. Therefore, while the antibody recognizes a

sequence downstream from the guide RNA target site, it is still appropriate for use in this study, especially as it failed to detect protein in the third experiment (Figure 4.6).

As each of the genotypes from experiment two presented the possibility for a wild-type allele, another round of targeting was required to eliminate the potential for expression of *PRDM14* from a wild-type allele. Due to the mosaicism seen in these animals (Table 4.3), we chose to perform SCNT to reduce the number of alleles found within the fetal gonad and further refine the experiment.

Experiment three: cloning of PRDM14 knockout fetuses from experiment one

Experiment 3 used fibroblasts from the male 2 fetus collected in experiment 2 for SCNT. This male was chosen based on the undetectable levels of *PRDM14* transcript seen via RT-PCR. Genotyping results showed that all 4 male fetuses had the same deletion: a 246 base pair deletion between the two CRISPR guide RNA target sites, which translates to a deletion of 82 amino acids. Interestingly, the transcript data showed two distinct expression profiles: 2 males showed expression of *PRDM14* transcript while the other 2 males had undetectable levels (Figure 4.5).

The two phenotypes were also reflected at the protein level, as the 2 males without detectable transcript also did not stain positively for protein. The reverse was also true: the 2 males that did show expression of transcript also showed expression of PRDM14 protein (Figure 4.6). The immunostaining of the negative controls was quite variable. While all staining was done at the same time and slides processed in the same order, the levels of background staining were different among samples. This

may be attributed to differing levels of endogenous peroxidases or uncontrollable differences in timing during various stages of the staining protocol.

These data indicate after reduction of *PRDM14* message and protein, that males still retain the ability to generate germ cells, as evidenced by morphology from gonadal tissue collected from E60 fetuses (Figure 4.7). This finding is in stark contrast to the phenotype observed in the mouse. In the mouse, ablation of *Prdm14* leads to sterility due to the complete absence of PGCs (Yamaji, Seki et al. 2008). While transcript and protein data presented here indicate ablation of *PRDM14* and the presence of germ cells, it is still unknown whether these animals are fertile.

The genotyping results of the animals generated from SCNT indicate an interesting phenomenon: 2 distinct phenotypes arising from the same genotype. The cloned male fetuses showed expression of *PRDM14* transcript and protein in 2 animals, and the absence of transcript and protein in the other 2 animals, despite being clones from the same cell line. This "same genotype, different phenotype" outcome in cloned animals has previously been reported in sheep (Rhind, King et al. 2003), mice (Tamashiro, Wakayama et al. 2003), pigs (Carter, Lai et al. 2002), cattle (Wells, Forsyth et al. 2004), and in a parthenogenetic species, the crayfish (Vogt, Huber et al. 2008). Most often, the phenotypic differences among cloned animals refer to animal mortality or disease state rather than individual gene expression. Some animals within a litter or cohort will die prematurely due to diseases that may have differences in heritability or penetrance (Wong, Gottesman et al. 2005). Besides disease state, growth and maturation differences also occur in SCNT animals, indicating other phenotypic components that are different among clones.

The most likely possibility to explain this phenomenon concerns the role of epigenetic reprogramming. SCNT relies on the oocyte environment to reprogram the introduced somatic cell. If this process does not occur appropriately, aberrant transcripts can be expressed from the donor cell lineage. In fact, this epigenetic memory has been reported in *Xenopus laevis* where transcripts characteristic of the donor cell were in abundance in an inappropriate lineage of the new embryo (Gurdon and Wilmut 2011). Therefore, it may be possible for differences in phenotype of the *PRDM14* SCNT fetuses to be attributed to incomplete reprogramming or an epigenetic memory from the donor cell, which may contribute to the absence of transcript and protein in males 1 and 2.

The differences in phenotype seen in the SCNT fetuses reveal ongoing technical issues that should be addressed in the future. In this current study, questions remain regarding the knockout status of the final SCNT animals due to the continued transcript and protein expression in 2 of the animals. It is likely that the continued expression is a trait unique to the pig system and that PRDM14 does not have the same function in mice and pigs. It is also possible that a true knockout was not achieved and the 2 fetuses that did not show transcript or protein did so based on the reasons discussed above. With this information, future experiments could be designed to answer those remaining questions.

