12-2017

Identifying Genetic Factors Influencing Sperm Mobility Phenotype in Chicken using Genome Wide Association Studies, Primordial Germ Cell Transplantation, and RNAseq.

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Identifying Genetic Factors Influencing Sperm Mobility Phenotype in Chicken using Genome Wide Association Studies, Primordial Germ Cell Transplantation, and RNAseq.

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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December 2017
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ABSTRACT

Sperm mobility is a major determinant of male fertility in chicken. In spite of low heritability of reproductive traits, sperm mobility has high heritability index which suggests presence of quantitative trait loci (QTLs) governing the trait. Our research focused on three objectives: i) to identify the QTLs affecting low mobility phenotype in chicken, ii) to understand the impact of Sertoli-cells and germ cells interactions in influencing the mobility phenotype and iii) to identify the genes and gene networks differentially expressed in male and female PGCs. To detect the QTLs, genome wide association studies (GWAS) was conducted which revealed the presence of multiple minor alleles influencing the trait and indicated the role of epistasis. The second section of research involved isolation, culture and transfer of primordial germ cells (PGCs) to create high line germ line chimera chicken carrying low line PGCs. We established the culture of chicken PGCs isolated from the embryonic blood in a feeder free culture conditions but could not detect the presence of low line genotype in the semen of transgenic males. Our final study involved RNA-sequencing (RNAseq) of male and female PGCs to identify differentially expressed genes from their transcriptomes. We identified five candidate genes: 3-hydroxy-3-methylglutaryl CoA reductase (HMGCA), germ cell-less (GCL), SWIM (zinc finger SWIM domain containing transcription factor), SLC1A1 (solute carrier family 1 member 1), UBE2R2L (ubiquitin conjugating enzyme) and validated their expression level in male and female PGCs by RT-qPCR. GCL was exclusively expressed in males while SLC1A1 & UBE2R2L were expressed only in female cPGCs. This present study provides novel gender specific germ cell markers in the broiler chicken. These results will help in elucidating the genetic programming of gender specific germ line development in broilers.

ACKNOWLEDGMENTS

I would like to pay my deepest gratitude and sincere thanks to my advisor and mentor, Dr. Douglas D. Rhoads without whom this arduous journey of PhD wouldn't have seen its day. From day one till the end of my graduate program, his constant guidance and support encouraged me to work innovatively towards my research. He played a pivotal role in developing my scientific aptitude that helped me evolve as a researcher. He is not only a great advisor but a protective father figure who supported and encouraged me in every step of my graduate life. I thank him from the core of my heart for believing in me and helping me to achieve my goals.

I am extremely grateful to Dr. David Froman from Oregon State University for providing crucial samples for my work and for constantly providing fresh ideas and advice that immensely helped me throughout my research. I am also thankful to Dr. Carl Schmidt and his lab for providing their valuable time and guidance in RNAseq techniques and data analysis.

I am thankful to Dr. Suresh Kumar Thallapuranam who has been a great source of inspiration. His enthusiastic approach towards teaching and research has always filled me with awe and inspired me to be a better student and researcher.

I am highly appreciative to Ms. Nhung Thi Nguyen who worked with me in this project. She has been a tremendous team player who was enthusiastic in her approach and provided crucial inputs that were pivotal in furthering this work. Besides, she has been a great friend that encouraged me to meet my goals and deadlines.

I was privileged to work with Dr. Adnan Ali Khalaf Al Rubaye who played the role of a helpful colleague, encouraging mentor, guide and protective brother from the day one of my graduate

life. My sincere thanks to him for his kind words of reassurance whenever I was in doubt and instilling confidence in me whenever I needed it the most.

I find myself extremely lucky to share my journey with awe-inspiring lab mates: Ms. Khaloud Al-Zahrani, Ms. Alia Parveen, Ms. Sura Faris Salih Zaki, Mr. Nnamdi Simon Ekesi, Mr. Abdulkarim Shwani, Mr. Amer Hasan, Dr. Shatovisha Dey, and Dr. Katy Tarrant. While working in such a healthy and cordial environment, I earned valuable friendships. My colleagues supported me, helped me and always had unwavering faith in my abilities. I thank them from the bottom of my heart.

I am indebted to my friends: Diksha, Gurpreet, Sonia, Alia, Julie, Prashanth, Syam, and Nnamdi who are my rock, my greatest critique and my inspiration. I would like to take this opportunity to pay my gratitude to them.

I am grateful to Dr. Ragupathy Kannan for his guidance that helped me to acquire PhD position in the University of Arkansas. I would also like to thank Becky, Heidi, Mike and rest of the staff of Biological Science Department who have been so kind and enduring with me. I am obliged to my defense committee for their supportive and encouraging attitude towards me.

I am indebted to my parents and my sisters without whom this whole journey wouldn’t have even started. Their unconditional love and support instilled immense confidence in me and made me the person I am today. I am privileged to have them as my family and would like to pay my deepest regards for their patience and faith in me.

Finally, I would like to thank all those who have contributed directly or indirectly in pursuing my goals.

LIST OF CONTENTS

LIST OF ABBREVIATIONS

ATP: Adenosine-tri-phosphate

bFGF: Basic fibroblast growth factor

BTB: Blood-testis barrier

BuDMF/O: Busulfan/DMF sesame oil emulsion

Busulfan: 1,4-Butanediol dimethylsulfonate

CEFs: Chicken embryonic fibroblasts.

CNS: Central nervous system

cPGCs: Chicken primordial germ cells

CPVs: Copy number variations

CVH: Chicken vasa homologue

DAPI: 4`, 6-Diamidino-2-phenylindole dihydrochloride

DAZL: Deleted in azoospermia like

DMEM: Dulbecco's modified Eagle medium

DMF: N, N-dimethylforamide

dpc: days post-coitum

ECs: Embryonic carcinoma cells

EGs: Embryonic germ cells

EMA-1: Embryonic mouse antigen -1

ESCs: Embryonic stem cells

FITC: Fluorescein isothiocyanate

FSH: Follicle stimulating hormone

GCL: Germ cell less spermatogenesis associated

GWAS: Genome wide association studies

HH stage: Hamburger and Hamilton stage

HL: Cross between high line male and low line female

HMGCA: 3-hydroxy-3-methylglutaryl CoA reductase

HWE: Hardy Weinberg equilibrium

IACUC: Institutional animal care and use committee

IGF-1: Insulin growth factor

ITGA-6: Integrin subunit alpha 6

ITGB-1: Integrin beta 1

LCFAs: Long chain fatty acids

LxH: Cross between low line male and high line female

LH: Leutinizing hormone

LIF: Leukocyte inhibitory factor

LMN: Chicken hepatocarcinoma line

MAF: Minimum allele frequency

Mbp: Mega base pairs

NGS: Next generation sequencing

NH: New Hampshire

OmA: Omphalomesenteric artery

PAS: Periodic-Acid-Schiff

PBS: Phosphate buffer saline

PMN leukocytes: Polymorphonuclear leukocytes

qPCR: Quantitative PCR

RT-qPCR: Quantitative reverse-transcriptase real time PCR

QTLs: Quantitative trait loci

Ribo: 18 S ribosomal sequence

RNA-seq: RNA sequencing

FPKM: Fragments per Kilobase of transcripts per Million mapped reads

S.D.: Standard deviation

SCF: Stem cell factor

SCTJs: Sertoli cells tight junctions

SDF-1: Stromal cell derived factor-1

SLC1A1: Solute carrier family 1 member 1

SMT: Sperm mobility test

SNPs: Single nucleotide polymorphisms

SSCs: Spermatogonial stem cells

SSEA: Stage specific embryonic antigen

SST: Sperm storage tubules

STO: Sandoz inbred mouse-derived thioguanine resistant and oubain resistant

TJs: Tight junctions

UBE2R2L: Ubiquitin conjugating enzyme

UVJ: Utero-vaginal junction

WL: White Leghorn

Wxho: W chromosome Xho-1 repetitive sequence

CHAPTER 1

Introduction

The rise of broiler industry in the United States (U.S.)

The Unites States (U.S.) broiler meat industry is one of the most profitable and thriving meat industries due to increased demand in both domestic and international markets. After Brazil, U.S. is the world's second largest exporter of broiler meat (Davis *et al*., 2013; Global Trade Information Services, 2012). Since 2004, the poultry meat industry has surpassed red meat (beef and pork) industries as the major meat product export for the U.S. (USDA, Economic Research Service, 2011). Increased health awareness by consumers, fewer religious restrictions on broiler meat consumption and higher efficiency to convert feed into meat has accentuated the pace of poultry production in the U.S. domestic market (Farrell, 2010; English *et al*., 2004; Davis *et al*., 2013). The escalating domestic broiler production was the stimulating factor for the exceptional rise in broiler meat exports. Intense genetic selection on production traits, improved management practices, and increased feed-conversion ratio has led to dramatic increases in the efficiency of broiler meat production (Havenstein *et al*., 2003; Schwean-Lardner *et al*., 2013; Askit *et al*., 2006).

Intensive selection vs reproduction

Genetic improvements in poultry production traits are the major force behind the exceptional growth of the broiler industry but there have also been negative effects on the heath issues owing to excessive body weight such as pulmonary hypertension (Julian, 1998), cardiovascular diseases (Julian, 1993), increased fat deposition (Griffin, 1996; Havenstein *et al*., 2003) and reproductive traits such as delayed sexual maturity and reduced male fertility (Goerzen *et al*., 1996; Barbato, 1999; Siegel and Dunnington, 1985; Hocking, 1990). It has been proposed that the intensive genetic selection of economically important traits has a negative impact on the selection of secondary sexual characters, libido and male mating potential ultimately leading to reduced flock

fertility (Pollock, 1999). Poor semen quality, and inability to copulate in the natural environments due to overweight directly jeopardizes the fertilization potential of male broilers (Hocking and Bernard, 2000). Decrease in fertility causes reduction in the hatchability percentage, which indirectly creates hindrance in the overall success of the poultry industry (Zakaria *et al.*, 2005).

Factors affecting hatchability

Analysis of the primary broiler breeder industry by Pollock revealed that increase of only 1% in hatchability of an integrator capable of retaining 15 million eggs set, could lead to an increase of $30,000/week (Pollock, 1999). According to a recent survey by the USDA (USDA, National Agriculture Statistics Service, 2017), the average hatchability percentage of broiler chicken was 83% during a week. The remaining 17% of the incubated eggs were trashed due to their inability to hatch. Many studies have focused on various putative factors affecting hatchability. These include the effects bird factors: hen age (Tona *et al*., 2004; Nowaczewski *et al*., 2016), light vs heavy breeds (Hudson *et al*., 2001), meat strain vs egg strain; egg factors: weight (Patra *et al*., 2016), shell thickness (Yamak *et al*., 2016), shell porosity, shape index, consistency of contents, size (Iqbal *et al*., 2017; Narushin and Romanov, 2002); egg incubation factors: temperature, humidity, ventilation, natural vs artificial incubation ; environmental factors: temperature and photoperiod; other factors: flock fertility (Zuidholf *et al.*, 2015), flock management practices (King'Ori, 2011), nutrition (Romero-Sanchez *et al.*, 2008). Reports have shown that the U.S. loses more than a billion eggs annually due to infertile eggs. This data points out the underlying problem of infertility because irrespective of best management and nutritional practices infertile eggs won't hatch.

Sperm mobility and fertility

The success of the broiler breeder industry depends largely on the fertility and hatchability of the incubated eggs. Fertility is a comprehensive term and is affected by many factors which include female and male attributes. Female attributes include egg quality, and physiological factors such as time spent by sperms in the sperm storage tubules (SSTs). SSTs are the specialized mucosal folds in the utero-vaginal junctions (UVJ), present at the caudal end of the oviduct where sperms are stored after insemination (Baksht, 1987). Unlike mammals, insemination is not always followed by fertilization in avian species (Ginsberg and Huck, 1989). After insemination, mature sperm are stored for prolonged duration in the SSTs and later are continuously released to the cranial end of oviduct, the site of fertilization (Wishart, 1987). The sperm quality and the protective microenvironment in the hen SSTs determine the survival efficiency of sperm to the point of successful fertilization in aves (Birkhead *et al*., 1999).

Male attributes specifically include semen quality traits like total sperm concentration, proportion of motile sperm, sperm morphology etc. At the industry level where the male to female ratio is generally 1:10, male contribution towards fertility becomes significantly greater than that of females (Parker and McDaniel, 2002). Both physical characteristics and semen quality traits are used to predict male fertility in the breeding industry. Selection based solely on male physical attributes is not a reliable tool to predict fertility (Wilson *et al*., 1979), the focus has been shifted towards semen traits to predict the fertilizing potential of males. One of the variable traits that significantly affects the semen quality and hence fertility is sperm mobility (Froman *et al*., 2002). Even though fertility is not a highly heritable trait (0.06-0.13) (Sapp *et al*., 2004), the heritability index of sperm mobility in chicken is high, $h^2 = 0.30$ (Froman *et al*., 2002). Being a quantitative trait and a direct determinant of male fertility (Froman and Rhoads,

2013), the sperm mobility phenotype provides an important tool to understand the underlying mechanisms that govern fertility. Froman and group created high and low sperm mobility lines by exploiting the high heritability of mobility trait (Froman *et al.*, 2000). Both lines contain males with high and low mobile sperms but the proportion of highly mobile sperm was significantly higher in the high line as compared to the low line. Previous studies on roosters selected for mobility trait, have pointed out defective mitochondrion, poor energy dynamics (Froman et al., 2011, 2013) and inability of motile sperm to effectively transit through SSTs as some of the reasons for poor mobility phenotype affecting fertility. These findings suggest the involvement of genetic components in influencing the mobility and hence fertility.

The objective of this research was to identify single nucleotide polymorphisms (SNPs) within the lines selected for mobility phenotype. The significant SNPs may provide genetic markers for selection for sperm mobility phenotype and hence could be used to detect highly fertile males at an early stage. Using Primordial Germ Cells (PGCs) as a tool, this study also attempts to understand the effect of somatic cell-germ cell interactions on the phenotype of developing sperms. This study could fill gaps in our understanding of male germ cell biology as to whether it is merely the genetic constitution of sperm cells that control its physical attributes, or it's the interaction between somatic cells and germ cells that determine the sperm mobility phenotype.

In the next chapter, a review is made on the effect of sperm mobility on fertility, spermatogenesis and the gaps in our knowledge, the concept of PGCs and their application with respect to this study, and last but not the least the use of PGCs as a tool to understand the influence of somatic cells in gamete physiology.

REFERENCES

Akşit, M., Yalcin, S., Özkan, S., Metin, K., & Özdemir, D. (2006). Effects of temperature during rearing and crating on stress parameters and meat quality of broilers. *Poultry Science*, *85*(11), 1867-1874.

Bakst, M. R. (1987). Anatomical basis of sperm-storage in the avian oviduct. *Scanning microscopy*, *1*(3), 1257-1266.

Barbato, G. F. (1999). Genetic relationships between selection for growth and reproductive effectiveness. *Poultry Science*, *78*(3), 444-452.

Birkhead, T. R., Martinez, J. G., Burke, T., & Froman, D. P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London B: Biological Sciences*, *266*(1430), 1759-1764.

Davis, C. G., Harvey, D., Zahniser, S., Gale, F., & Liefert, W. (2013). Assessing the growth of US broiler and poultry meat exports. *A Report from the Economic Research Service. USDA*, 1-28.

English, D. R., MacInnis, R. J., Hodge, A. M., Hopper, J. L., Haydon, A. M., & Giles, G. G. (2004). Red meat, chicken, and fish consumption and risk of colorectal cancer. *Cancer Epidemiology and Prevention Biomarkers*, *13*(9), 1509-1514.

Farrell, D. (2010). The role of poultry in human nutrition. Poultry development review. Food and Agriculture Organization of the United Nations, Rome, Italy, pp: 2. http://www.fao.org/docrep/013/al709e/al709e00.pdf

Froman, D. P., & Feltmann, A. J. (2000). Sperm mobility: phenotype in roosters (Gallus domesticus) determined by concentration of motile sperm and straight line velocity. *Biology of Reproduction*, *62*(2), 303-309.

Froman, D. P., & Rhoads, D. D. (2013). Breeding and genetics symposium: A systems biology definition for chicken semen quality. *Journal of animal science*, *91*(2), 523-529.

Froman, D. P., Feltmann, A. J., Pendarvis, K., Cooksey, A. M., Burgess, S. C., & Rhoads, D. D. (2011). Physiology and endocrinology symposium: a proteome-based model for sperm mobility phenotype. *Journal of animal science*, *89*(5), 1330-1337.

Froman, D. P., Pizzari, T., Feltmann, A. J., Castillo-Juarez, H., & Birkhead, T. R. (2002). Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, Gallus gallus domesticus. *Proceedings of the Royal Society of London B: Biological Sciences*, *269*(1491), 607-612.

Ginsberg, J. R., & Huck, U. W. (1989). Sperm competition in mammals. *Trends in Ecology & Evolution*, *4*(3), 74-79.

Global Trade Information Services (GTIS). (2012). *World Trade Atlas*. Machine readable database. Internet edition 4.7e. Columbia, South Carolina. www.gtis.com.

Goerzen, P. R., Julsrud, W. L., & Robinson, F. E. (1996). Duration of fertility in ad libitum and feed-restricted caged broiler breeders. *Poultry Science*, *75*(8), 962-965.

Griffin, H. D. (1996). Understanding genetic variation in fatness in chickens. *Annual report*, *95*(96), 35-38.

Havenstein, G. B., Ferket, P. R., & Qureshi, M. A. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poultry Science*, *82*(10), 1500-1508.

Hocking, P. M. (1990). The relationships between dietary crude protein, body weight, and fertility in naturally mated broiler breeder males. *British Poultry Science*, *31*(4), 743-757.

Hocking, P. M., & Bernard, R. (2000). Effects of the age of male and female broiler breeders on sexual behaviour, fertility and hatchability of eggs. *British Poultry Science*, *41*(3), 370-376.

Hudson, B. P., Lien, R. J., & Hess, J. B. (2001). Effects of body weight uniformity and pre-peak feeding programs on broiler breeder hen performance. *Journal of Applied Poultry Research*, *10*(1), 24-32.

Iqbal, J., Mukhtar, N., Rehman, Z. U., Khan, S. H., Ahmad, T., Anjum, M. S., Pasha, R. H., & Umar, S. (2017). Effects of egg weight on the egg quality, chick quality, and broiler performance at the later stages of production (week 60) in broiler breeders. *The Journal of Applied Poultry Research*, *26*(2), 183-191.

Julian, R. J. 1993. Ascites in Poultry. *Avian Pathol*ogy, *22*(3), 419-454.

Julian, R. J. 1998. Rapid growth problems: Ascites and skeletal deformities in broilers. *Poult*ry *Science*, *77*(12), 1773-1780.

King'Ori, A. M. (2011). Review of the factors that influence egg fertility and hatchability in poultry. *International Journal of Poultry Science*, *10*(6), 483-492.

Narushin, V. A., & Romanov, M. N. (2002). Egg physical characteristics and hatchability. *World's Poultry Science Journal*, *58*(3), 297-303.

Nowaczewski, S., Babuszkiewicz, M., & Kaczmarek, S. (2016). Effect of broiler breeders' age on eggshell temperature, embryo viability and hatchability parameters. *Annals of Animal Science*, *16*(1), 235-243.

Parker, H. M., & McDaniel, C. D. (2002). Selection of young broiler breeders for semen quality improves hatchability in an industry field trial. *The Journal of Applied Poultry Research*, *11*(3), 250-259.