In the future, it would be wise to design the CRISPR guide site to target an area containing key domains required for activity. This project sought to edit the DNA sequence as early as possible to interrupt or change a large area of downstream translation or folding. While the current project generated large deletions in the

coding sequence, these deletions were all before key structural components of the gene. A future attempt should plan to target the PR/SET domain or the zinc fingers required for binding to target DNA. This would likely improve chances of generating an interruption to an important functional domain, and generate a better candidate for knockout. Additionally, gonads should be collected earlier in development. PGCs exist for a short period of time in mice and this time period in pigs is still being refined, but is around E28. However, because PRDM14 protein was still expressed at E60 when these fetuses were collected, it may be expressed for a longer time period in pig than it is in mouse, making E60 or E28 a reasonable time point to use.

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Chapter 5: Summary and Future Directions

The overall objective of this dissertation was to analyze the role of *Prdm14* in a rescue experiment using a chimeric mouse model, as well as to determine its function within the pig model system. Due to the extensive research regarding the role of *Prdm14* in mice, we first sought to determine if the CRISPR/Cas9 system could be used to interrupt its function because it is a large multi-exon gene. Our lab has had previous success in generating knockouts of single exon genes (Park, Kaucher et al. 2017, Sheets, Park et al. 2018), but had yet to generate presumptive knockouts of a multi-exon gene. Therefore, the first study (Chapter 2) focused on building upon the breadth of knowledge in the mouse system by applying an aggregation technique in order to generate chimeras that had a *Prdm14* rescue phenotype. This study used the CRISPR/Cas9 system to interrupt the function of *Prdm14* in one embryo and aggregated that embryo with pluripotent cells from either another embryo or embryonic stem cells. Donor cells consisting of either blastomeres from a wild-type embryo or control R1 ESC were able to contribute to the germ cell population of chimeric mice. This demonstrated that these pluripotent cells were able to give rise to germ cells in a *Prdm14*-deficient embryo, and the founder animals were fertile. Unfortunately, when *Cwc15*$^{+/-}$ ESC were used as the donor cell type, the outcome was not the same. These *Cwc15*$^{+/-}$ cells were unable to contribute to the germline, and therefore unable to rescue the *Prdm14* knockout phenotype.

The inability for *Cwc15*$^{+/-}$ ESC to contribute to the germline is unsurprising, as they had been shown previously in our lab to only generate somatic chimeras. It was hypothesized that the blastocyst complementation technique would be able to

facilitate the incorporation of the *Cwc15*$^{+/-}$ into the PGC niche as competition from endogenous PGC had been removed. However, this approach was unable to overcome the limitations of the *Cwc15*$^{+/-}$ ESC line. The mouse stem cell field is lacking a technique to allow for the generation of germline chimeras from ESC lines that are questionable in their characterization as fully pluripotent. Previous research has shown a need for such a technique, as there have been numerous reports of ESC that have been unable to contribute to the germline in chimeras (Pacholczyk, Suhag et al. 2008, Coleman, Brennan et al. 2015, Cotton, Meilak et al. 2015).

Recently, blastocyst complementation has become a popular technique to direct cells toward a specific lineage. There have been numerous reports of mouse-rat chimeras made via blastocyst complementation in order to generate organs and specific cell types from one species within the fetus of the other species (Isotani, Hatayama et al. 2011, Isotani, Yamagata et al. 2016, Yamaguchi, Sato et al. 2017). A landmark publication in this field was the generation of rat pancreas in a pancreatogenesis-disabled mouse (Kobayashi, Yamaguchi et al. 2010). The blastocyst complementation technique is coming of use for biomedical applications, as researchers are seeking a way to grow human organs in other species for potential use as transplants (Wu, Platero-Luengo et al. 2017). While it is a well-developed technique to generate mouse-rat chimeras, it is still being refined for use in generating interspecies chimeras between higher order mammals (Wu, Greely et al. 2016).

The biomedical field is rapidly developing techniques to attempt generating human organs inside large animals, most notably the pig. Introductory studies using the mouse and rat to determine the feasibility of interspecies chimeras have

determined that this technique is possible. The first report of human-pig chimeras shows that it is possible to have human iPSC incorporated into a porcine fetus up until E28, albeit at low frequency and low levels of chimerism (Wu, Greely et al. 2016). The low level of contribution by the human cells within the pig embryo may be due to the evolutionary distance between pig and human, and studies between human and non-human primates may show a higher degree of success, though laws in many countries currently prohibit such studies (Wu, Greely et al. 2016).