Patra, M. K., Sanchu, V., Ngullie, E., Hajra, D. K., & Deka, B. C. (2016). Influence of egg weight on fertility and hatchability of backyard poultry varieties maintained under institutional farm conditions. *The Indian Journal of Animal Sciences*, *86*(8).

Pollock, D. L. (1999). A geneticist's perspective from within a broiler primary breeder company. *Poultry Science*, *78*(3), 414-418.

Romero-Sanchez, H., Plumstead, P.W., Leksrisompong, N., Brannan, K.E. & Brake, J. (2008) Feeding Broiler Breeder Males. 4. Deficient Feed Allocation Reduces Fertility and Broiler Progeny Body Weight. *Poultry Science*, 87 (4), 805–811.

Sapp, R. L., Rekaya, R., Misztal, I., & Wing, T. (2004). Male and female fertility and hatchability in chickens: A longitudinal mixed model approach. *Poultry Science*, *83*(8), 1253-1259.

Schwean-Lardner, K., Fancher, B. I., Gomis, S., Van Kessel, A., Dalal, S., & Classen, H. L. (2013). Effect of day length on cause of mortality, leg health, and ocular health in broilers. *Poultry Science*, *92*(1), 1-11.

Siegel, P. B., & Dunnington, E. A. (1985). Reproductive complications associated with selection for broiler growth. *Poultry genetics and breeding*, *1985*, 59-72.

Tona, K., Onagbesan, O., De Ketelaere, B., Decuypere, E., & Bruggeman, V. (2004). Effects of age of broiler breeders and egg storage on egg quality, hatchability, chick quality, chick weight, and chick posthatch growth to forty-two days. *Journal of Applied Poultry Research*, *13*(1), 10-18.

U.S. Department of Agriculture, Economic Research Service (ERS). (2011). "Red Meat and Poultry Yearbooks: U.S. Beef, Pork, and Broiler Per Capita Consumption - Retail Weight, 1997–2010."

USDA (2017). Broiler Hatchery. National Agricultural Statistics Service. http://usda.mannlib.cornell.edu/usda/current/BroiHatc/BroiHatc-05-17-2017.pdf. Accessed May, 2017.

Wilson, H. R., Piesco, N. P., Miller, E. R., & Nesbeth, W. G. (1979). Prediction of the fertility potential of broiler breeder males. *World's Poultry Science Journal*, *35*(02), 95-118.

Wishart, G. J. (1987). Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. *Journal of reproduction and fertility*, *80*(2), 493-498.

Yamak, U. S., Sarica, M., Boz, M. A., & Ucar, A. (2016). The effect of eggshell thickness on hatching traits of partridges. *Revista Brasileira de Ciência Avícola*, *18*(SPE), 13-18.

Zakaria, A. H., Plumstead, P. W., Romero-Sanchez, H., Leksrisompong, N., Osborne, J., & Brake, J. (2005). Oviposition pattern, egg weight, fertility, and hatchability of young and old broiler breeders. *Poultry Science*, *84*(9), 1505-1509.

Zuidhof, M. J., Holm, D. E., Renema, R. A., Jalal, M. A., & Robinson, F. E. (2015). Effects of broiler breeder management on pullet body weight and carcass uniformity. *Poultry Science*, *94*(6), 1389-1397.

CHAPTER 2

Literature Review

Sperm mobility and fertility

Sperm motility, a semen quality attribute, has been used widely in the livestock industries, and human medicine, to estimate the fertilization potential for males. Sperm motility, as the name indicates, is the ability of sperm to move. This movement can be progressive (the net gain in distance due to unidirectional movement) or non-progressive (no net gain in distance due to circular movement). Although sperm motility assays are widely used due to their simplicity and quick results, it does not account for factors that hamper the net movement of sperm in female reproductive tract. Hence, a more reliable trait to determine semen quality is sperm mobility.

Sperm mobility is a quantitative trait in chicken and is defined as the net movement of sperm against physiological resistance at body temperature with straight line velocity of $\geq$ 30 μm/sec (VSL; the straight-line distance from beginning to end of a sperm track divided by the time taken) (Froman and Kirby., 2005). Sperm cells spend significant time in the SSTs in females after insemination (Fig.2.1). Duration in the SSTs can be from a few days to weeks. Physiological parameters within the SSTs play critical roles in sperm mobility. Even though a sperm is motile, it may not be progressively motile in native conditions, but a mobile sperm will have a definitive progressive movement.

Studies have shown that in competitive mating scenarios both ejaculate quantity and quality play a significant role in determining the paternity but in a time dependent manner (Birkhead *et al*., 1995; Colegrave *et al*, 1995; Birkhead and Biggins 1998; Pizzari *et al*., 2008). Prolonged sperm storage time in SSTs will result in ejaculates containing a higher proportion of highly mobile sperm. Whereas, the low mobile sperm tend to rapidly lose fertilizing potential irrespective of their quantity as they are rapidly lost from the SSTs (Froman *et al*.,2008) (Fig 2.2). Ejaculate quantity is certainly important because the more sperm cells the higher the chances of a few

mobile sperm to fertilize the ovum. This advantage of quantity over quality is highly time sensitive because only the high quality, mobile sperm can persist in SSTs for longer times and hence get more opportunities to fertilize the egg upon ovulation (Dzuik, 1996; Donoghue *et al*., 1998; Brillard & Antoine, 1990; Brillard and Baksht, 1990).

Out of various factors that affect successful fertilization in a domestic fowl, sperm mobility appears to be the most important factor in addressing fertility. To use the sperm mobility trait as a tool to categorize highly fecund males, Froman and group developed an *in vitro* sperm penetration assay that quantified the proportion of mobile sperm in semen samples. The assay uses a 6% w/v Accudenz solution, a non-ionic, biologically inert separation medium, that, owing to the differential medium density, forms an interface when a semen suspension is overlaid upon it (Froman and Feltmann, 2000). As sperm cells pass into the Accudenz layer, the absorbance of the Accudenz solution increases. After 5-min. incubation at 41^0C, the absorbance is measured, where absorbance is directly proportional to the number of mobile sperm cells in the sample. Using this assay, Froman and group demonstrated that sperm mobility is directly correlated with fertility (Froman *et al*., 1999) (Fig. 2.3).

Generation of high and low mobile sperm lines

Male fertility in domestic fowl is a function of sperm mobility (Birkhead *et al*., 1995; Froman *et al*., 1999). Owing to the high heritability index (h^2=0.30) of sperm mobility, Froman and group used selection based on sperm mobility to produce two divergent chicken lines with widely different sperm mobility phenotypes. Furthermore, studies on the mobility trait revealed that it is (i) a normally distributed trait (Fig. 2.4), (ii) age-independent, (iii) positively correlated with

motile sperm concentration (r=0.71) and sperm ATP content (Froman and Feltmann, 1998). The sperm mobility lines were also categorized based on quantitative parameters. Roosters having the mobility mean >1.5 S.D. (Standard deviation) above the population mean were considered as high mobility and those 1.5 S.D. below the population mean were termed as low mobility (Fig. 2.4) (Froman and Feltmann, 1998; Bowling *et al*., 2003).

Sperm mobility and mitochondria

The relationship between sperm motility and mitochondria has been extensively studied in both vertebrates and invertebrates (Wishart, G. J., 1982; Ford, W.C.L., 2006; Miki *et al*., 2004; Miki, K., 2006). Glycolysis and oxidative phosphorylation are two metabolic processes that generate energy in the form of ATP in sperm cells (Goldberg & Norman, 1961; Nascimento *et al*., 2008). The role of mitochondria becomes important since oxidative phosphorylation takes place in this organelle (Ruiz-Pesini *et al*., 2007). There is still debate about the relative contribution of glycolysis and oxidative phosphorylation in providing energy for sperm motility but mitochondrial contribution in generating oxidative energy in combination with its strategic position in the sperm is noteworthy. Mitochondria are situated in the sperm mid-piece and directly transfer energy to the tail filaments that ultimately facilitate in sperm motility (O'Connell *et al*., 2002).

Previous studies have focused their attention in determining the molecular cause of differential sperm mobility. Mitochondrial dysfunction came out as one of the prominent reasons (Froman and Kirby, 2005; Froman *et al*., 2011; Froman and Feltmann, 2005). To further illustrate the role of mitochondria in sperm motility, semen from the high and low mobility chicken lines was

analyzed (Froman *et al*., 2005). One of the physiological differences between these lines was the proportion of mobile sperm in their ejaculates. Semen from the high line roosters carried higher proportion of mobile sperm cells as compared to the low line roosters (Fig. 2.5). Mass spectrometric analyses of sperm ejaculates between the experimental high and low mobile lines showed high correlation between sperm ATP content, O_2 consumption and sperm motility (Froman and Kirby, 2005). The prevalence of aberrant mitochondrial ultrastructure was found to be significantly higher (40%) in immotile sperm (Froman and Kirby, 2005).

Sperm is dependent on mitochondrial oxidative phosphorylation and cytoplasmic glycolysis for fulfilling its energy needs (Bishop, 1962; Bedfort & Hoskins, 1990). To be reproductively efficient, the sperm must exhibit optimum motility in both male and female reproductive tracts. In avian species, sperm are immotile in the male reproductive tract (epididymis and deferent duct) but after ejaculation show differential motility (Ashizawa & Sano, 1990). Sperm penetration and storage in the hen's reproductive tract depends heavily on the mitochondrial integrity of sperm as only mobile sperm can transverse the vagina and enter into SSTs (Steele, 1992; Birkhead *et al*., 1999). The sperm storage in SSTs allows sustained release of sperm cells during the hen's egg production phase which ensure maximum fertility between inseminations (Bakst *et al*., 1994). The ascension of sperm in the SSTs is an active process. In the primary SSTs, the required energy is provided by the oxidation of endogenous long chain fatty acids (LCFAs) in the sperm's mitochondria. After reaching secondary SSTs, the epithelial cells of the infundibulum provide exogenous LCFAs which are metabolized in the outer mitochondrial membrane of the sperm. Once the energy reserves get exhausted or the oxidation of LCFAs discontinue due to either mitochondrial malfunction or its senescence, efflux of sperm from the secondary SSTs occurs which then move forward to fertilize the egg (Froman *et al*., 2003).

As previously discussed, semen of both high and low mobile line roosters contained different proportions of high and low mobile sperm (Fig.2.5). Ejaculates of low mobile roosters were found to have a higher proportion of immobile sperm with defective mitochondria. The fertilization potential of low mobile roosters was significantly reduced due to poor semen quality (Froman and McLean, 1996; Froman *et al*., 1997; Donoghue *et al*., 1998; Froman and Feltmann, 1998; Froman *et al*., 1999). In the female reproductive tract, the proportion of sperm ascending the vagina to reach SSTs was greatly diminished due to prevalence of immotile sperm in the low line ejaculates. Those sperm that managed to reach the secondary SSTs were prematurely released due to mitochondrial failure, hence drastically reducing the fecundity of the low sperm mobility line males (Froman *et al*.,2006). The propensity of premature mitochondrial failure in the low mobile sperm lines raises question as to whether this attribute is the result of underlying genetic predisposition that puts the mature sperm at risk within the excurrent system of rooster testes or whether the inherent factors in the male reproductive system causes delay in the movement of semen thus leading to mitochondrial senescence (Froman *et al*., 2010).

Spermatogenesis in avian species

Higher organisms procreate through sexual reproduction. This involves fusion of specialized reproductive cells called gametes from two sexually differentiated individuals. Gamete production occurs in the sexually mature individuals and the process of gametogenesis differs between male and females. In males, this process of gametogenesis is known as spermatogenesis while in females it is referred as oogenesis. In this section, we will focus on spermatogenesis and factors that influence this process.

Spermatogenesis is the process of specialized cell division that generates haploid gametes in the testes of sexually mature males. This process is divided into three phases: (i) spermatocytogenesis, where stem cells known as spermatogonia proliferate and get renewed by the process of mitotic cell division, (ii) meiosis, where primary spermatocytes (2n) proceed through a reductive cell division to form four spermatids (1n), and (iii) spermiogenesis, where spermatids differentiate into spermatozoa (Fig. 2.6) (Jones and Lin, 1992).

Spermatogenesis has been extensively studied in mammals especially in the context of the role of seminiferous tubule epithelial cells in the maintenance and proliferation of germ cells (Clemont, 1972; Lacy *et al*., 1969). Somatic cell types present in the testis which are involved in germ cell differentiation and proper development of testis includes Leydig cells (produce testosterone) (Mendis-Handagama, 1997), myoid cells (secrete basal lamina components) (Maekawa et al., 1996) and Sertoli cells. Sertoli cells form direct contact with the proliferating and differentiating germ cells and provide nutrition and structural support to them throughout spermatogenesis in the seminiferous tubules (Griswold, 1998). Sertoli cells form desmosome junctions with germ cells (Russell, 1977b) and control the movement of molecules and hormones between cells (Meng *et al*., 2005). Required steroidal and peptide hormones of the germ cells are provided by the Sertoli cells (Jones and Lon,1992). Sertoli cells also form the tight junctions (TJs) that form the blood- testis barrier (BTB) that creates an immunologically safe adluminal compartment for the haploid meiotic and post meiotic germ cells during differentiation process (Zhou *et al.*, 2002; Dym & Fawcett, 1970; Russell, 1977a; Bremner *et al*., 1994; Tsukita *et al*., 2001; Smith and Braun, 2012) (Fig.2.7). This provides the microenvironment for spermiogenesis and protect haploid spermatozoa from autoimmunity.

The division of germ cells is unique in various aspects: (i) it is the only cell division that reduces the chromosomal content to half, hence it needs to be sequestered in the immunologically privileged adluminal compartment of the seminiferous tubules across the blood-testis barrier, and (ii) the cell divisions followed by mitosis are incomplete in a way that the daughter cells after each division maintain connections between each other via cytoplasmic bridges (Jones and Lin, 1992) (Fig. 2.6). These daughter cells, besides maintaining contact with each other, are also intimately associated with the Sertoli cells which in turn regulate the developmental processes of stem spermatogonium through successive divisions until the final stage of spermiation (Russell, 1977b; Meng *et al*., 2005).

We can conclude from the literature that sperm mobility, a direct determinant of fertility in chicken, is a quantitative trait with high heritability index. This suggests that whatever genetic elements are responsible for this trait get transferred from one generation to another by germ cells. Since there is intimate contact of germ cells with somatic cells of seminiferous epithelium especially the Sertoli cells throughout spermatogenesis, there arises a question as to whether the mobility trait is solely determined by genetic components of the developing sperm or whether this trait is influenced by somatoplasm to germplasm interactions?

Primordial germ cells: precursors of germ cells

Sexually reproducing organisms transfer their genetic information through gametes; sperms for males and eggs for females. The primitive germ cells, also known as primordial germ cells (PGCs) that give rise to germ cells are set aside early in embryogenesis to separate them from the mortal somatic lineages (Shim *et al*., 1997). These cells are so important that the process of

sequestering germ cells early in embryonic development is conserved evolutionarily throughout the animal phylogeny. Extensive studies on both invertebrates and vertebrates has confirmed PGC presence at the earliest stages of embryogenesis (Illmensee and Mahowald, 1974; Fujimoto *et al*., 1977; McLaren, 2003). In some genera, like *Xenopus,* germ cell aggregates are present in the eggs even before fertilization (Haesman *et al*., 1984), in mouse they are observed 8.5 days post–coitum (dpc) (Ginsberg *et al*., 1990) while in species, like zebrafish, they appear as early as the first few cellular divisions after fertilization (Raz, 2003). In chicken, it has been hypothesized that the fate of the germ line lineage is maternally predetermined and hence follows a preformation model (Kagami *et al*., 1997). Detection of Cvh (Chicken Vasa homologue) protein, a germ cell marker, in chicken oocytes prior to fertilization supported this hypothesis (Laval *et al*., 2009).

The precise origin of PGCs, their migration to the genital ridge and their division rate after migration varies among species and is time dependent. The following section reviews the origin, migration pattern, cellular characteristics, and cultural characteristics of chicken primordial germ cells (cPGCs).

Origin, migration pattern, and cellular characteristics of cPGCs

The origin and migration pattern of PGCs varies between species. In amniotes like chicken, PGCs originate from the epiblast (pluripotent cellular mass that ultimately gives rise to extraembryonic mesoderm and embryonic ectoderm) (Karagenc *et al*., 1996). Hamburger and Hamilton have defined different development stages of the embryonic chicken (Hamburger and Hamilton, 1951). At HH stage 4 (Hamburger and Hamilton stage 4; 18-19 hrs. of incubation) the

PGCs migrate to the area of the zona pellucida, multiply with a doubling time of approximately 6.6-6.8 h, then move to the germinal crescent and enter the blood stream between HH stage 10 to 12 (40-50 hrs. of incubation) (Fig. 2.8) (Ukeshima *et al*., 1991; Han, 2009).

The cPGCs use the circulatory system as a migratory route and settle in the genital ridges (Nakamura *et al*., 2007). At stage 14 (50-53 hrs. of incubation), cPGCs reach their highest population in the blood stream (Tajima *et al*., 1999). Studies have shown that at HH stage 17 (52-64 hrs. of incubation), cPGCs emerge from capillaries posterior to the omphalomesenteric arteries, the area between the splanchnopleure and the open-gut endoderm (Ando and Fujimoto, 1983; Nakamura et al., 2007). The cPGCs enter the genital ridges where they accumulate as gonadal germ cells (Ando and Fujimoto, 1983). By stage 22 (31/2 days of incubation) almost all cPGCs arrive in the developing genital ridges and differentiate into either oogonia (females) or spermatogonia (males) later in embryonic development (Fig.2.9). It has been hypothesized that cPGC migration is controlled by evolutionarily conserved chemokines such as SDF-1/CXCL-12 and follow the same migratory pattern as followed by Polymorphonuclear (PMN) leukocytes towards the site of inflammation (Stebler *et al*., 2004). The migration pattern of avian and mammalian PGCs is very well described in tabular form by Han (Table 2.1) (Han, 2009).

Once cPGCs reach the genital ridges, there are marked changes in their properties. Expression of germ cell specific antigens such as EMA-1, and SSEA-1 change based on the cPGC sex. Studies have shown that in females, the expression of EMA-1 and SSEA-1 disappears after 8 days of incubation whereas it disappears after 11 days of incubation in males (Maeda *et al*., 1994). In general, the expression of germ-cell-specific genes is upregulated and the expression of pluripotent genes is downregulated after PGCs establish in the gonads. After gonadal development, PGCs differentiation into either spermatozoa or ova is influenced by the

interaction between PGCs and the gonadal somatic cells (Urven *et al*., 1988; Maeda *et al*., 1994; Park & Han, 2013). In females, PGCs differentiate into primary oocytes, enter meiosis (stage 34 or 8 days of incubation) and get arrested in the diplotene phase of prophase I at the time of birth (Swift, 1915). Once a female attains sexual maturity, the oocytes get released from meiotic arrest and start dividing further. In contrast, male PGCs proliferate and differentiate into spermatogonia (stage 39 or 13 days of incubation), enter into a premeiotic stage by upregulating meiotic genes and ultimately end up in mitotic arrest in G0/G1 phase till the time of birth (Swift, 1916). Germ cells remain quiescent until males attain sexual maturity after which spermatogenesis begins, leading to extensive proliferation and development of mature sperm. It has been reported that in mice, irrespective of their sex chromosome constitution PGCs enter into the meiotic prophase (Upadhyay and Zamboni, 1982; Mclaren, 1995). The process of oogenesis is considered to be a cell-autonomous response, whereas the entry of PGCs into the spermatogenesis pathway is an induced response heavily influenced by male gonadal Sertoli cells (McLaren, 2003). In chicken, studies on mixed-sex germline chimeras have demonstrated that the cPGCs entry into gametogenesis is influenced by their chromosomal sex while successful gametogenesis is influenced by the surrounding gonadal somatic cells (Naito *et al*., 1999; Kagami *et al*., 1995, 1997; Nakamura *et al*., 2013).

These findings throw light on the changing behavior of cPGCs (gene expression, differentiation pattern, morphology) after their entry into the genital ridges. It also strengthens the regulatory role of Sertoli cells and the surrounding somatic tissue microenvironment in defining germ cell fate in males. Whether these complex interactions of somatic cells and germ cells (especially in males) can change the expression profile in developing germ cells and influence the phenotype of spermatozoa, is a question that needs to be addressed. In chicken, the migratory pattern of

cPGCs is well documented but little is known about the mechanisms behind the sex specific differentiation of cPGCs. Furthermore, the cross talk between Sertoli cells and cPGCs and its influence on phenotype of the differentiating germ cells is interesting to probe with respect to the sperm mobility trait.

Morphology, gene expression and cultural characteristics of cPGCs

There have been extensive studies of cPGCs for various purposes including understanding germ cell differentiation, germ plasm conservation, transgenic chicken production (Nakamura *et al*., 2013). For manipulation of cPGCs it is imperative to study their migratory pattern in developing embryos, differentiation dynamics of germ cells, and their cultural characteristics. Moreover, interest in chicken transgenics pushed research in the direction of finding a robust culture system for maintenance and propagation of cPGCs. Decades of research and studies resulted in defining salient morphological features and biochemical characteristics of cPGCs *in-vitro* (Yang and Fujihara, 1999; Han *et al*., 2002).

There are characteristic features of cPGCs that set them apart from blastodermal cells and early somatic cells. Morphologically, cPGCs are usually larger in size approximately 10-20 µm in diameter, spherical in shape, rich in cytoplasmic lipid content and glycogen (Fig. 2.10) (Song *et al*., 2014). The cytoplasmic projections on the surface of PGCs acts as pseudopodia for locomotion (Han *et al*., 2010). Being pluripotent cells, they express high levels of stem cell specific genes like *Nanog*, *Sox 2*, *Pouv* and *Oct-4*. Furthermore, the expression of germ cell specific genes like Cvh and Dazl has been shown to be crucial for cPGCs survival (Kito *et al*., 2010). There are very distinct surface cPGC glycoproteins used as cPGCs markers such as

SSEA-1 (stage specific embryonic antigen-1), SSEA-3 (stage specific embryonic antigen-3), SSEA-4 (stage specific embryonic antigen-4), EMA-1 (embryonic mouse antigen-1), ITGA6 (integrin subunit alpha 6) and ITGB1 (integrin beta 1) (Jung *et al.*, 2004).

Long term maintenance of cPGCs in-vitro

Studies conducted so far on cPGC culture have delineated the use of different feeder lines for cPGC proliferation and maintenance. Feeder cell lines have included irradiated Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) feeder layer, primary chick embryonic fibroblast (CEF) cell lines, chicken hepatocarcinoma line (LMN) and Buffalo rat liver cells (Kawaguchi *et al*., 1987; Choi *et al*.,2010). Feeder layers are proposed to provide growth factors that helps in cPGC growth in culture. Deliberate studies on defining the specific growth factors to replace the feeder cell lines have focused on greater control over culture conditions and which growth factors are critical for proliferation and maintenance of an undifferentiated state for cPGCs (Song *et al*., 2014). These growth factors are leukemia inhibitory factor (LIF), stem cell factor (SCF), and basic fibroblast growth factor (bFGF) (Lavoir *et al*., 2006). Growth factor bFGF belongs to the fibroblast growth factor orthologs that promote growth of blastodermal, embryonic stem cells and PGCs in chicken (Park *et al*., 2006; Park *et al*.,2000; Lavoir *et al*.,2006). Survival of cPGCs is critically dependent on bFGF inducing the MEK/ERK signaling pathway to enable cPGCs division in culture conditions (McDonald *et al*., 2010). LIF is necessary for long term culture of cPGCs and is also shown to be involved in increased expression of stem cell markers like SSEA-1, EMA-1 (Petitte *et al*., 2004). SCF is required for inhibiting differentiation in proliferating cPGCs. The combination of these growth factors is crucial for the maintenance of long term culture of cPGCs in feeder-less conditions.

Even though culture characteristics, growth factors, feeder layers, culture medium, etc., are well defined for cPGC culture, there is disparity between culture patterns of male and female PGCs. As demonstrated in some studies, male cPGCs are more efficient in proliferating as compared to female cPGCs in culture. Between male and female cultured cPGCs, the frequency to inhabit chimeric chickens is considerably less for female cPGCs (van de Lavoir *et al*.,2006).

Understanding the behavioral pattern of cPGCs under culture conditions, essential factors required for their survival and proliferation, causes of differential growth pattern between male and female cPGCs is not only required but essential for culturing cPGCs effectively for longer duration and easy manipulation. Studies conducted so far stressed on defining media components, growth factors, supplemental feeder cell lines and temperature-time conditions, yet very few studies have reported cPGC culture on feeder-less conditions and their growth characteristics. Different cultural characteristics and behavior of male and female cPGCs laid the foundation to do the in-depth analysis of gene expression pattern between them. To achieve this goal, it is important to have pure cultures of cPGCs without any feeder layer so that harvested RNAs for RNAseq analysis are not contaminated from the feeder layer.

SYNOPSIS

Sperm mobility, a quantitative and heritable trait in chicken is one of the paramount parameter used in the poultry industry to detect the fertilizing potential of males (Froman *et al*., 2002; Parker and McDaniel, 2002). Furthermore, it is known to be influenced by genetic components with more relevance provided by the maternal genetic composition due to mitochondrial effect (Froman and Feltmann, 2005). Even though extensive studies have been done to identify the genes influencing sperm mobility trait, genes responsible for the trait remains obscure (Froman and Rhoads, 2013). Our purpose of this research is first, to identify genetic markers contributing to sperm mobility trait by conducting GWAS analyses on three different generations of high line and low line reciprocal and double reciprocal crosses created by Dr. Froman, and second, to use cPGCs as a tool to understand the impact of interactions between somatic cells and germ cells in influencing the mobility phenotype.

FIGURES

Figure 2-1: Pictorial representation of sperms in sperm storage tubules in female reproductive tract. (a) Light microscope image of sperms in the sperm storage tubules, and (b) transmission electron microscope. Adapted from Yoshimura et al., 2008.

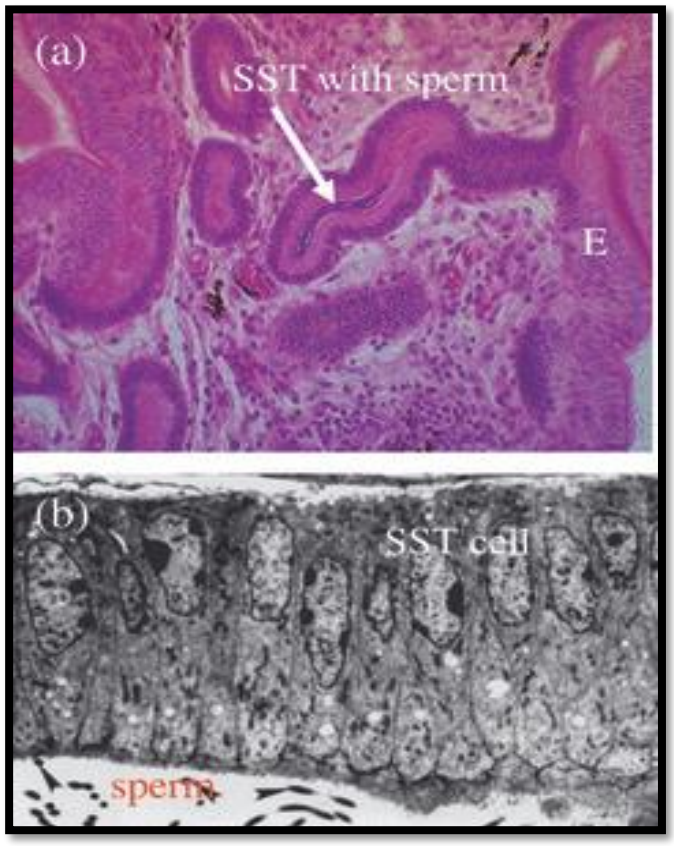


Figure 2.2: Changes in the fertilizing efficiency of low-mobile ejaculate with respect to time. (a) Low mobility ejaculates when enriched in higher proportion (4:1, white data points) than high mobility ejaculates, tend to compete equally in fertilizing eggs, but the fertilization potential decreases rapidly over time after insemination. The proportion of paternity by low-mobility male (Y-axis) decreases linearly over successive days (X-axis) which is much sharper in lower enriched pair (2:1, black data points) than higher enriched one (4:1). (b) The slope of probability of paternity over mobility ratio becomes steeper over laying sequence, which is more pronounced in 2:1 treatment. This indicates that as the laying duration increases, the probability of paternity by low mobile sperms decreases sharply (Froman *et al*., 2008).

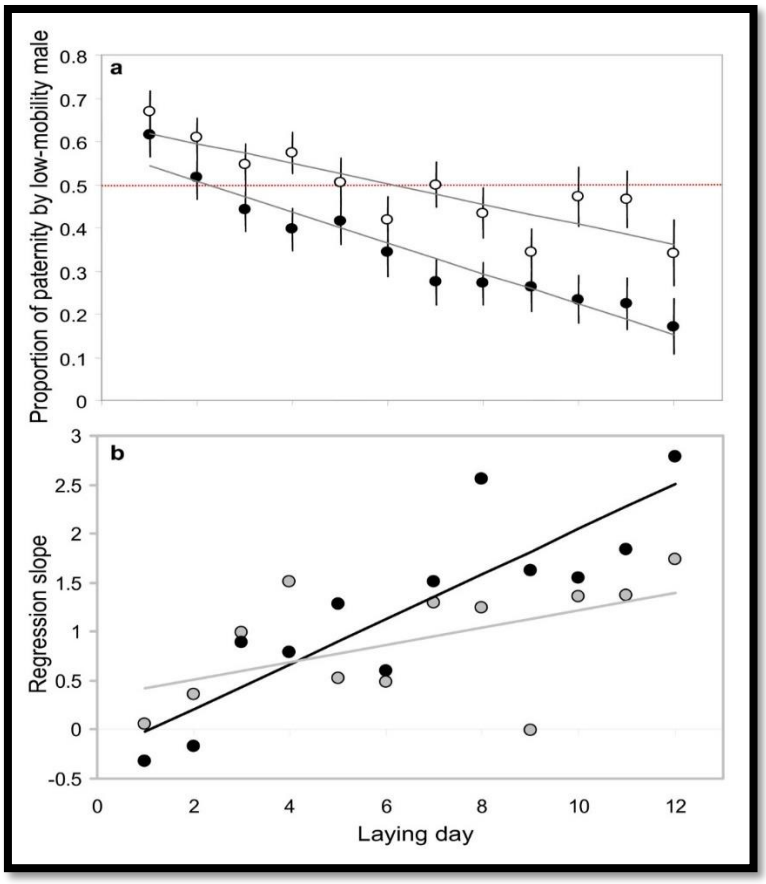


Figure 2.3: Fertility plotted as function of sperm mobility. Sperm suspension was overlaid on w/v (6%) Accudenz layer and sperm mobility was accessed by observing the change in absorbance @550nm (X-axis). Higher the absorbance, higher is the fertility. Fertility (Y-axis) is determined by inseminating hens with respective semen ejaculates. Open circles denote broiler males used in the experiment (Froman *et al*., 1999).

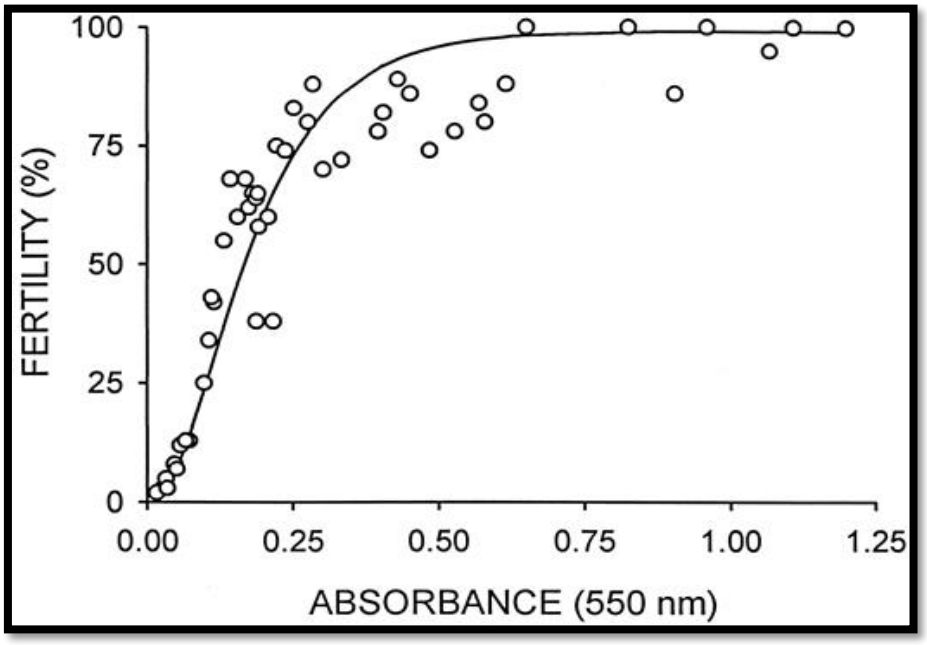


Figure 2.4: Normal probability density function of sperm mobility trait. Sperm mobility was predicted using 6% w/v Accudenz assay by measuring the absorbance of sperm suspension @550nm (X-axis) at body temperature. The bell-shaped curve represents the distribution of sperm mobility of domesticated fowl. Dashed lines marked the limits of standard deviation from the mean. Mobility phenotypes are represented by regions marked by the dashed lines (Froman and Feltmann, 2000).

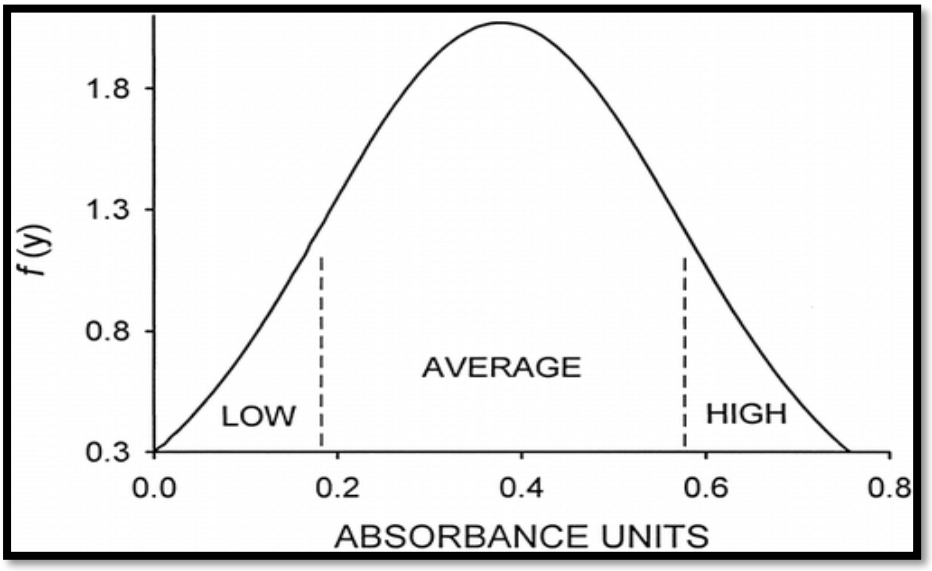


Figure 2.5: Differential mobility phenotype within high and low sperm mobility lines as observed in New Hampshire chickens. The mobility distribution in low sperm mobility line is highly skewed and depicts very high proportion of immobile sperm whereas ejaculates from high mobility lines are normally distributed. Adapted from Froman *et al*., 2010.

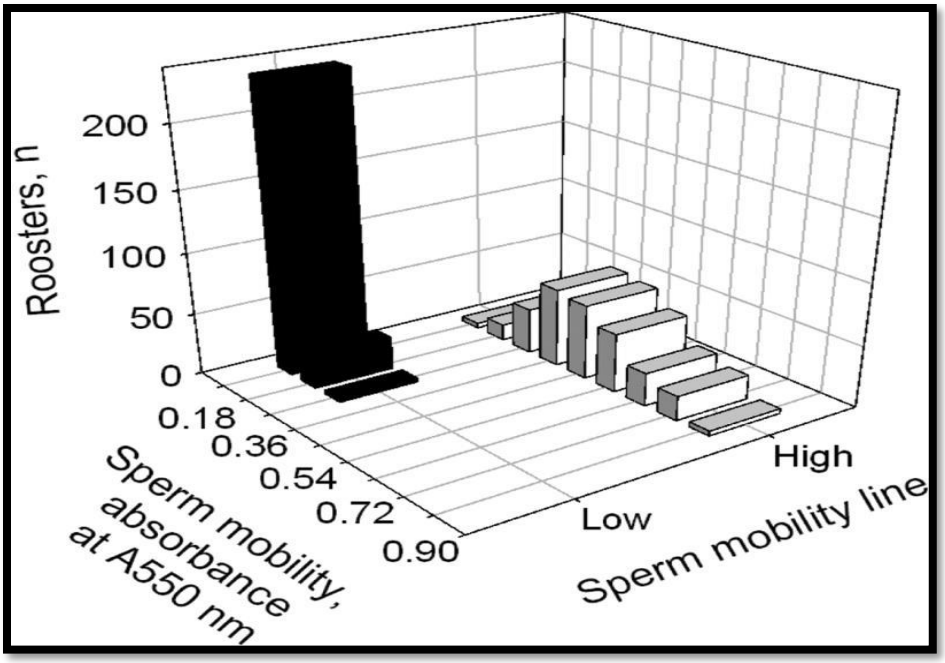


Figure 2.6: Different stages of spermatogenesis in the Japanese quail represented through diagram. (1) spermatocytogenesis, the mitotic cell division of stem spermatogonia for its proliferation and renewal, (2) meiosis, reductional cell division generating spermatids(n) from primary spermatocytes(2n), (3) spermiogenesis, the final differentiation stage generating spermatozoa from spermatids (Jones and Lin, 1992)