There are ethical concerns that human pluripotent cells injected into non-human animals have the possibility to contribute to germ cell and neuronal lineages. This could be addressed by limiting the contribution of such stem cells to certain lineages, similar to the *Prdm14* chimeric mouse study presented here. One approach to do this is to limit the ability of the pluripotent cells to contribute to only the organ of interest by modifying their gene expression (Rashid, Kobayashi et al. 2014, Wu, Greely et al. 2016). Preliminary mouse studies using pluripotent cells that have been committed to the endodermal lineage via expression of *Mix1* have been able to guide the directed cells toward endodermal organs (Kobayashi, Kato-Itoh et al. 2015).

There remains a variety of editing techniques that may be used in order to reduce the potential for human pluripotent cells to contribute to non-target lineages: the use of committed progenitor cells instead of pluripotent cells or the introduction of suicide genes under neural- or germ cell-specific promoters (Wu, Greely et al. 2016). These techniques may allow for generation of humanized organs with little chance for off-target humanized tissue.

As evidenced by the ongoing studies using large animals for biomedical research, there is a need for basic biological research in such animals. Because pigs in particular are becoming widely used as a bridge model species between rodents and humans, it is first necessary to determine if they exhibit similar developmental biology as compared to humans. To that end, the second study (Chapter 3) sought to determine if *PRDM14* had the same role in early embryo development in pig as it does in mouse.

In order to address this question, embryos from various stages of development were collected from artificially inseminated gilts. These embryos from zygote, 2-cell, 4-cell, morula, blastocyst, and E26 stages of development were analyzed for gene expression of *PRDM14* as well as other germ cell-related genes. Because *Prdm14* is expressed as early as the 2-cell stage in the mouse, it was hypothesized that it may also be functional at a similar time point in pig development. In addition to *PRDM14*, several other genes were included for analysis based on their role in the germ cell program (*DAZL*, *VASA*, *STRA8*, *PRDM1*, *TFAP2C*), their interactions with *PRDM14* (at least as shown in the mouse system; *CARM1*, *TET1*, *TET2*), or their involvement in pluripotency (*POU5F1*, *NANOG*, *SOX2*, *ESRRB*). The data included here indicate that *PRDM14* and other PGC and germ cell-related genes are not expressed during early pig development. For *PRDM14* in particular, this is in contrast to the expression pattern seen in the mouse system, indicating that the role in early embryo development may not be conserved between these two species (Burton, Muller et al. 2013, Grabole, Tischler et al. 2013).

There have been relatively few studies describing the transcription profile of early pig embryos. Some have focused on differences in transcription between manipulated and non-manipulated embryos (Zhou, Dobrinsky et al. 2014), while others have sought to characterize the normal transcription profile of a specific area of interest such as metabolomics, small RNAs, or pluripotency (Yang, Du et al. 2012, Hall and Hyttel 2014, Krisher, Heuberger et al. 2015). However, none have focused on the role of germ cell factors during these early preimplantation time points.

The data collected from the second study suggest a different role for *PRDM14* within the early pig embryo, as expression was low during these early time points. Based on these findings, the next study (Chapter 4) was performed to determine the role of *PRDM14* within the porcine germ cell program. This was done by using the CRISPR/Cas9 system to generate functional knockouts by disrupting the coding sequence of the gene early in exon 1. In the first experiment, a single guide RNA was used to interrupt the gene. After animals were grown to puberty, they still retained germ cells.

Therefore, a second attempt was made using two guide RNAs in case the first experiment had not resulted in a functional disruption to the gene. In the second attempt, PRDM14 protein was still detected in gonads of both male fetuses, although RNA was undetectable in male 2. However, due to the mosaicism present in these fetuses, a third attempt at generating a knockout was made by performing SCNT using fibroblasts from male 2. In this third attempt at generating a phenotypic knockout for *PRDM14*, 2 males exhibited an absence of protein while 2 males still expressed protein. This also correlated with the transcript data. Overall, the knockout

animals made in this experiment all still had germ cells present, indicating that while *PRDM14* was successfully inhibited, it is not required for germ cell formation in the pig.

While this is the first report of the knockout of *PRDM14* in pigs, it is not the first report of a gene within the germ cell lineage that has been targeted for knockout. Pigs have also been targeted for *NANOS2* ablation (Park, Kaucher et al. 2017); *NANOS2* is responsible for male fertility in mice, *Drosophila*, and humans (Kobayashi, Yamada et al. 1996, Forbes and Lehmann 1998, Tsuda, Sasaoka et al. 2003, Kusz, Tomczyk et al. 2009, Sada, Suzuki et al. 2009). Unlike *PRDM14*, *NANOS2* contains a single exon which when targeted by the CRISPR/Cas9 system generates a more sizeable interruption to the gene's function. *NANOS2* has been shown to have a conserved function among these 4 species (Park, Kaucher et al. 2017).

In contrast to *NANOS2*, *PRDM14* does not seem to have conserved function from mouse to pig. Recent studies have demonstrated that while *PRDM14* is present in pig fetal gonads, its expression is faint and cytoplasmic (Kobayashi, Zhang et al. 2017). In contrast, *Prdm14* expression in the mouse system is strong and localized to the nucleus from the time of specification through migration and residence in the fetal gonad (Yamaji, Seki et al. 2008).

Evidence presented here in combination with recent studies describing its role in both humans and pigs suggest that *PRDM14* is not required for PGC development in pigs (Kobayashi, Zhang et al. 2017). At the time of PGC specification in pigs, *SOX17* is expressed at the posterior primitive streak, and a subset of SOX17 positive

cells also show PRDM1 expression. TFAP2C is also expressed in porcine embryos 1.5 days after initial SOX17 expression (Kobayashi, Zhang et al. 2017). Similar results were found in differentiation of human ESCs to PGCs *in vitro*, as *PRDM14* was also not required for this process (Kobayashi, Zhang et al. 2017). In combination with data from this pivotal publication, the data presented from the current *PRDM14* knockout study demonstrate another example of the failure of the mouse model system to mirror the developmental biology of pigs.

Future studies in the area of PGC development in pigs and humans are likely to be centered around the role of *SOX17* and *PRDM1*, as there are multiple reports of their expression within human PGCLCs, and their use in inducing the PGCLC fate *in vitro* (Lin, Chiu et al. 2014, Irie, Weinberger et al. 2015, Irie and Surani 2017, Mitsunaga, Odajima et al. 2017, Gomes Fernandes, Bialecka et al. 2018). These two genes have been identified as excellent candidates required for specification of the PGC fate in pigs, and future knockout studies should focus on either these to fully determine their role in the germ cell lineage.

In conclusion, the studies explored in this dissertation have contributed to our understanding of the role of *PRDM14* in both pigs and mice. While it is an essential gene in mice that is required for PGC development, it is dispensable within the pig system. This research demonstrates that gene function in one species does not always mirror gene function in another species. In this case, both humans and pigs have a lessened need for *PRDM14* within the germ cell program. The results of these studies demonstrate that a bridge model is needed between rodent and human, as gene function is not always recapitulated between these two species. In the last 15 years,

there has been a recent surge in the field of pig biomedical research as more researchers have recognized this need. Further research using pigs as a biomedical research model can help bridge this gap and elucidate their similarities to human reproductive biology.