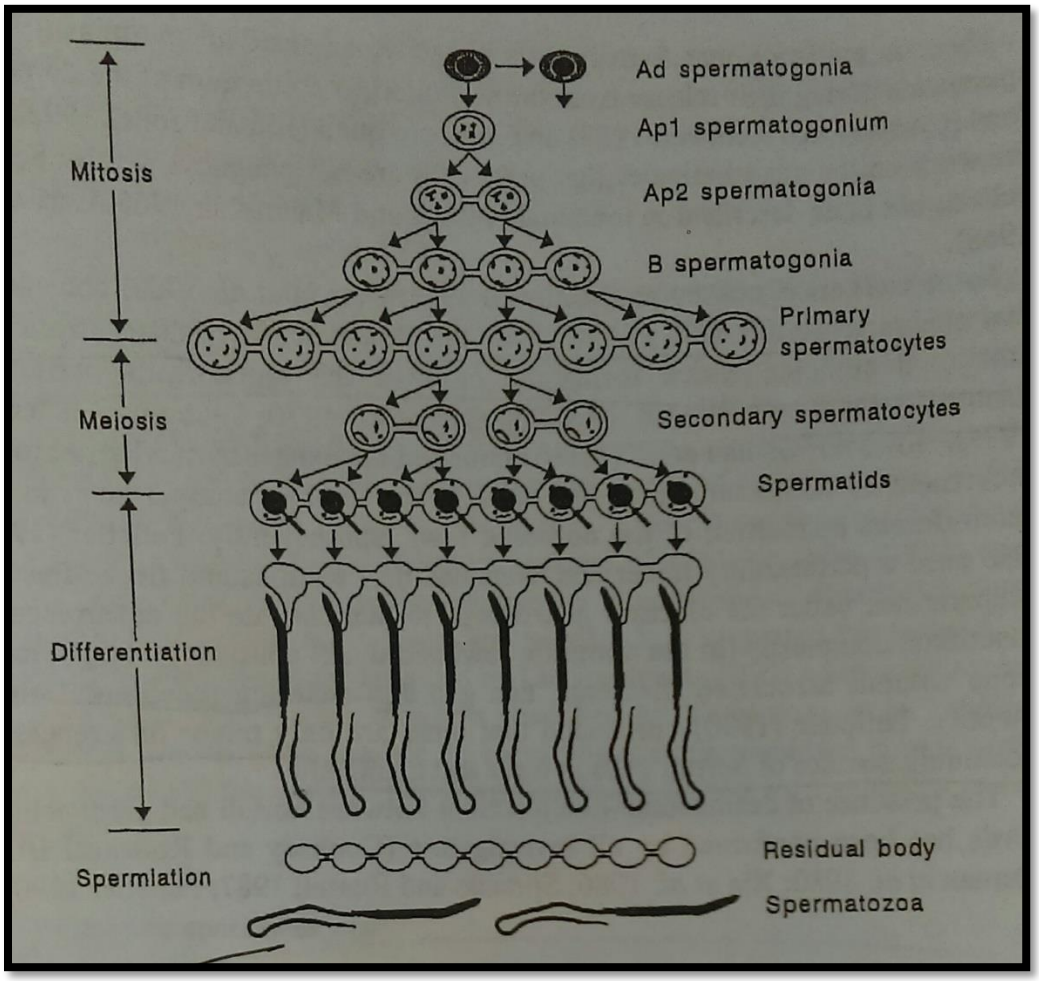


Figure 2.7: Pictorial representation of tight junction amongst Sertoli cells and between differentiating spermatocytes. (A) relative position of germ cells at different stages of differentiation in the section of seminiferous epithelium. The association between Sertoli cells and spermatocytes via tight junctions is clearly represented. (B) Confocal imaging of SCTJs (Sertoli cells tight junctions) using two different markers F-actin and CLDN11. (C) pictorial illustration of Sertoli cells and CLDN11-containing tight junctions and interconnections between pre-leptotene spermatocytes through tight junctions (Smith and Braun, 2012).

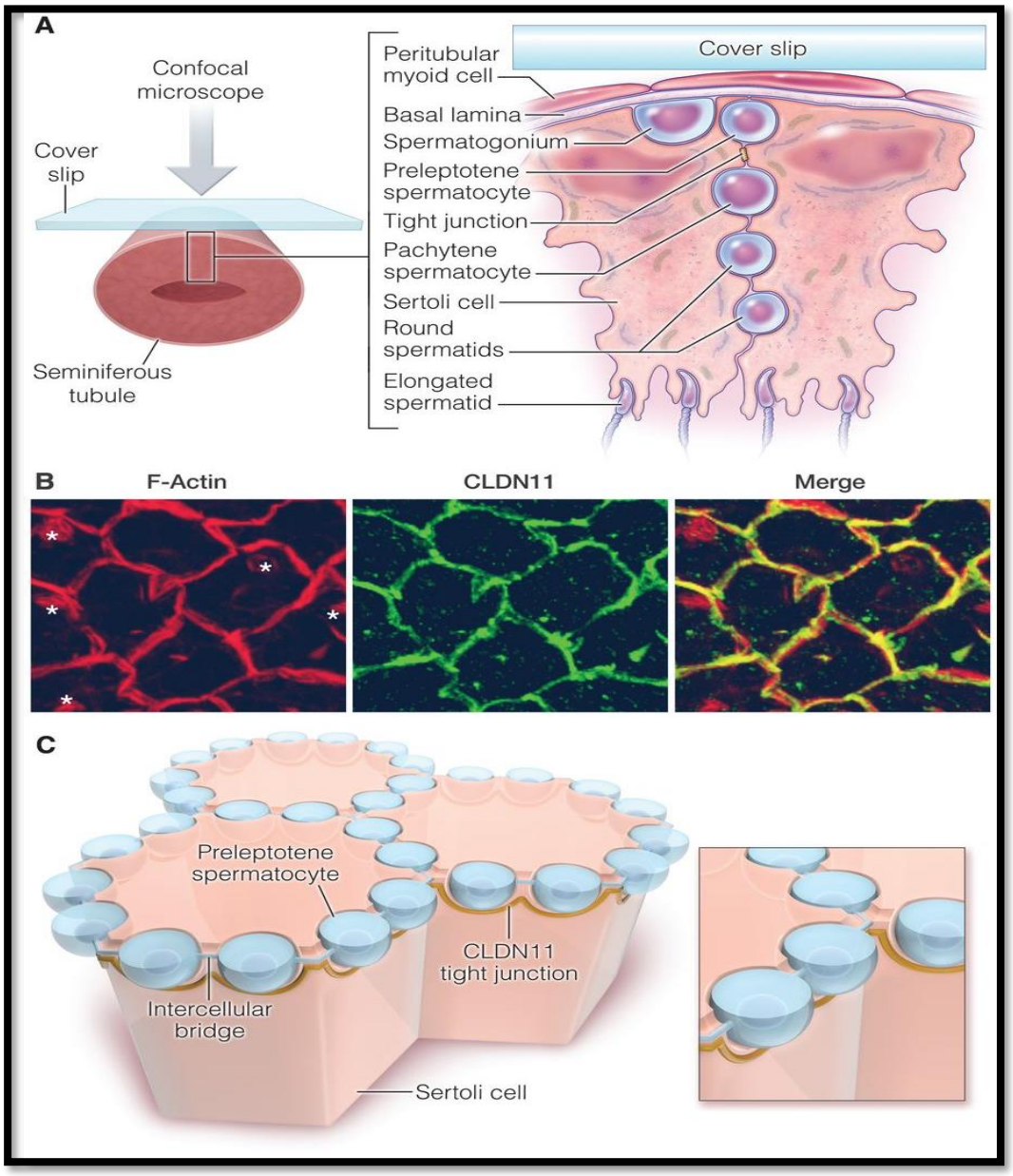


Figure 2.8: Hamburger and Hamilton stages of chicken embryonic development. Stage 4(18-19 hrs. of incubation) is marked by appearance of primitive streak and zone of area pellucida where PGCs migrate from epiblast (4). At HH stages 10-12 (40-50 hrs. of incubation) PGCs enters in the blood stream (10). At stage 17 (52-64 hrs. of incubation) PGCs start escaping from the capillaries and migrate into the developing genital ridge (12). By the end of stage 22 (31/2 days of incubation) almost all PGCs inhabits the genital ridge and very few remains in the circulation (17) (Ando and Fujimoto, 1983). Pictures are adapted from Hamburger and Hamilton, 1951.

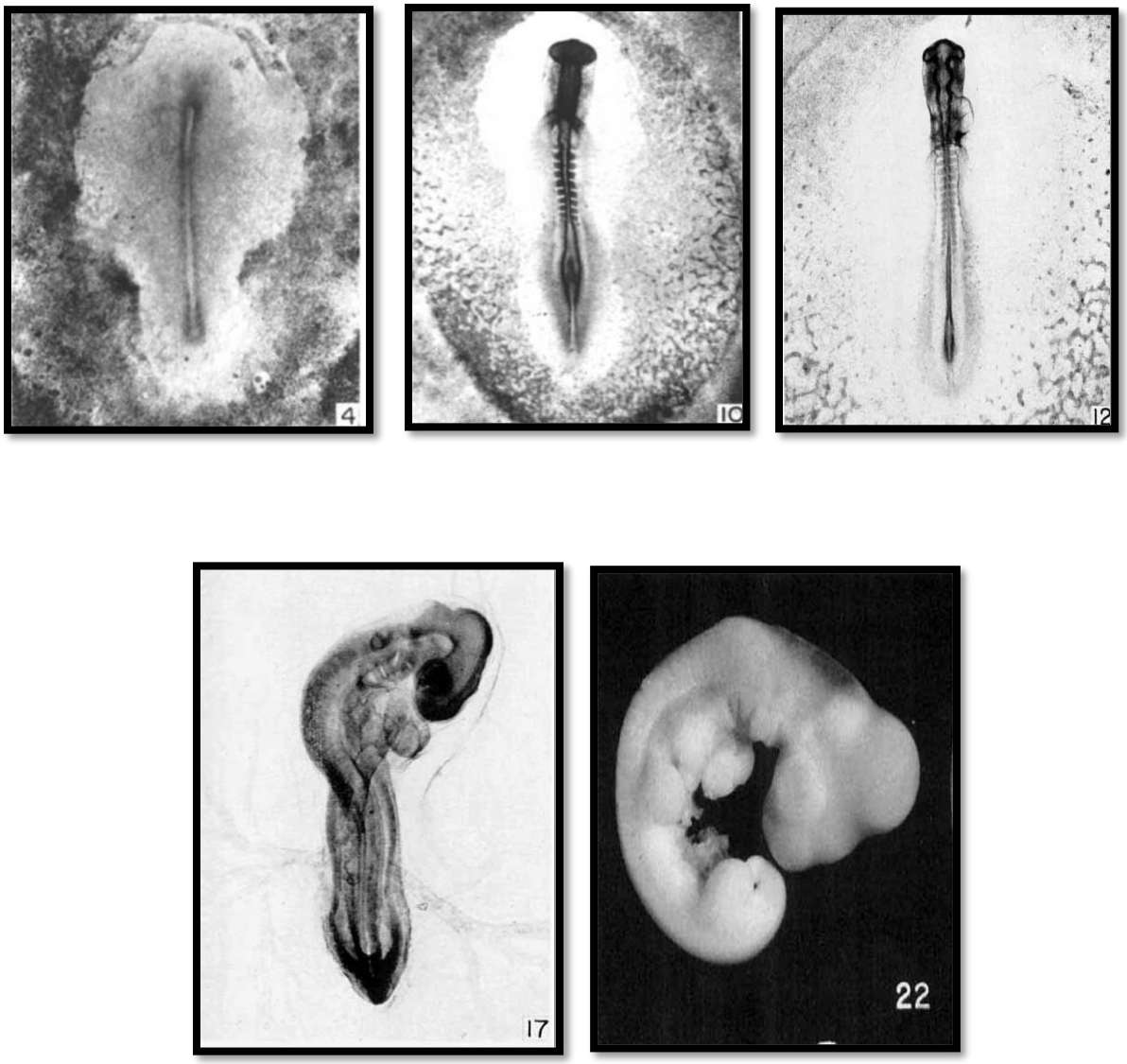


Figure 2.9: Pictorial representation of primordial germ cells (PGCs) distribution in chick embryo. (A) At HH stage 17, the PGCs (black dots) start accumulating in the area posterior to the omphalomesenteric artery (OmA). (B) A transverse section of developing chick embryo depicting HH stage 22. PGCs transverse the capillaries (Cp) and migrate to the developing genital ridge in this stage (GR) (Ando and Fujimoto, 1983).

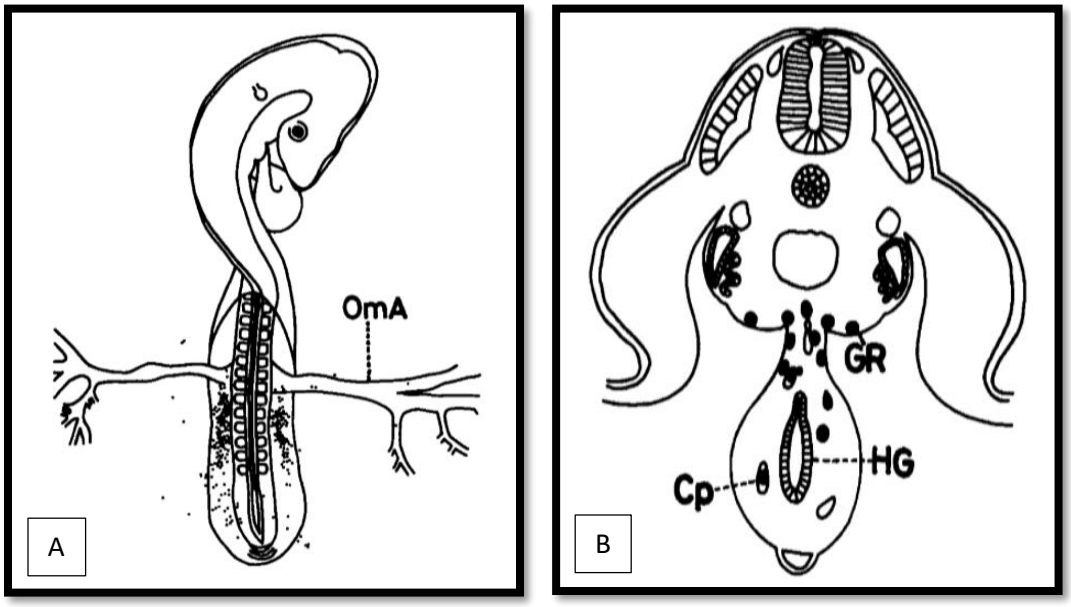
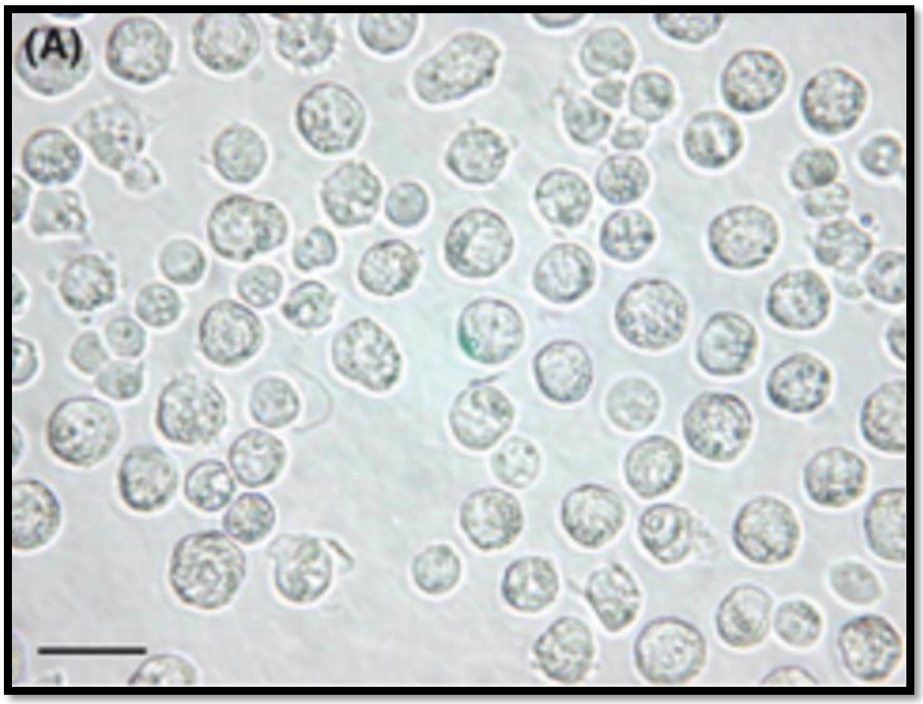


Figure 2.10: Morphology of cPGCs in culture. (A) PGCs are larger in size, spherical in shape and has high cytoplasmic lipid content (40X magnification). Adapted from Nakamura *et al*.,2013



TABLES

Table 2.1: Comparison between migratory pattern of avian and mammalian PGCs. Adapted from Han, 2009. In both species, epiblasts give rise to PGCs early in the embryonic developmental but there is slight variation in their migration patterns.

The migration pattern of primordial germ cells (PGCs) in avian and mammal

EG & K, H & H stage	Avian (chicken)
Stage X	Central region of area pellucida
Stage 4	Germinal crescent
Stage 10–17	Move and circulate in extra-embryonic blood vessel
Stage 20–26	Migration and colonization into embryonic gonad
Hatch	Proliferation or differentiation in sex organ
Embryonic day	Mammal (mouse)
E6-6.5	First arise from epiblast
E7-7.5	First seen at region of forming hindgut
E9.5	Leave hindgut and migration to urogenital ridge
E11.5	Migration to genital ridge
Birth	Proliferation or differentiation in sex organ

REFERENCES

Ando, Y., & Fujimoto, T. (1983). Ultrastructural Evidence that Chick Primordial Germ Cells Leave the Blood-Vascular System Prior to Migrating to the Gonadal Anlagen. *Development, growth & differentiation*, *25*(4), 345-352.

Aramaki, S., Tanaka, K., Tomoki, S. O. H., Yamauchi, N., & Hattori, M. A. (2010). Temporal and spatial differential expression of chicken germline-specific proteins cDAZL, CDH and CVH during gametogenesis. *Journal of Reproduction and Development*, *56*(3), 341-346.

Ashizawa, K., & Sano, R. (1990). Effects of temperature on the immobilization and the initiation of motility of spermatozoa in the male reproductive tract of the domestic fowl, Gallus domesticus. *Comparative Biochemistry and Physiology Part A: Physiology*, *96*(2), 297-301.

Bakst, M. R. (1987). Anatomical basis of sperm-storage in the avian oviduct. *Scanning microscopy*, *1*(3), 1257-1266.

Bedford, J. M., & Hoskins, D. D. (1990). The mammalian spermatozoon: morphology, biochemistry and physiology. *Marshall's physiology of reproduction*, *2*, 379-568.

Birkhead, T. R., & Biggins, J. D. (1998). Sperm competition mechanisms in birds: models and data. *Behavioral Ecology*, *9*(3), 253-260.

Birkhead, T. R., Martinez, J. G., Burke, T., & Froman, D. P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London B: Biological Sciences*, *266*(1430), 1759-1764.

Birkhead, T. R., Wishart, G. J., & Biggins, J. D. (1995). Sperm precedence in the domestic fowl. *Proceedings of the Royal Society of London B: Biological Sciences*, *261*(1362), 285-292.

Bishop, D. W. (1962). Sperm motility. *Physiological reviews*, *42*(1), 1-59.

Bowling, E. R., Froman, D. P., Davis, A. J., & Wilson, J. L. (2003). Attributes of broiler breeder males characterized by low and high sperm mobility. *Poultry Science*, *82*(11), 1796-1801.

Bremner, W. J., Millar, M. R., Sharpe, R. M., & Saunders, P. T. (1994). Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology*, *135*(3), 1227-1234.

Brillard, J. P., & Antoine, H. (1990). Storage of sperm in the uterovaginal junction and its incidence on the numbers of spermatozoa present in the perivitelline layer of hens' eggs. *British Poultry Science*, *31*(3), 635-644.