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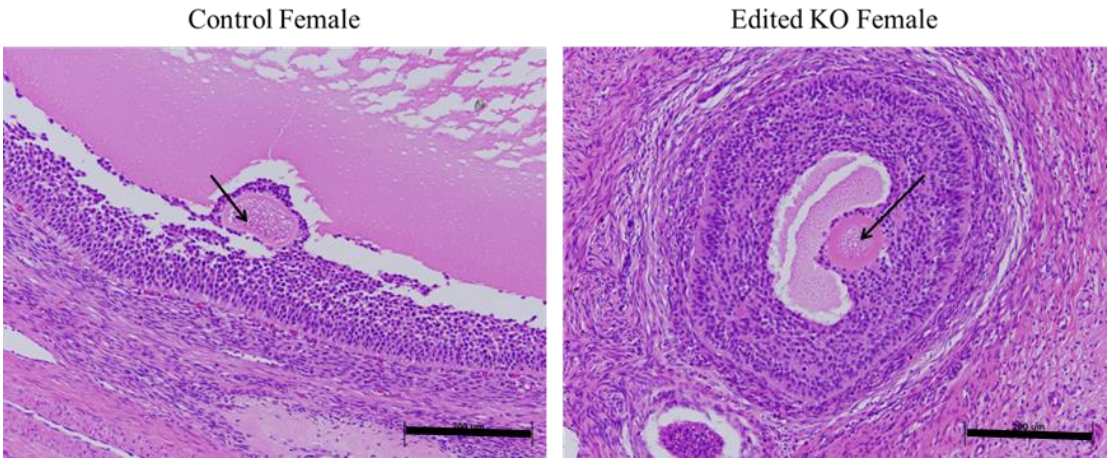
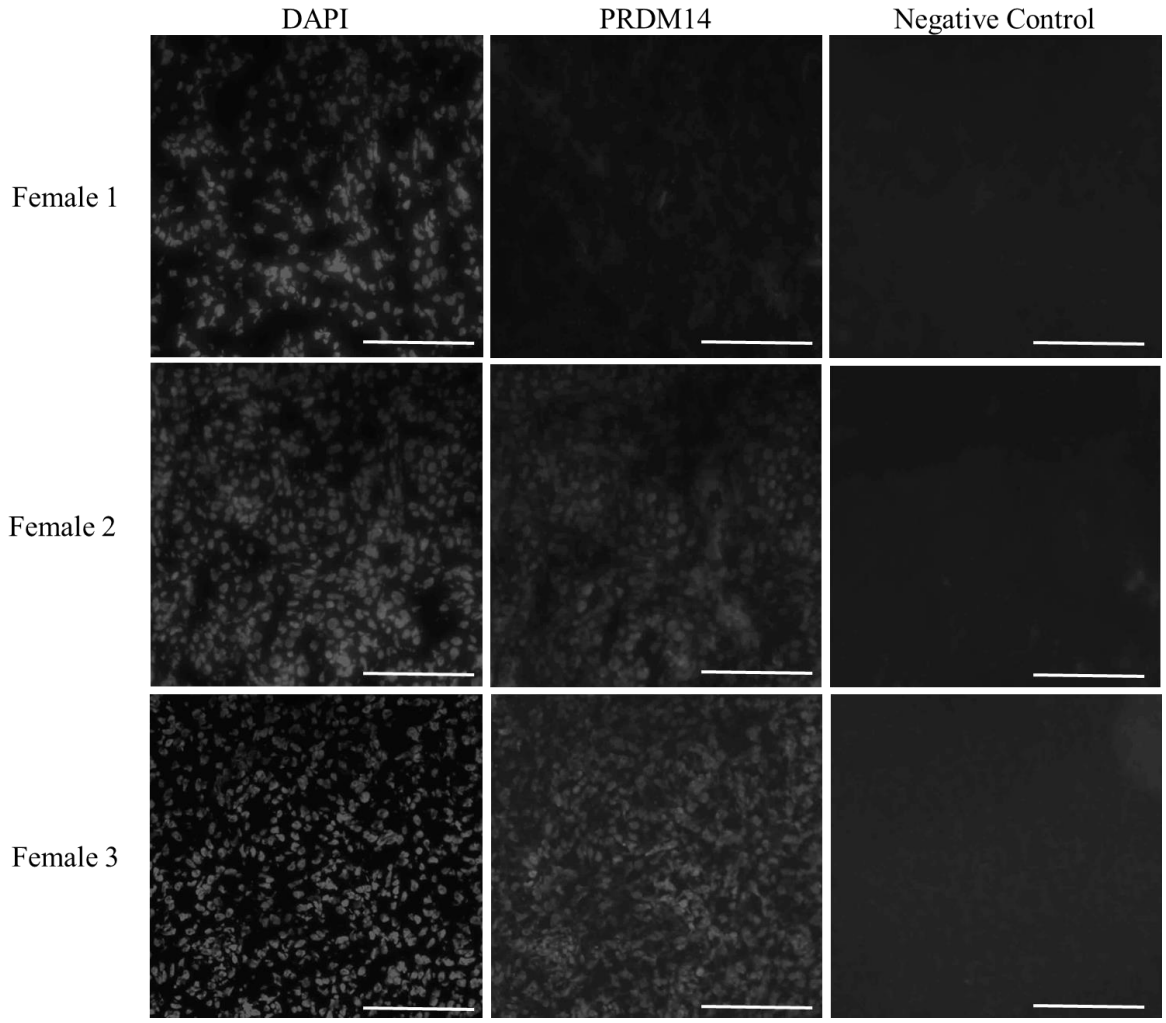
Appendix