Brillard, J. P., & Bakst, M. R. (1990). Quantification of spermatozoa in the sperm-storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. *Biology of Reproduction*, *43*(2), 271-275.

Choi, J. W., Kim, S., Kim, T. M., Kim, Y. M., Seo, H. W., Park, T. S., & Han, J. Y. (2010). Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells. *PloS one*, *5*(9), e12968.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiological reviews*, *52*(1), 198-236.

Colegrave, N., Birkhead, T. R., & Lessells, C. M. (1995). Sperm precedence in zebra finches does not require special mechanisms of sperm competition. *Proceedings of the Royal Society of London B: Biological Sciences*, *259*(1355), 223-228.

Das, S. C., Isobe, N., & Yoshimura, Y. (2008). REVIEW ARTICLE: Mechanism of Prolonged Sperm Storage and Sperm Survivability in Hen Oviduct: A Review. *American Journal of Reproductive Immunology*, *60*(6), 477-481.

Donoghue, A. M. (1999). Prospective approaches to avoid flock fertility problems: predictive assessment of sperm function traits in poultry. *Poultry Science*, *78*(3), 437-443.

Donoghue, A. M., Holsberger, D. R., Evenson, D. P. & Froman, D. P. (1998). Semen donor selection by in vitro sperm mobility increases fertility and semen storage in the turkey hen. *Journal of andrology*, *19*(3), 295-301.

Dym, M., & Fawcett, D. W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biology of Reproduction*, *3*(3), 308-326.

Dziuk, P. J. (1996). Factors that influence the proportion of offspring sired by a male following heterospermic insemination. *Animal Reproduction Science*, *43*(2-3), 65-88.

Ford, W. C. L. (2006). Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round? *Human reproduction update*, *12*(3), 269-274.

Froman, D. P., & Feltmann, A. J. (1998). Sperm mobility: a quantitative trait of the domestic fowl (Gallus domesticus). *Biology of Reproduction*, *58*(2), 379-384.

Froman, D. P., & Feltmann, A. J. (2000). Sperm mobility: phenotype in roosters (Gallus domesticus) determined by concentration of motile sperm and straight line velocity. *Biology of Reproduction*, *62*(2), 303-309.

Froman, D. P., & Feltmann, A. J. (2005). Fowl (Gallus domesticus) sperm motility depends upon mitochondrial calcium cycling driven by extracellular sodium. *Biology of Reproduction, 72 (1)*, 97-101.

Froman, D. P., & McLean, D. J. (1996). Objective measurement of sperm motility based upon sperm penetration of Accudenz®. *Poultry Science*, *75*(6), 776-784.

Froman, D. P., & Rhoads, D. D. (2013). Breeding and genetics symposium: A systems biology definition for chicken semen quality. *Journal of animal science*, *91*(2), 523-529.

Froman, D. P., Feltmann, A. J., Pendarvis, K., Cooksey, A. M., Burgess, S. C., & Rhoads, D. D. (2011). Physiology and endocrinology symposium: a proteome-based model for sperm mobility phenotype. *Journal of animal science*, *89*(5), 1330-1337.

Froman, D. P., Pizzari, T., Feltmann, A. J., Castillo-Juarez, H., & Birkhead, T. R. (2002). Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, Gallus gallus domesticus. *Proceedings of the Royal Society of London B: Biological Sciences*, *269*(1491), 607-612.

Fujimoto, T., Miyayama, Y., & Fuyuta, M. (1977). The origin, migration and fine morphology of human primordial germ cells. *The Anatomical Record*, *188*(3), 315-329.

Ginsberg, J. R., & Huck, U. W. (1989). Sperm competition in mammals. *Trends in Ecology & Evolution*, *4*(3), 74-79.

Ginsburg, M. A. L. K. A., Snow, M. H., & McLaren, A. N. N. E. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development*, *110*(2), 521-528.

Goldberg, E., & Norman, C. (1961). The metabolism of ejaculated spermatozoa from the fowl. *Journal of Cellular Physiology*, *58*(2), 175-180.

Griswold, M. D. (1998, August). The central role of Sertoli cells in spermatogenesis. In *Seminars in cell & developmental biology* (Vol. 9, No. 4, pp. 411-416). Academic Press.

Hamburger, V., & Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *Journal of morphology*, *88*(1), 49-92.

Han, J. Y. (2009). Germ cells and transgenesis in chickens. *Comparative immunology, microbiology and infectious diseases*, *32*(2), 61-80.

Han, J. Y., Park, T. S., Hong, Y. H., Jeong, D. K., Kim, J. N., Kim, K. D., & Lim, J. M. (2002). Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained in vitro for an extended period. *Theriogenology*, *58*(8), 1531-1539.

Heasman, J., Quarmby, J., & Wylie, C. C. (1984). The mitochondrial cloud of Xenopus oocytes: the source of germinal granule material. *Developmental biology*, *105*(2), 458-469.

Holsberger, D. R., Donoghue, A. M., Froman, D. P., & Ottinger, M. A. (1998). Assessment of ejaculate quality and sperm characteristics in turkeys: sperm mobility phenotype is independent of time. *Poultry Science*, *77*(11), 1711-1717.

Illmensee, K., & Mahowald, A. P. (1974). Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg. *Proceedings of the National Academy of Sciences*, *71*(4), 1016-1020.

Jones, R. C., & Lin, M. (1992). Spermatogenesis in birds. *Oxford reviews of reproductive biology*, *15*, 233-264.

Jung, J. G., Kim, D. K., Park, T. S., Lee, S. D., Lim, J. M., & Han, J. Y. (2005). Development of novel markers for the characterization of chicken primordial germ cells. *Stem Cells*, *23*(5), 689-698.

Kagami, H., Tagami, T., Matsubara, Y., Harumi, T., Hanada, H., Maruyama, K., Sakurai, M., Kuwana, T., & Naito, M. (1997). The developmental origin of primordial germ cells and the transmission of the donor-derived gametes in mixed-sex germline chimeras to the offspring in the chicken. *Molecular reproduction and development*, *48*(4), 501-510.

Karagenc, L., Cinnamon, Y., Ginsburg, M., & Petitte, J. N. (1996). Origin of primordial germ cells in the prestreak chick embryo. *Genesis*, *19*(4), 290-301.

Kawaguchi, T., Nomura, K., Hirayama, Y., & Kitagawa, T. (1987). Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. *Cancer research*, *47*(16), 4460-4464.

King'Ori, A. M. (2011). Review of the factors that influence egg fertility and hatchability in poultry. *International Journal of Poultry Science*, *10*(6), 483-492.

Lacy, D., Vinson, G. P., Collins, P., Bell, J., Fyson, P., Pudney, J., & Pettitt, A. J. (1969). The Sertoli cell and spermatogenesis in mammals.

Lavial, F., Acloque, H., Bachelard, E., Nieto, M. A., Samarut, J., & Pain, B. (2009). Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate. *Developmental biology*, *330*(1), 73-82
.

Maeda, S., Ohsako, S., Kurohmaru, M., Hayashi, Y., & Nishida, T. (1994). Analysis for the stage specific antigen of the primordial germ cells in the chick embryo. *Journal of Veterinary Medical Science*, *56*(2), 315-320.

Maekawa, M., Kamimura, K., & Nagano, T. (1996). Peritubular myoid cells in the testis: their structure and function. *Archives of histology and cytology*, *59*(1), 1-13.

McLaren, A. (2003). Primordial germ cells in the mouse. *Developmental biology*, *262*(1), 1-15.

McLaren, A., Mittwoch, U., & Josso, N. (1995). Germ cells and germ cell sex. *Philosophical Transactions: Biological Sciences*, 229-233.

Mendis-Handagama, S. M. (1997). Luteinizing hormone on Leydig cell structure and function. *Histology and histopathology*, *12*(3), 869-882.

Meng, J., Holdcraft, R. W., Shima, J. E., Griswold, M. D., & Braun, R. E. (2005). Androgens regulate the permeability of the blood–testis barrier. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(46), 16696-16700.

Miki, K. (2006). Energy metabolism and sperm function. *Society of Reproduction and Fertility supplement*, *65*, 309-325.

Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F.,Perreault, S. D., Eddy, E. M., & O'Brien, D. A. (2004). Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16501-16506.

Nakamura, Y., Kagami, H., & Tagami, T. (2013). Development, differentiation and manipulation of chicken germ cells. *Development, growth & differentiation*, *55*(1), 20-40.

Nakamura, Y., Yamamoto, Y., Usui, F., Mushika, T., Ono, T., Setioko, A. R., Takeda, K., Nirasawa, K., Kagami, H., & Tagami, T. (2007). Migration and proliferation of primordial germ cells in the early chicken embryo. *Poultry Science*, *86*(10), 2182-2193.

Nascimento, J. M., Shi, L. Z., Tam, J., Chandsawangbhuwana, C., Durrant, B., Botvinick, E. L., & Berns, M. W. (2008). Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. *Journal of cellular physiology*, *217(3)*, 745-751.

O'Connell, M., McClure, N., Lewis S.E.M. (2002). The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Human Reproduction*, 17(3): 704-709.

Park, H. J., Park, T. S., Kim, T. M., Kim, J. N., Shin, S. S., Lim, J. M., & Han, J. Y. (2006). Establishment of an in vitro culture system for chicken preblastodermal cells. *Molecular reproduction and development*, *73*(4), 452-461.

Park, T. S., & Han, J. Y. (2000). Derivation and characterization of pluripotent embryonic germ cells in chicken. *Molecular reproduction and development*, *56*(4), 475-482.

Parker, H. M., & McDaniel, C. D. (2002). Selection of young broiler breeders for semen quality improves hatchability in an industry field trial. *The Journal of Applied Poultry Research, 11(3)*, 250-259.

Petitte, J. N., Liu, G., & Yang, Z. (2004). Avian pluripotent stem cells. *Mechanisms of development*, *121*(9), 1159-1168.

Pizzari, T., Worley, K., Burke, T., & Froman, D. P. (2008). Sperm competition dynamics: ejaculate fertilising efficiency changes differentially with time. *BMC Evolutionary Biology*, *8*(1), 332.

Pollock, D. L. (1999). A geneticist's perspective from within a broiler primary breeder company. *Poultry Science*, *78*(3), 414-418.

Raz, E. (2003). Primordial germ-cell development: the zebrafish perspective. *Nature Reviews. Genetics*, *4*(9), 690.

Ruiz-Pesini, E., Díez-Sánchez, C., López-Pérez, M. J., & Enriquez, J. A. (2007). The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? *Current topics in developmental biology*, *77*, 3-19.

Russell, L. (1977a). Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. *Developmental Dynamics*, *148*(3), 313-328.

Russell, L. (1977b). Desmosome-like junctions between Sertoli and germ cells in the rat testis. *Developmental Dynamics*, *148*(3), 301-312.

Sapp, R. L., Rekaya, R., Misztal, I., & Wing, T. (2004). Male and female fertility and hatchability in chickens: A longitudinal mixed model approach. *Poultry Science*, *83*(8), 1253-1259.

Sexton, T. J. (1974). Oxidative and glycolytic activity of chicken and turkey spermatozoa. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *48*(1), 59-65.

Shim, H., Gutiérrez-Adán, A., Chen, L. R., BonDurant, R. H., Behboodi, E., & Anderson, G. B. (1997). Isolation of pluripotent stem cells from cultured porcine primordial germ cells. *Biology of reproduction*, *57*(5), 1089-1095.

Smith, B. E., & Braun, R. E. (2012). Germ cell migration across Sertoli cell tight junctions. *Science*, *338*(6108), 798-802.

Stebler, J., Spieler, D., Slanchev, K., Molyneaux, K. A., Richter, U., Cojocaru, V., Tarabykin, V., Wylie, C., Keseel, M., & Raz, E. (2004). Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Developmental biology*, *272*(2), 351-361.

Steele, M. G. (1992). A study of the influence of sperm surface proteins on the activity of avian spermatozoa in vitro and in vivo [PhD thesis]. Dundee, Scotland, United Kingdom: Dundee Institute of Technology.

Swift, C. H. (1915). Origin of the definitive sex-cells in the female chick and their relation to the primordial germ-cells. *Developmental Dynamics*, *18*(3), 441-470.

Swift, C. H. (1916). Origin of the sex-cords and definitive spermatogonia in the male chick. *Developmental Dynamics*, *20*(3), 375-410.

Tajima, A., Hayashi, H., Kamizumi, A., Ogura, J., Kuwana, T., & Chikamune, T. (1999). Study on the concentration of circulating primordial germ cells (cPGCs) in early chick embryos. *Journal of experimental zoology*, *284*(7), 759-764.

Tsukita, S., Furuse, M., & Itoh, M. (2001). Multifunctional strands in tight junctions. *Nature reviews. Molecular cell biology*, *2*(4), 285.

Ukeshima, A., Yoshinaga, K., & Fujimoto, T. (1991). Scanning and transmission electron microscopic observations of chick primordial germ cells with special reference to the extravasation in their migration course. *Journal of electron microscopy*, *40*(2), 124-128.

Urven, L. E., Erickson, C. A., Abbott, U. K., & McCarrey, J. R. (1988). Analysis of germ line development in the chick embryo using an anti-mouse EC cell antibody. *Development*, *103*(2), 299-304.

Van de Lavoir, M. C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L.T., Gessaro, T. M., Swanberg, S. E., & Delany, M. E. (2006). Germline transmission of genetically modified primordial germ cells. *Nature*, *441(7094)*, 766-769.

Wilson, H. R., Piesco, N. P., Miller, E. R., & Nesbeth, W. G. (1979). Prediction of the fertility potential of broiler breeder males. *World's Poultry Science Journal*, *35*(02), 95-118.

Wishart, G. J. (1982). Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. *Journal of reproduction and fertility*, *66*(2), 457-462.

Wishart, G. J. (1987). Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. *Journal of reproduction and fertility*, *80*(2), 493-498.

Yang, G., & Fujihara, N. (1999). Long-term proliferation of chicken primordial germ cells cultured in vitro. *Journal of Reproduction and Development*, *45*(2), 161-166.

Zamboni, L., & Upadhyay, S. (1983). Germ cell differentiation in mouse adrenal glands. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *228*(2), 173-193.

Zhou, Q., Nie, R., Prins, G. S., Saunders, P. T., Katzenellenbogen, B. S., & Hess, R. A. (2002). Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *Journal of andrology*, *23*(6), 870-881.

CHAPTER 3

Multi-generational genome wide association studies reveal the movement of quantitative trait loci (QTLs) within the low line phenotype over the course of three generations

ABSTRACT

A genome wide association study (GWAS) was conducted to detect significant chromosomal regions affecting sperm mobility trait in chicken. Sperm mobility is a quantitative production trait that defines semen quality in chicken. The GWAS was performed on progeny from reciprocal F_1, F_2 and F_3 crosses between the high and low mobility chicken lines to identify quantitative trait loci (QTLs) affecting the mobility phenotype. DNA was from semen samples collected from the lower and upper tail of the mobility distribution. The samples were genotyped using 60k SNP chip. Both F_2 and F_3 populations showed regions of significant associations on the chromosome Z but the regions varied between them. Previous GWAS analysis conducted on the parental low sperm mobile line identified regions at 32 and 63 Mbp on chromosome Z showing association with low sperm mobility phenotype but the three single nucleotide polymorphisms (SNPs) chosen from the respective regions showed no association with the mobility phenotype. Our study indicates that the QTLs for the mobility phenotype share multiple regions on the genome and suggests a significant role of epistasis for the mobility phenotype.

INTRODUCTION

Sperm mobility is a biologically significant trait that is a major determinant of semen quality in the poultry breeding industry (Froman *et al*., 1998, Bowling *et al*., 2003). Sperm mobility is the ability of sperm to travel with a straight-line velocity of > 30μm/sec against resistance at body temperature (Birkhead *et al*., 1999, Froman *et al*., 2002, Bowling *et al*., 2003). It is a quantitative trait and is positively correlated with fertility in chicken (Froman and Feltmann, 1998). Little to no information is available about the genetic loci in the chicken genome governing this trait. Molecular and proteomic studies conducted so far have concluded that sperm mobility is a multifactorial trait influenced by sperm interaction with its environment (Labas et al., 2014, Froman and Rhoads, 2014). The need to identify genes and gene networks that control mobility phenotype is important to understand male gamete biology. The relatively high heritability index (h^2=0.3) of this reproductive trait made it possible to design a study to identify the underlying factors affecting the mobility phenotype. Identification of the genetic factors affecting sperm mobility trait is important to understand the molecular basis of the mobility phenotype and to curb losses due to infertility in the poultry breeding industries that accounts for the loss of ~1 billion eggs /year (USDA, 2017).

A sperm mobility test (SMT) was developed by Froman and his research group at Oregon State University (OSU). Literature on the sperm mobility assay defines a robust method for detecting the mobility profile of the semen samples in avian species particularly in chicken (Holsberger *et al*., 1998; Birkhead *et al*., 1999). For SMT, a semen sample is layered on top of 6% (w/v) Accudenz medium for 5 minutes at 41^0C (avian body temperature) (Froman and McLean, 1996; Holsberger *et al*., 1998). After the incubation, absorbance of the Accudenz layer is measured @

550nm. The absorbance is directly proportional to the number of sperm penetrating the medium. The higher the absorbance value, the higher the proportion of mobile sperm in the test sample (Donoghue *et al*., 1998; Froman and Feltmann, 2000; Froman *et al*., 2003). This assay was and still is widely used in phenotyping males for their mobility profiles in both poultry and turkey industries.

The high heritability index (h^2=0.30) and the easy phenotypic assay for the sperm mobility trait allowed Froman and Feltmann (2000) to divergently select chicken lines based on their mobility scores. The high mobile line exhibited normal mobility distribution with both low and high mobile sperm in their ejaculates, with the majority falling in between. For the low mobile lines, mobility distribution was highly skewed with high proportion of immobile sperm (Froman and Feltmann, 2011; Froman and Rhoads, 2013). The average mobility score of low mobile sperm cells from the high line was higher than the high mobility sperm cells from the low line roosters. Premature mitochondrial failure in the low mobile sperm has been indicated to cause their untimely exit from the sperm storage tubules (SSTs) in hens and thus a predisposing factor of infertility (Froman et al., 2011; Froman and Kirby, 2005). Proteomic studies on sperm from the high and low sperm mobility lines have confirmed the differential expression of glycolytic enzymes and the proteins involved in ATP metabolism between the lines (Froman et al., 2014). Multiple studies were conducted to determine the causative factors of differential mobility phenotype in chickens (Bakst *et al*., 1994; Froman and Feltmann, 1998; Bowling *et al*., 2003; Froman and Kirby, 2005; Froman *et al*., 2006; Pizzari *et al*., 2008; Froman *et al*. 2014). These studies pointed towards the role of genetic elements and their interactions at the molecular level in predisposing sperm cells to the mitochondrial aberrations ultimately leading to the low mobility/immobility. Therefore, the goal of the current study was to locate the genetic loci

involved in this phenotypic trait, to identify the molecular pathways and gene network that affect the mobility trait.

Previously, GWAS analysis of test subjects from the subpopulations of low mobile lines (the mode and the lower tail of the low mobile lines) had identified multiple loci of interest scattered throughout the genome (Froman and Rhoads, 2013). Special emphasis was given to loci on chromosome Z because: 1) avian males are homozygous for the sex chromosome (ZZ) whereas females are heterozygous (ZW); and 2) a strong maternal effect on the sperm mobility heritability was reported in earlier studies (Froman *et al*., 2002). In this study, two SNPs were selected from the susceptible loci on chromosome Z based on the previous GWAS work (Froman *et al*., 2013) and tested on the parental DNAs by TaqMan assay. Furthermore, CLEX lines (F_1 population) were generated by crossing high line males with low line females and vice versa. F_1 sires from high x low cross were mated with F_1 hens from low x high cross constituting F_2 population. Two separate GWAS analyses using a 60k SNP chip were done on the F_2 and F_3 roosters (progeny of the F_2 population) selected from the upper and the lower tail of the mobility distribution.

MATERIALS AND METHODS

Experimental animals

The New Hampshire chicken lines were bred and maintained by Dr. Froman in Oregon State University (OSU) as per *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010) guidelines. The reciprocal and double reciprocal crosses between high and low mobile lines were generated and phenotyped as described by Froman *et al*. (2013).

Briefly, for the CLEX line (F_1 reciprocal cross) one sire from the mode of each line was bred to 9 hens of the opposite line. For the double reciprocal cross (F_2 population), F_1 sire from the mode of low x high cross (low line male x high line female) was bred with F_1 dams from the high x low cross (high line male x low line females). The progeny of the F_2 cross constituted F_3 test population. In all three cases eggs were collected and incubated until hatch. Chicks were reared and males were phenotyped in triplicates at 27, 28 and 29 weeks of age using the SMT (Froman and Feltmann, 1998). Single classification ANOVA (Sokal and Rohlf, 1969a) was used to analyze average mobility scores for the F_1 males while nested ANOVA (Sokal and Rohlf, 1969) was used for the F_2 and F_3 populations. At 30 weeks of age all birds were euthanized by cervical dislocation.

Semen sample collection and DNA isolation

For the first GWAS, semen was collected from the 3 subpopulations within low line males: mode (n=10), upper tail (n=18) and lower tail (n=18) of the phenotypic distribution. In contrast, for the F_2 and F_3 GWAS analyses, semen was collected from males within the upper (n=30) and lower tail (n=30) of the mobility distribution. Frozen semen samples were shipped to the University of Arkansas for DNA isolation. Bailes et al. (2007) protocol for DNA isolation was used to extract DNA from the sperm cells. DNAs were further purified by organic extraction using phenol-chloroform-isoamyl alcohol procedure, ethanol precipitated, and dissolved in Te buffer (Tris-Cl 10 mM, EDTA 0.1 mM, pH 7.5). DNAs were quantified using Hoechst 33258 fluorescence measured by fluorimetry (model TKO, Hoefer Scientific Instruments, San- Francisco, CA).

60k SNP chip SNPlotyping

DNAs were shipped to DNA Landmarks (Saint-Jean-sur-Richelieu, Quebec, Canada) for SNPlotyping using a moderate density Illumina 60k SNP chip panel (Groenen et al., 2011). To analyze the GWAS results, allele frequencies for each phenotypic group were calculated separately in Microsoft Excel (Microsoft Corp., Redmond, Wa). Loci were filtered to remove SNPs with: 1) minor allele frequency (MAF) less than 0.05, 2) monomorphic SNPs, 3) unknown chromosomal position, or 4) deviation from Hardy Weinberg equilibrium (HWE) at $P<0.05$. Expected genotype counts for each locus were calculated using allele frequencies and total counts. Chi-square test was implemented for identifying any significant difference between the 2 subpopulations (Sokal and Rohlf, 1969d). Furthermore, the chi-square test computed P-values were transformed and plotted as $1\text{-}\log_{10} P$ value. A sliding window of 10 consecutive SNPs was used to calculate the average of $1\text{-}\log_{10} P$ for each SNP position, to minimize the chances of false positives and for visualizing the data.

Genome data

ICGSC Gallus-gallus-4.0/gal Gal4 (GCA_000002315.2) assembly was used for all chromosomal map positions.

Real-Time PCR

The probes used in the TaqMan assay for quantitative PCR (qPCR) genotyping were designed for specific SNPs on the Z chromosome. The primer-probe sequences, annealing temperatures

and the SNPs information are provided in Table 3.1. Probes were incorporated with Zen modifications quenched with Iowa black and were synthesized by Integrated DNA Technologies (IDT; Coralville, IA). CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Richmond, CA) was used to perform qPCR genotyping. Each reaction comprised of 20 µL reaction volume including 1x Taq-Buffer (50 mM Tris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/mL of BSA), 0.2 mM $MgCl_2$, 0.2 mM dNTP, 1.0 µM each forward and reverse primers, 0.05 µM each probe, 2.5 units of Taq polymerase, and 2 µL of DNA. A two-step PCR procedure was used as follows: 90°C for 30 seconds, 10 cycles of 90°C for 15 seconds and primer specific annealing temperature for 30 seconds; followed by 30 cycles of 90°C for 15 seconds and primer-pair specific annealing temperature for 30 seconds, and a plate read. The TaqMan assay for the SNP Z:32.626 was done with the help of Dr. Rhoads while two undergraduate students, Caroline Daniels and Lauren Locklear, performed TaqMan assays on the SNPs Z:32.900 & Z:63.270 respectively.

Statistical Methods

Nested ANOVA was used for evaluating the variations within males selected from the distribution mode for the study. Single classification ANOVA was used for males within the upper and lower tail of the mobility distribution in all test populations (Froman *et al*., 2013). Mobility phenotype was used to evaluate the genotyped individuals. For the first GWAS where two analyses were made from the same test population, genotype frequencies and allele frequencies were calculated 1) for the individuals from the lower tail and the mode and upper tail combined and 2) for the upper tail individuals vs individuals from the mode and lower tail. For the rest of test populations, genotype and allele frequencies were calculated for males from the

lower tail and the upper tail of the mobility distribution. A chi-square test was performed for each locus and the loci exhibiting P-value < 0.05 were considered for further analysis.

Variations between the observed and calculated allele frequency for the tested SNPs were evaluated by the chi-square test and the chi-square values were corrected for false positives by applying a simple Bonferroni correction; the calculated P values were multiplied by the number of chi square tests performed. A SNP was considered significant having a P-value of <0.05.

RESULTS

The aim of this study was to identify QTLs contributing to the sperm mobility trait in roosters. Chicken lines selected for low and high mobility traits were used as experimental subjects. Reciprocal crosses between the lines were generated to identify major alleles segregating with the mobility phenotype and to identify SNPlotypes that were differentially represented with respect to phenotype. GWAS analyses used a medium density 60k SNP panel. The focus was to examine roosters from subpopulations within the range of sperm mobilities to find SNPs associated with low mobility. Specifically, we wanted to identify SNPs which contribute to the extremely low phenotype because 1) mobility distribution within the line was highly skewed towards the upper ranges of mobility and 2) sperm population within the upper tail of the low mobility line exhibited poorer mobility scores as compared to sperm within the lower tail of high mobile chicken lines (Figure 3.1, Froman and Rhoads, 2013). Previous GWAS analysis conducted comparing the parental low line had identified multiple regions of interest on different chromosomes (Froman and Rhoads, 2013). That same study identified two loci on the Z chromosome near 13 and 16 Mbp that were likely associated with the mobility phenotype

(Froman and Rhoads, 2013). These loci were previously reported to be associated with low sperm mobility in pedigree lines of meat-type chickens (D. D. Rhoads and D. P. Froman, unpublished data). Emphasis was on chromosome Z to identify SNPs that segregate between the lines for two reasons. First, avian males are homozygous for the sex chromosome Z whereas females are heterozygous carrying Z and W chromosomes. Second, an exclusive maternal additive effect has been observed on the heritability of sperm mobility trait (Froman *et al*., 2002). Based on this observation, F_2 population was created by selecting F_1 sire from the low line male x high line female reciprocal cross and F_1 dam from the high line male x low line female reciprocal cross. If the maternal component plays a significant role in determining low mobility phenotype, the associated SNPs would become apparent within F_2 subpopulations based on the direction of the cross. Further, progeny of F_2 and F_3 population was generated to observe the QTLs pattern of segregation within the generations.

The 60k Illumina SNP Bead Chip contained 57,636 total SNPs, out of which 4,353 SNPs were removed due to no or incomplete data, 18,756 SNPs were excluded due to MAF <0.05, 842 SNPs were not included in the study due to non-compliance with HWE (HWE P<0.05). Nearly 680 SNPs were excluded due to their mapping to undefined chromosomal locations. A total of 32,996 informative SNPs were obtained after quality control filtering and were mapped to chromosomes 1-28, and chromosome Z. Loci having the average $1\text{-}\log_{10}P$ value greater than 2.5 were identified as possible candidates for sperm mobility QTLs. For the first GWAS analysis, roosters were selected from two subpopulations of the parental low sperm mobile line. Males from the lower tail, upper tail, and mode of the mobility distribution were selected to identify the associated SNPs. Roosters from the modal distribution were incorporated in the study to include the allelic frequencies of the males representing majority of the low line population. Chi-square

statistical test was employed where genotype and allele frequencies of males from the lower tail and mode were grouped together and were compared with that from the upper tail. Loci that showed significant association in the first GWAS were near 32 and 63 Mbp on chromosome Z (Figure 3.2). To further analyze the role of these regions in affecting the mobility phenotype, we used exonuclease assays (TaqMan) to genotype for the SNPs that differed between the mobility lines. We used three SNPs located at GgaZ:32.626, GgaZ:32.900, & GgaZ:63.270 (Table 3.1). TaqMan assays designed for the SNPs were used to genotype additional DNAs from roosters exhibiting high and low sperm mobility phenotype within the parental high and low sperm mobility lines and CLEX lines (F_2 and F_3 populations; progeny of the reciprocal cross between the parental lines and F_2 cross respectively).

The genotype data from the SNPs GgaZ:32.900 and GgaZ:63.270 did not conform with HWE in both parental high and low sperm mobility lines. In the high sperm mobility line, the genotype data from the SNP GgaZ:32.900 produced nearly equal representation of both alleles 1 and 2 in the population (Table 3.2). Both alleles had high homozygosity frequencies but the heterozygote genotype frequency was extremely low (3%), thus deviating significantly from HWE. In the low sperm mobile line, the genotype data for SNP GgaZ:32.900 revealed allele 2 as the major allele (84%) (Table 3.3). Like high line, there was under representation of the heterozygous genotype (1%) in the parental low line. Near absence of heterozygous genotype frequency in both parental lines suggest the presence of null alleles for the SNP in the parental lines. We did not perform TaqMan assay on the CLEX lines due to the inability of GgaZ:32.900 SNP to conform with HWE in either parental lines. For SNP GgaZ:63.270, allele 2 was the predominant allele in both parental lines (Table 3.2, Table 3.3). The frequency of the homozygous allele 1 genotype was low in both parental lines but in the low line it was less than 10% (Table 3.3). Interestingly, in

both high and low mobile lines, the frequency of heterozygous genotype was higher than expected and thus was not in accord with HWE. The higher frequency of heterozygous genotype was intriguing as homozygous allele 1 frequency was low frequency in both lines (Table 3.3). Unlike for the parental lines, the genotype data from GgaZ:63.270 for both CLEX populations (high x low cross; low x high cross) was in conformation with HWE. In both CLEX populations, allele 2 was the major allele representing 47 % and 41% of total genotypes in high x low and low x high cross respectively (Table 3.4, Table 3.5). We did not observe any significant association between any genotype with the low mobility phenotype in either CLEX population. As allele 1 for GgaZ:63.270 is a minor allele in CLEX this further impedes utility for detecting a QTL due to low genetic diversity.

The genotype data from GgaZ:32.626 was in conformation with HWE for the high line. Allele 1 was the major allele with 60% of the tested population carrying the homozygous genotype whereas just 5% were observed to carry homozygous allele 2 genotype (Table 3.2). Interestingly, the heterozygous genotype showed significant association with the low mobility phenotype within the high mobility lines ($p<0.01$). In the parental low mobility line, genotype data from SNP GgaZ:32.626 was in conformation with HWE. For this SNP, both alleles were equally represented and the heterozygous genotype was represented in higher percentage (45%) (Table 3.3). Unlike high sperm mobility line where we found significant association between the heterozygous genotype with low mobility phenotype ($p=0.006$), we did not observe significant association with phenotype in the low mobility line. The low representation of males expressing high mobility phenotype (n=12 out of 248) within low line could explain the observation regarding association of heterozygous genotype with mobility phenotype. Studies having equal representation of both phenotypes within the line/population could give a clearer picture on the

association of heterozygous genotype with low mobility trait for SNP GgaZ:32.626. To further analyze the segregation of alleles with mobility phenotype the CLEX (reciprocal cross F_1) population were tested for the respective SNP. For GgaZ:32.626 SNP, the high x low CLEX genotype data deviated significantly from HWE. There was low homozygosity for both alleles with a concomitant increase in the heterozygosity (Table 3.4). The low x high CLEX genotype data for the same SNP followed HWE. In high x low CLEX line, where low line female was used as dam the frequency of heterozygous genotype was predominant in the population (61%) (Table 3.4). This observation was intriguing as the calculated frequency of heterozygous genotype was also high (45%) in the low line males (Table 3.3). For the low x high CLEX line (high line female as dam) the distribution of heterozygous genotype frequency followed the same pattern as observed in the high line males for this SNP. In both CLEX crosses, allele 1 was the major allele (Table 3.5). This observation is important because in both CLEX crosses, genotype frequencies of the males followed the same distribution of genotype frequency distributions for their respective dam lines. We did not observe significant association between any genotypes and low mobility phenotype for the GgaZ:32.626 SNP in either CLEX lines.

The SNPs identified in the first GWAS analysis on the parental low line were not found to be associated with the low mobility phenotype in either of the CLEX populations. To detect whether any loci identified in the first GWAS segregated with mobility phenotype, two separate GWAS analyses were conducted on the subpopulations within F_2 and F_3 generations. After the SNP data were filtered (as for the previous GWAS), the allelic and genotypic frequencies were calculated for both generations. The expected and observed SNP data obtained from GWAS, were analyzed using chi-square test to detect significant regions associated with the mobility phenotype. We expected to see similar loci as were detected in the parental low line. Both F_2 and

F_3 generations showed multiple regions of interests spread throughout the genome. Significant peaks were observed for several regions on the Z chromosome in both generations (Figure 3.3). Unfortunately, the regions of significance on chromosome Z shifted between generations. The genotypic frequencies found to have most significant association with the low mobility phenotype were located around 8 Mbp in the F_2 population whereas the region of significance identified in the F_3 generation was at 55 Mbp. The regions identified in both generations were different from the loci identified from the parental low line. When the GWAS data was analyzed with respect to allele or genotype counts we observed the same pattern. For the F_2 generation using allele counts, there was a region around 54 Mbp that showed highest significance whereas for the F_3 generation there was a region near 55 Mbp (Figure 3.4). Similarly, when the GWAS was analyzed for genotype counts, regions of significance were shifted from the parental low mobile line in both F_2 and F_3 generation (Figure 3.4).

The comparative analyses of the GWAS using genotype and allele counts from the three separate GWAS studies on the low line, the progeny of CLEX line (F_2) and the progeny of F_2 (F_3) revealed that the regions of significance kept shifting their genomic locations (Figure 3.3, Figure 3.4).

DISCUSSION

This study was designed to locate consistent, heritable genetic loci affecting sperm mobility trait and genes within those loci to understand the molecular mechanism behind the different sperm mobility phenotypes. Three separate GWAS studies involving 60k SNP chip were used on three different generations differing in their mobility scores. Previously, GWAS study conducted on the two subpopulations within the low line males revealed multiple region of significance over multiple chromosomes (Froman and Rhoads, 2014). SNPs on chromosome Z were chosen for

further analyses as i) significant variations were observed between the low and high mobile males within the low line population, ii) the heritability of sperm mobility was observed to have a strong maternal additive effect (Froman *et al*., 2002), and iii) previous studies on the pedigree lines of meat type chicken and F_2 cross of a broiler breeder and leghorn hens, located 2 loci near 13 and 16 Mbp on the chromosome Z that were found to be strongly associated with the low sperm mobility phenotype. Subpopulations of high and low sperm mobility within the lines were selected for GWAS analyses. The lines chosen for SNPs identification were the low mobile chicken line, the F_2 and F_3 populations. Semen DNA of roosters from the test populations were evaluated on 29 chromosomes including Z chromosome. P-value for each locus was transformed into $1\text{-}\log_{10}P$ for better evaluation. The average sliding window of 10 SNPs was utilized for each locus to reduce the false positives and to increase the confidence level of discovered SNPs. In the first SNPlotyping assay, regions on chromosomes 1, 8, and Z had an average $1\text{-}\log_{10}P$ value > 2.5. SNPs on the chromosome Z were selected for further analysis and were found to have no significance when tested on the high line, low line, and the CLEX line males. Furthermore, two successive GWAS analyses on the F_2 and F_3 males identified different regions of significance spanned over different chromosomes. In all three populations, chromosome Z continued to show consistency in variations but the regions of significance varied. This inconsistency in the genomic regions of significance between the tested lines suggest the role of additional genetic elements like copy number variations (CPV), rare variants, and epistasis in governing the QTLs for the mobility phenotype in roosters

GWAS have been used widely in both humans and animals to explain the heritability of complex diseases, phenotypic variations, and the production traits (Manolio *et al*., 2009). Different SNP arrays of varying depths been used previously to identify significant trait-associated SNPs both

in humans and in livestock species. In chickens, 60k SNP chip array have been used to identify CNVs associated with economically important traits, mapping Mendelian traits between wild and commercial lines, detecting genetic markers for egg production and the quality traits (Jia *et al*., 2013, Wragg *et al*., 2013, Goeren *et al*., 2011, Dorshorst *et al*., 2011, Liu *et al*., 2011). In the current study, a 60k SNP chip was utilized to detect SNPs associated with the sperm mobility trait. Stringent quality control measures and a sliding window of 10 SNPs was applied for each locus to avoid Type 1 and Type 2 errors, common errors encountered in the genotyping technology. The inconsistency of significant SNPs in the inter-generational GWAS assays using medium density 60k SNP chip highlights the importance of high density SNP arrays and/or whole genome sequencing methods to identify SNPs for complex reproductive trait like sperm mobility. Application of quality control measures, on one hand, improved the SNP calling and decreased the false positives but on the other hand drastically decreased the total SNPs to work with. For instance, nearly 33% of the SNPs were discarded because of monomorphic alleles and in total 43% of the total GWAS data was not included in the analyses due to stringent statistical criteria applied in the current study. The SNPs identified on chromosome Z, although they did not produce the affirmative data in TaqMan assays, shouldn't be discarded. Previous studies have shown the importance of non-significant SNPs in predicting the heritability of complex diseases and traits (Makowsky *et al*., 2011, Eleftherohorinou *et al*., 2009). If the sperm mobility trait is controlled by SNPs that have small effect size (every allele contributes towards poor mobility phenotype) then the probability of retrieving significant loci through medium density GWAS analysis becomes very low.