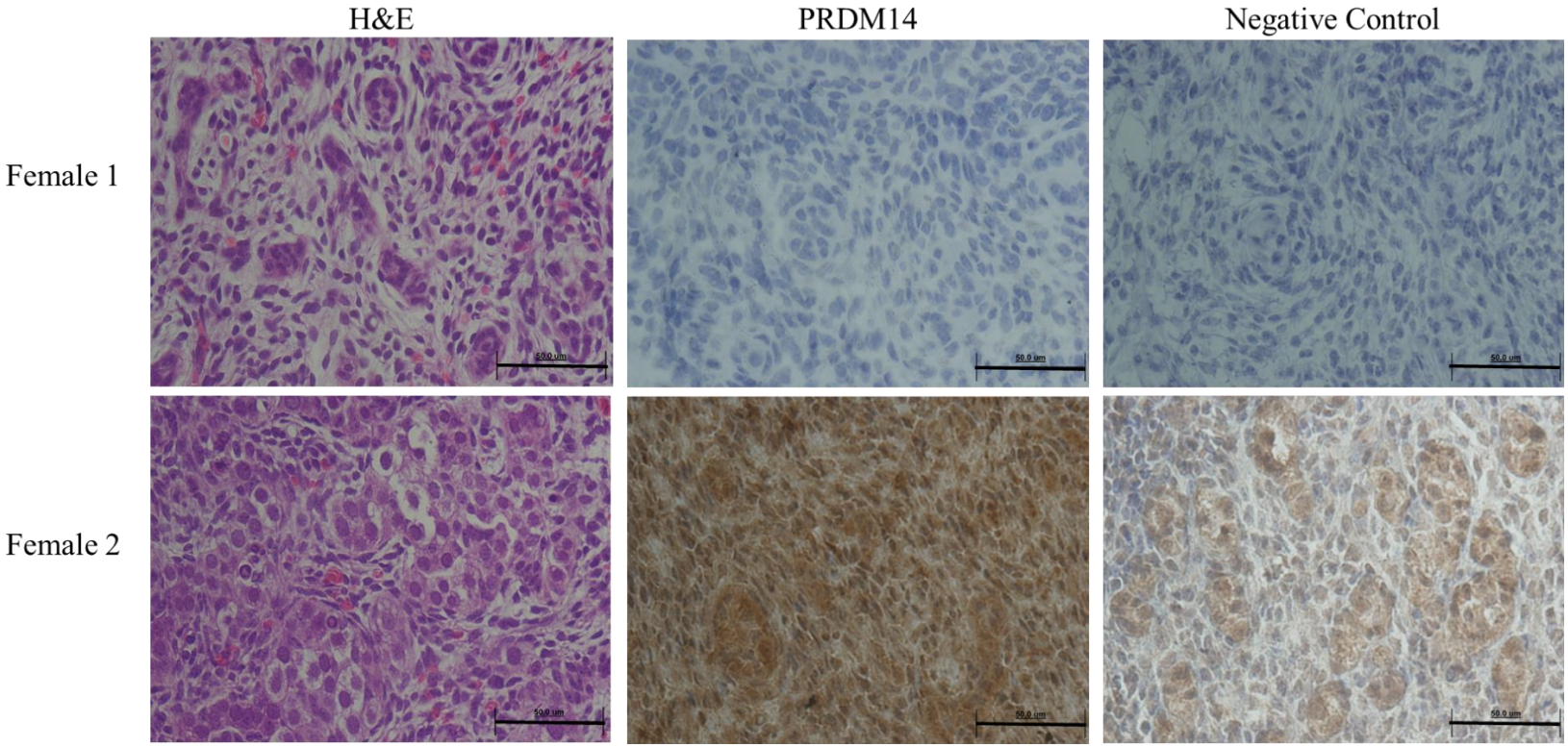
Supplementary Figure 1. Control and KO female gonads; H&E staining. Arrows indicate oocytes. Scale bar: 200 µm.



Supplementary Figure 2. Fluorescent immunohistochemistry images of PRDM14 from female fetuses in experiment two. Scale bar: 500 μm.

Supplementary Table 1. Genotypes of female fetuses generated from SCNT. bp: base pair; aa: amino acid.

Fetus	Sex	Genotype	Edits (nucleotide)	Edits (amino acid)
1	F	Heterozygous	246 bp deletion; 186 bp deletion	82 aa deletion 62 aa deletion
2	F	Heterozygous	246 bp deletion; 186 bp deletion	82 aa deletion 62 aa deletion



Supplementary Figure 3. H&E and immunohistochemical staining of cloned female fetuses. Scale bar: 50 μm.

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