This study used 60k SNP chip method for GWAS analyses in the test generations which yielded nearly 36,000 informative SNPs per generation. This information proved insufficient to

determine the QTL for the sperm mobility. Some of the limiting factors in this study include use of medium density SNP array that has a lower predictive power in detecting SNPs of lower effect sizes. If the contribution of SNPs or loci towards a quantitative trait is small and these SNPs span all over the genome, the low density GWAS may not pick them up (Morota and Gianola, 2014). Secondly, limited sequence information on the micro-chromosomes and the sex chromosomes in the chicken genome assembly utilized decreased the probability to scan the probable SNPs affecting the trait. Chicken micro-chromosomes tend to be more gene rich than the macro-chromosomes (Hillier *et al*., 2004). The possibility of epistatic interactions between the genetic loci affecting the mobility phenotype cannot be ignored. Recently, application of high density SNP arrays and whole genome sequencing have been shown to solve some of the aforementioned issues. In chicken, the next generation sequencing (NGS) techniques has tremendously improved the chances to detect rare SNPs due to increased coverage depth generating millions of SNPs spanning all over the genome, higher representation of intergenic and intronic SNPs, and higher performance in conferring SNPs in micro-chromosomes (Pertille *et al*., 2016). There is growing evidence that shows the efficacy of NGS technique for GWAS detecting QTLs in the intergenic regions, not detected previously using 60k SNP chip, and these SNPs play pivotal role in the associated phenotype (S. Dey manuscript in review). This work highlights the inadequacy of low and medium density SNP chip methods to predict rare genetic elements that contribute towards complex quantitative traits.

SUMMARY AND CONCLUSION

Sperm mobility is a complex quantitative trait positively correlated with male fertility in chicken which makes it an important production trait (Froman et al., 1999). In avian species, sperm mobility has high heritability index suggesting the involvement of genetic elements in influencing the mobility phenotype. Identification of the genetic loci contributing towards mobility phenotype is important for commercial selection programs and to enhance the knowledge of male gamete biology. To predict genomic locations of probable SNPs we used 60k SNP chip GWAS on three test populations selected for low mobility phenotype to remove the founder effect bias. Regions of significance were identified on chromosome Z, but in further analyses the regions appeared to be non-significant and regions of significance shifted between generations. Recent studies using whole genome sequencing to identify informative QTLs and CNVs in humans and livestock species have revolutionized the ability to detect rare SNPs and capture signals that were difficult to notice by using low to moderate density SNP arrays. In future, the implementation of whole genome sequencing approach in chicken lines differing in mobility phenotype will help in better visualization of the variations which may lead to the detection of small effect size SNPs affecting sperm mobility phenotype.

FIGURES

Figure 3.1: Sperm mobility distribution within low and high sperm mobility New Hampshire chicken lines. Mobility scores of sperm exhibiting high mobility within low mobility chicken lines are lower than the mobility scores of sperm exhibiting low mobility within high mobility chicken lines. Adapted from Froman and Rhoads (2013).

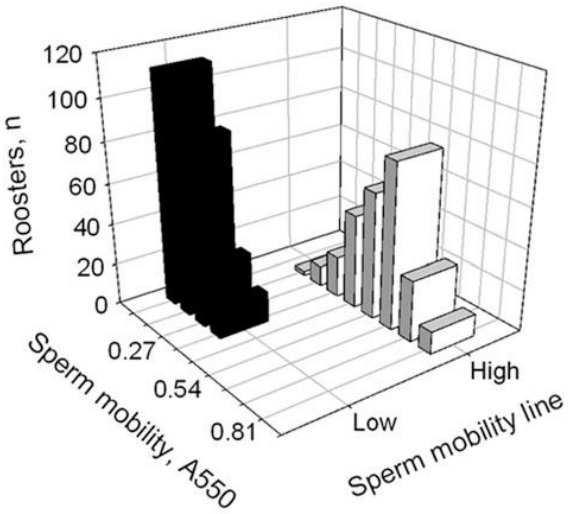


Figure 3.2: A comparative analysis of genotype frequencies and allele frequencies obtained from Genome wide association studies conducted on the subpopulations within the low mobile parental males. Multiple regions of interests were found on the chromosome Z. Association of SNP loci to mobility was visualized as an average of 1-logP value. Y axis represents average (1-log P) value whereas X axis denotes the positions of SNPs in the chromosome Z.

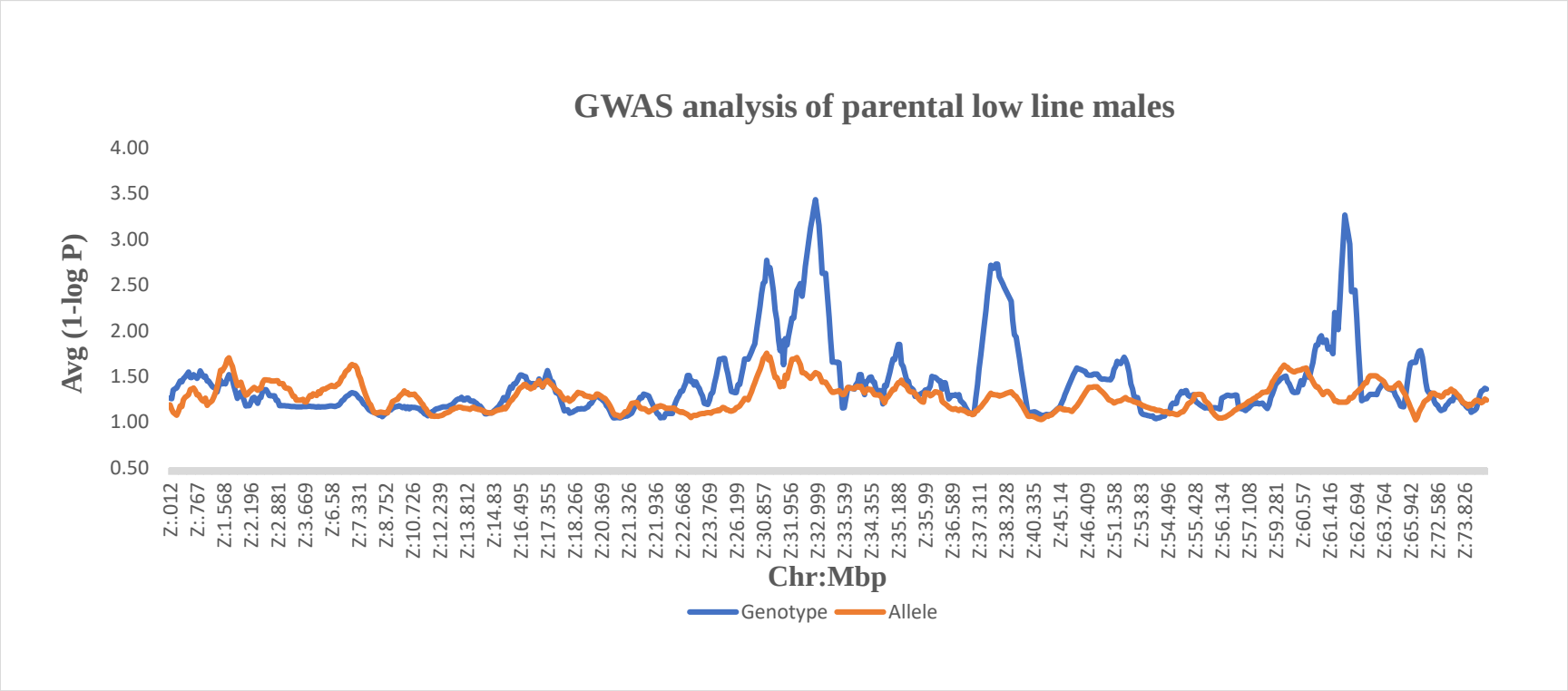


Figure 3.3 A comparative analysis of genotype frequencies obtained from Genome wide association studies conducted within the low line males, first filial generation of CLEX lines males (F_2) and F_3 males (progeny of F_2) on the chromosome Z. Multiple regions of interests were found on the chromosome Z but the regions varied between the three test subjects. 3 regions were identified; 2 at 32 Mbp and one at 63 Mbp in the low line males and were studied further. Association of SNP loci to mobility was visualized as an average of 1-$\log_{10}$P value.

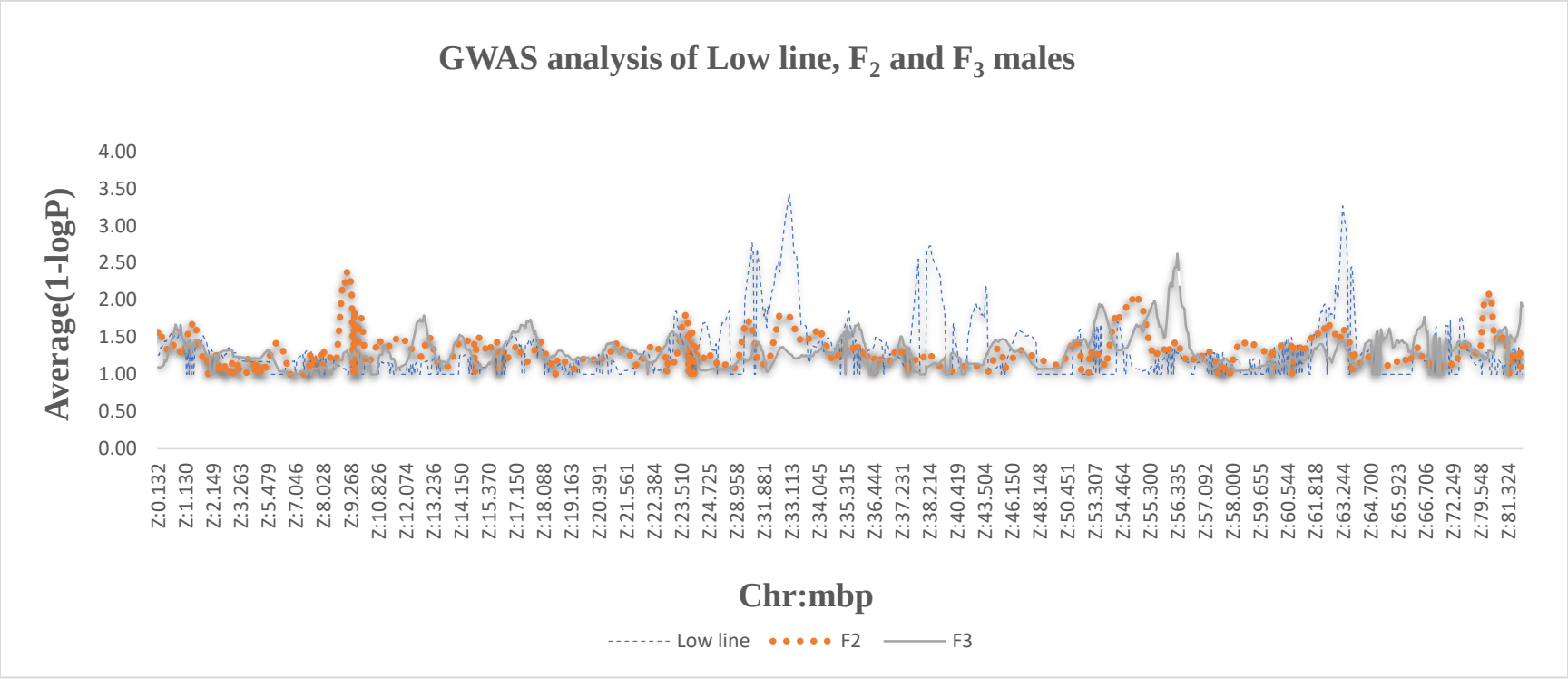
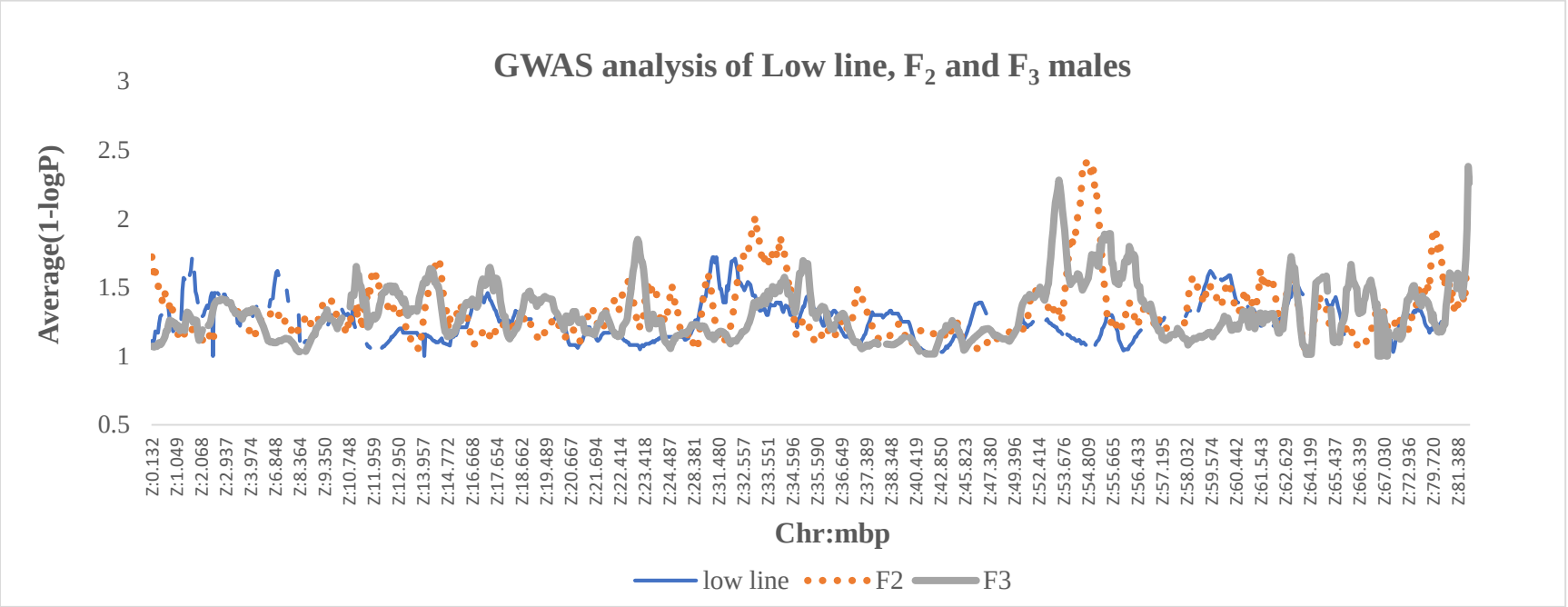


Figure 3.4 A comparative analysis of allele frequencies obtained from Genome wide association studies conducted within the low line males, first filial generation of CLEX lines males (F_2) and F_3 males (progeny of F_2). Multiple regions of interests were found on the chromosome Z but the regions varied between the three test subjects. Association of SNP loci to mobility was visualized as an average of 1-$\log_{10}$P value.



TABLES

Table 3.1 Location of SNPs identified from GWAS. Sequences of forward and reverse primers, probes, and conditions of qPCR are also enlisted. The orientation of sequences of both forward (F) and reverse (R) primers and probes are in 5`-3`. Probe 1 to detect allele 1 is labelled with FAM while Probe 2 to detect allele 2 is labelled with HEX.

SNP ID	**SNP location (Chr:Mbp)**	**Refernce/ Alternative Allele strand**	**Annealing Temp (^{0}C)**	**Primer**	**Probe**
32.626	GgaZ:32.626	C/T (Fwd)	62.2	**F**: CATTGTGGACTGAGGGAAAATAAAACT **R:** GCAGCTCAAGAGGTCAGTGAGCATA	**Probe 1** TT**c**ATCTGACATTGGGTGTGTTGTG[a] **Probe 2** TT**t**ATCTGACATTGGATGTGTTGTGGATAA
32.900	GgaZ:32.900	A/G (Fwd)	66.5	**F**: ACGAGCAAATAGCAACCTAGTGAACGA **R**: GATTCAAGCTGGAAGACATCGAGGAGA	**Probe 1** TG**a**TGCTGTAACTCTACAAGTCAGCTAG **Probe 2** TG**g**TGCTGTAACTCTACAAGTCAGCTAG
63.270	GgaZ:63.270	C/T (Fwd)	66.3	**F**: CTTCCATAGCGAGGCCTAAACAGCTTTAACA **R**: GGTTGGAAAGCTTTACAACTCCATTGCTGGT	**Probe 1** TC**c**TAAAATGAAGCCTAATACTGGTGCTTC **Probe 2** TC**t**TAAAATGAAGCCTAATACTGGTGCTTCAG

[a] Lower-case letters indicate loci specific for SNP.

Table 3.2 Genotype data for GgaZ:32.626, GgaZ:32.900, and GgaZ:63.270 for roosters from high sperm mobility line. Within high line, males from the upper tail (high sperm mobility phenotype) and lower tail (low sperm mobility phenotype) of the mobility distribution were genotyped using each assay. Genotype 1 is homozygous for the allele 1, 1+2 is heterozygous and 2 is homozygous for allele 2 (Table 3.1). Genotype frequencies (Genotype Freq.) were calculated for the parental high line and the high and low mobile subpopulations with in the line. The total number of genotypes (Count) within the high line subpopulations is listed in separate columns. Chi-square test was conducted and P-values are presented for genotypes with frequency $\geq$0.10. Data for high line, low line and CLEX line males are presented separately.

High line males

SNP ID	SNP location (Chr:Mbp)	Genotype	Genotype %	High count[a]	Low count[b]	High freq.	Low freq.	P-value
32.626	GgaZ:32.626	1	60%	249	20	0.63	0.25	0.109
		1+2	35%			0.32	0.75	0.006*
		2	5%			0.05	0.00	-
32.900	GgaZ:32.900	1	54%	220	21	0.56	0.33	0.178
		1+2	3%			0.03	0.05	-
		2	43%			0.41	0.62	0.160
63.270	GgaZ:63.270	1	14%	254	21	0.14	0.14	0.990
		1+2	30%			0.31	0.19	0.347
		2	56%			0.55	0-.67	0.467

a: number of males having high mobility score; b: number of males having low mobility score.

Table 3.3 Genotype data for GgaZ:32.626, GgaZ:32.900, and GgaZ:63.270 for roosters from low sperm mobility line. Within low line, males from the upper tail (high sperm mobility phenotype) and lower tail (low sperm mobility phenotype) of the mobility distribution were genotyped using each assay. Genotype 1 is homozygous for the allele 1, 1+2 is heterozygous and 2 is homozygous for allele 2 (Table 3.1). Genotype frequencies (Genotype Freq.) were calculated for the parental low line and the high and low mobile subpopulations with in the line. The total number of genotypes (Count) within the low line subpopulations is listed in separate columns. Chi-square test was conducted and P-values are presented for genotypes with frequency ≥0.10. Data for high line, low line and CLEX line males are presented separately.

Low line males

SNP ID	SNP location (Chr:Mbp)	Genotype	Genotype %	High count[a]	Low count[b]	High freq.	Low freq.	P-value
32.626	GgaZ:32.626	1	21%	12	236	0.17	0.21	0.760
		1+2	47%			0.67	0.46	0.314
		2	32%			0.17	0.33	0.330
32.900	GgaZ:32.900	1	15%	11	238	0.09	0.16	0.592
		1+2	1%			0.00	0.01	-
		2	84%			0.91	0.84	0.796
63.270	GgaZ:63.270	1	6%	12	237	0.08	0.06	-
		1+2	18%			0.17	0.18	0.907
		2	76%			0.75	0-.76	0.971

a: number of males having high mobility score; b: number of males having low mobility score.

Table 3.4 Genotype data for GgaZ:32.626 and GgaZ:63.270 for roosters from CLEX line (High line male x Low line female). Within CLEX line, males from the upper tail (high sperm mobility phenotype) and lower tail (low sperm mobility phenotype) of the mobility distribution were genotyped using each assay. Genotype 1 is homozygous for the allele 1, 1+2 is heterozygous and 2 is homozygous for allele 2 (Table 3.1). Genotype frequencies (Genotype Freq.) were calculated for the CLEX line and the high and low mobile subpopulations with in the line. The total number of genotypes (Count) within the low line subpopulations is listed in separate columns. Chi-square test was conducted and P-values are presented for genotypes with frequency $\geq$0.10. Data for high line, low line and CLEX line males are presented separately.

H x L males

SNP ID	SNP location (Chr:Mbp)	Genotype	Genotype %	High count[a]	Low count[b]	High freq.	Low freq.	P-value
32.626	GgaZ:32.626	1	34%	119	2	0.34	0.50	0.693
		1+2	61%			0.62	0.50	0.828
		2	5%			0.04	0.00	-
63.270	GgaZ:63.270	1	10%	102	2	0.10	0.00	0.658
		1+2	43%			0.42	1.00	0.218
		2	47%			0.48	0.00	0.327

a: number of males having high mobility score; b: number of males having low mobility score.

Table 3.5 Genotype data for GgaZ:32.626 and GgaZ:63.270 for roosters from CLEX line (Low line male x High line female). Within CLEX line, males from the upper tail (high sperm mobility phenotype) and lower tail (low sperm mobility phenotype) of the mobility distribution were genotyped using each assay. Genotype 1 is homozygous for the allele 1, 1+2 is heterozygous and 2 is homozygous for allele 2 (Table 3.1). Genotype frequencies (Genotype Freq.) were calculated for the CLEX line and the high and low mobile subpopulations with in the line. The total number of genotypes (Count) within the low line subpopulations is listed in separate columns. Chi-square test was conducted and P-values are presented for genotypes with frequency $\geq$0.10. Data for high line, low line and CLEX line males are presented separately.

L x H males

SNP ID	SNP location (Chr:Mbp)	Genotype	Genotype %	High count[a]	Low count[b]	High freq.	Low freq.	P-value
32.626	GgaZ:32.626	1	90%	116	18	0.89	1.00	0.642
		1+2	10%			0.11	0.00	0.156
		2	2%			0.00	0.00	-
63.270	GgaZ:63.270	1	6%	98	10	0.06	0.00	0.434
		1+2	54%			0.57	0.20	0.127
		2	41%			0.37	0.80	0.041*

a: number of males having high mobility score; b: number of males having low mobility score.

REFERENCES

Abasht, B., & Lamont, S. J. (2007). Genome-wide association analysis reveals cryptic alleles as an important factor in heterosis for fatness in chicken F2 population. *Animal genetics*, *38*(5), 491-498.

Bailes, S. M., Devers, J. J., Kirby, J. D., & Rhoads, D. D. (2007). An inexpensive, simple protocol for DNA isolation from blood for high-throughput genotyping by polymerase chain reaction or restriction endonuclease digestion. *Poultry Science*, *86*(1), 102-106.

Bakst, M. R., Wishart, G., & Brillard, J. P. (1994). Oviducal sperm selection, transport, and storage in poultry. *Poultry Science Review*, *5*, 117-143.

Birkhead, T. R., Martinez, J. G., Burke, T., & Froman, D. P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London B: Biological Sciences*, *266*(1430), 1759-1764.

Bowling, E. R., Froman, D. P., Davis, A. J., & Wilson, J. L. (2003). Attributes of broiler breeder males characterized by low and high sperm mobility. *Poultry Science*, *82*(11), 1796-1801.

Bowling, E. R., Froman, D. P., Davis, A. J., & Wilson, J. L. (2003). Attributes of broiler breeder males characterized by low and high sperm mobility. *Poultry Science*, *82*(11), 1796-1801.

Dorshorst, B., Molin, A. M., Rubin, C. J., Johansson, A. M., Strömstedt, L., Pham, M. H., Chen, C.F., Hallbook, F., Ashwell, C., & Andersson, L. (2011). A complex genomic rearrangement involving the endothelin 3 locus causes dermal hyperpigmentation in the chicken. *PLoS genetics*, *7*(12), e1002412.

Eleftherohorinou, H., Wright, V., Hoggart, C., Hartikainen, A. L., Jarvelin, M. R., Balding, D., Colin, L. & Levin, M. (2009). Pathway analysis of GWAS provides new insights into genetic susceptibility to 3 inflammatory diseases. *PloS one*, *4*(11), e8068.

Froman, D. P. (2006). Application of the sperm mobility assay to primary broiler breeder stock. *Journal of applied poultry research*, *15*(2), 280-286.

Froman, D. P., & Feltmann, A. J. (1998). Sperm mobility: a quantitative trait of the domestic fowl (Gallus domesticus). *Biology of Reproduction*, *58*(2), 379-384.

Froman, D. P., & Kirby, J. D. (2005). Sperm mobility: phenotype in roosters (Gallus domesticus) determined by mitochondrial function. *Biology of Reproduction*, *72*(3), 562-567.

Froman, D. P., & McLean, D. J. (1996). Objective measurement of sperm motility based upon sperm penetration of Accudenz®. *Poultry Science*, *75*(6), 776-784.

Froman, D. P., & Rhoads, D. D. (2013). Breeding and genetics symposium: A systems biology definition for chicken semen quality. *Journal of animal science*, *91*(2), 523-529.

Froman, D. P., Feltmann, A. J., Pendarvis, K., Cooksey, A. M., Burgess, S. C., & Rhoads, D. D. (2011). Physiology and endocrinology symposium: a proteome-based model for sperm mobility phenotype. *Journal of animal science*, *89*(5), 1330-1337.

Froman, D. P., Pizzari, T., Feltmann, A. J., Castillo-Juarez, H., & Birkhead, T. R. (2002). Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, Gallus gallus domesticus. *Proceedings of the Royal Society of London B: Biological Sciences*, *269*(1491), 607-612.

Groenen, M. A., Megens, H. J., Zare, Y., Warren, W. C., Hillier, L. W., Crooijmans, R. P., Vereijken, A., Okimoto, R., Muir, W.M. & Cheng, H. H. (2011). The development and characterization of a 60K SNP chip for chicken. *BMC genomics*, *12*(1), 274.

Hillier, L. W., Miller, W., Birney, E., Warren, W., Hardison, R. C., Ponting, C. P., Bork, P., Burt, D.W., Goeren, M.A., Delany, M.E. & Dodgson, J. B. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature*, *432*(7018), 695-716.

Holsberger, D. R., Donoghue, A. M., Froman, D. P., & Ottinger, M. A. (1998). Assessment of ejaculate quality and sperm characteristics in turkeys: sperm mobility phenotype is independent of time. *Poultry Science*, *77*(11), 1711-1717.

Jia, X., Chen, S., Zhou, H., Li, D., Liu, W., & Yang, N. (2013). Copy number variations identified in the chicken using a 60K SNP BeadChip. *Animal genetics*, *44*(3), 276-284.

Labas, V., Grasseau, I., Cahier, K., Gargaros, A., Harichaux, G., Teixeira-Gomes, A. P., Alves, S., Bourin, M., Gerard, N. & Blesbois, E. (2015). Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *Journal of proteomics*, *112*, 313-335.

Li, G., Li, D., Yang, N., Qu, L., Hou, Z., Zheng, J., Xu, G. & Chen, S. (2014). A genome-wide association study identifies novel single nucleotide polymorphisms associated with dermal shank pigmentation in chickens. *Poultry Science*, *93*(12), 2983-2987.

Liu, W., Li, D., Liu, J., Chen, S., Qu, L., Zheng, J., Xu, G., & Yang, N. (2011). A genome-wide SNP scan reveals novel loci for egg production and quality traits in white leghorn and brown-egg dwarf layers. *PloS one*, *6*(12), e28600.

Magee, D. A., Park, S. D. E., Scraggs, E., Murphy, A. M., Doherty, M. L., Kijas, J. W., & MacHugh, D. E. (2010). Technical note: High fidelity of whole-genome amplified sheep deoxyribonucleic acid using a high-density single nucleotide polymorphism array-based genotyping platform. *Journal of animal science*, *88*(10), 3183-3186.

Makowsky, R., Pajewski, N. M., Klimentidis, Y. C., Vazquez, A. I., Duarte, C. W., Allison, D. B., & de Los Campos, G. (2011). Beyond missing heritability: prediction of complex traits. *PLoS genetics*, *7*(4), e1002051.

Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., McCarthy, M.I., Ramos, E.M., Cardon, L.R., Chakravarti, A. & Cho, J. H. (2009). Finding the missing heritability of complex diseases. *Nature*, *461*(7265), 747-753.

Matukumalli, L. K., Lawley, C. T., Schnabel, R. D., Taylor, J. F., Allan, M. F., Heaton, M. P., O'Connell, J., Moore, S.S., Smith, T.P., Sonstegard, T.S. & Van Tassell, C. P. (2009). Development and characterization of a high-density SNP genotyping assay for cattle. *PloS one*, *4*(4), e5350.

Morota, G., & Gianola, D. (2014). Kernel-based whole-genome prediction of complex traits: a review. *Frontiers in genetics*, *5*.

Pértille, F., Guerrero-Bosagna, C., Da Silva, V. H., Boschiero, C., da Silva Nunes, J. D. R., Ledur, M. C., Jensen, P. & Coutinho, L. L. (2016). High-throughput and cost-effective chicken genotyping using next-generation sequencing. *Scientific Reports*, *6*, 26929.

Ramos, A. M., Crooijmans, R. P., Affara, N. A., Amaral, A. J., Archibald, A. L., Beever, J. E., Bendixen, C., Churcher, C., Clark, R., Dehais, P., & Hansen, M. S. (2009). Design of a high-density SNP genotyping assay in the pig using SNPs identified and characterized by next generation sequencing technology. *PloS one*, *4*(8), e6524.

Sun, Y., Liu, R., Zhao, G., Zheng, M., Sun, Y., Yu, X., Li, P. & Wen, J. (2014). Genome-wide linkage analysis and association study identifies loci for polydactyly in chickens. *G3: Genes| Genomes| Genetics*, *4*(6), 1167-1172.

USDA (2017). Broiler Hatchery. National Agricultural Statistics Service. http://usda.mannlib.cornell.edu/usda/current/BroiHatc/BroiHatc-05-17-2017.pdf. Accessed May, 2017.

Wragg, D., Mwacharo, J. M., Alcalde, J. A., Hocking, P. M., & Hanotte, O. (2012). Analysis of genome-wide structure, diversity and fine mapping of Mendelian traits in traditional and village chickens. *Heredity*, *109*(1), 6-18.

CHAPTER 4

Cultural characterization of chicken primordial germ cells (cPGCs) grown without feeder-layer in well-defined culture medium and germline transmission of cultured cPGCs from the low sperm mobile lines into the recipient high mobile White Leghorn (WL) embryos

ABSTRACT

The objective of this study is to demonstrate whether the somatic-germ cell interactions influence the sperm mobility phenotype using germ-line chimeric chicken as a research model. Chicken primordial germ cells (cPGCs) were used as a tool to create germline chimeric chickens. Low line New Hampshire (NH) embryos were used to isolate donor cPGCs. Busulfan treated 3-days old White Leghorn (WL) embryos were used as recipients. Isolated blood cPGCs were cultured and maintained in feeder-less culture conditions. Cultural characteristics and germ line specificity of cPGCs were tested by immunocytochemistry, biochemical tests, and real time PCR. Cultured donor cPGCs were injected into the vasculature of busulfan treated WL embryos which were incubated until hatch. After hatch, putative male chimera chicks were reared until sexual maturity. Eight separate trials were conducted in which the hatching percentage ranged from 9% to 43%. Nearly 52% of the hatched chicks were males. Semen of the reared males were tested for the presence of an A/G SNP in the mitochondrial gene for $tRNA^{ARG}$. The representation of donor derived sperms was determined to be less than 3 % in the recipient semen; and below the level of sensitivity of the assay. Due to the low proportion of low line genotype in the recipient semen, the sperm mobility assay to determine the phenotype of donor derived sperm was not performed. This study represented a novel approach to answer the question of sperm mobility by utilizing PGCs model. In future, the demonstration of cellular interactions between germ cells and somatic cells can be clarified by utilizing more robust methods to reduce resident cPGCs in the recipient embryos and using genetically modified donor cPGCs with reporter gene to track their presence in the recipient roosters.

INTRODUCTION

Transgenic animals are considered as a great resource in research and in biotechnology industries due to their wide applications and practical feasibility. After the first report on the transgenic chicken generation using avian retrovirus (Salter *et al*., 1987), there has been a rapid surge in the number of publications reporting transgenic chicken as it serves as an excellent model for developmental biology (Smith and Sinclair, 2001; Mozdiac and Pettite, 2004; Rashidi and Sottile, 2009; Vergara and Canto-Solar 2012) and as a bioreactor in generating pharmaceutical proteins (Lillico *et al*., 2005; Ivarie 2006). Various approaches have been applied to generate transgenic chickens including use of lentiviral vectors (McGrew *et al*., 2004; Zhu *et al*, 2005), PGC culture (Naito *et al*., 1996; van de Lavoir *et al*., 2006), transposons (McDonald *et al*., 2012; Park and Han, 2012), gene targeting in cultured PGCs by homologous recombination (Schusser et al., 2013), precise genome editing using CRISPR/Cas technology (Park *et al*., 2014). After the report on use of cultured PGCs in generating germ line chicken chimera by Naito (1996), research was intensified in defining cultural characteristics of cPGCs *in vitro* (Naito *et al*., 2010; Song *et al.,* 2014). Chimera chicken generation has been achieved using cPGCs isolated from chicken embryos at different developmental stages such as embryonic blood, developing gonads, embryonic germ cells (EG), and blastodermal cells etc., cultured *in vitro* before transferring into recipient embryos (Naito *et al*., 1994; Chang *et al*., 1995; Han *et al*., 2002; Park *et al*., 2003; Watanabe *et al*., 1992).

In all species in the animal kingdom, PGCs are segregated from the somatic lineages however the mode and timing of germ cell segregation varies. There are two distinct modes of PGCs segregation that have been well documented in the animal kingdom so far. In some species, maternally inherited determinants determine the formation of germ cells, hence PGCs are

segregated and identified very early in the embryonic development whereas in mammals germ cells are segregated later in the embryonic development and are induced by epigenetic signals from the surrounding somatic tissues hence following an epigenetic mode of PGC segregation (Extavour and Akam, 2003). Some of the species that follow predetermined mode include *Drosophila*, *Caenorhabditis elegans*, *Xenopus levis*, *Danio rerio*, *Gallus gallus* (Ephrussi and Lehmann, 1992; Hird et al., 1996; Ikenishi et al., 1986; Braat et al., 1999; Tsunekawa et al., 2000).

In chickens, PGC originate from the epiblast and are initially localized in the central zone of the area pellucida (stage X as documented by Eyal-Giladi et al. 1981). At HH stage 4 (18-19 h of incubation) nearly 200 cPGCs migrate to the germinal crescent, proliferate and passively enter into and circulate in the blood stream until HH stage 10-12 (40-50 h of incubation) (Ando & Fujimoto, 1983; Ukeshima *et al*., 1991). At stage 14 (51-53 h of incubation) PGCs reach their highest number in the bloodstream. The cPGCs exit the bloodstream at the developing genital ridges at stage 17 (52-64 h of incubation) (Meyer, 1964), where they actively colonize the future gonads. In females, cPGCs enter into meiosis after 8 days of incubation whereas in males, PGCs differentiate into spermatogonial stem cells (SSCs) after 13 days of incubation (Howarth, 1995).

In both sexes, germ cells maintain intimate contact with the gonadal somatic cells which is crucial for their survival and successful gametogenesis. In males, cellular interactions between the testicular somatic cells (Leydig cells, interstitial epithelial cells and Sertoli cells) and germ cells is crucial for the maintenance of spermatogenesis (Pointis and Segretain, 2005). Both endocrine factors such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and intercellular contacts mediate the interactions that occur between the germ cells and the gonadal

cells (McLachlan *et al*., 2002; Huleihel & Lunenfeld, 2004). In the seminiferous tubules, Sertoli cells maintain intimate contact with each other and with the germ cells through gap junctions. These junctions allow movement of nutrients (≤1 kDa molecular mass) and signaling molecules between the cells (Bruzzone *et al*., 1996). The importance of cellular interactions between Sertoli-Sertoli cells and Sertoli-germ cells in spermatogenesis is very well elucidated in a review by Mruk and Cheng (2004). Changes in the activity of proteases and protease inhibitors within germ cells have been reported after they attach with the Sertoli cells through gap junctions (Mruck *et al*., 1997). Reports have shown that any interference in these cellular interactions disrupts the germ cell movement from the basal lamina to the adluminal compartment of the seminiferous tubule (Cheng and Mruk, 2002). The integral role of Sertoli cells in synchronizing proliferation and differentiation of male germ cells by unidirectional signaling through gap junctions is well documented (Decrouy et al., 2004; Risley et al., 2002). There remain unanswered questions regarding genes and signaling pathways controlling these processes. Whether these interactions can influence the phenotype of the differentiated germ cells is still unclear. Studies in mice have demonstrated that when donor testicular cells were transplanted into recipient testes, the recipient generated normal spermatozoa with donor genotype in nearly one-third of the experimental males (Brinster and Zimmermann, 1994). Since then germ cell transplantation techniques have been implemented with different success rates in many mammalian species (Brinster and Avarbock, 1994; Honaramooz et al., 2002; Shinohara et al., 2003; Herrid et al., 2009), fishes (Lacerda et al., 2006, 2013; Majhi et al., 2009) and birds (Benesova *et al*., 2013; Pereira *et al*., 2013). Furthermore, germ cell transplantation provides a unique opportunity to tweak the genetic resource and ask questions pertaining to germ cell biology.

In birds, especially in chicken, use of primordial germ cells in germline chimera production has been established (Mozdziak et al., 2006; Park & Han, 2012). Multiple studies have defined cPGCs culture characteristics (Jung et al., 2006, Naito et al., 2010), optimum culture conditions for cPGCs growth and proliferation (McDonald et al., 2010; Miyahara et al., 2012), use of different feeder layers for cPGCs maintenance *in-vitro* (Raucci et al., 2014), and growth factors required for retaining cPGC commitment towards germ-line lineages (Choi et al., 2010; Lavoir et al., 2006; Lu et al., 2014). In the current study, we have i) used a minimalistic approach of culturing cPGCs from embryonic blood in a defined feeder-less culture medium, ii) characterized the cultured cPGCs for PGC specific genes and stem cell specific markers, through biochemical assays, and immunocytochemical staining, and iii) transplanted cPGCs into the blood stream of PGC depleted White Leghorn embryos. Recipient embryos were grown to maturity and tested for the percentage of donor derived sperms in the semen of recipient males by measuring the relative presence of a diagnostic mitochondrial A/G SNP in the tRNA arginine gene.

MATERIALS AND METHODS

Fertilized eggs and animal care

Dr. Froman (Oregon State University; OSU) provided the low line New Hampshire (NH) chicken embryos, for donor cPGCs culture. The poultry research farm at the University of Arkansas (UofA) provided fertile White Leghorn (WL) eggs. Putative chimeric chickens were maintained in the poultry farm at the UofA until sexual maturity. All animal research was approved (#15002) by the UofA Institutional Animal Care and Use Committee (IACUC) and experimentation guidelines were followed throughout the study.

Isolation and culture of donor cPGCs in feeder-less culture conditions

One-day old NH embryos were incubated (NatureForm® hatchery systems) maintained at 99^0F temperature and a relative humidity of 50-60% for optimum embryonic development. The racks were tilted at opposing 45^0 angles every 30 minutes to maintain optimum conditions for developing embryos. At HH stage 14 (52-54 h of incubation), whole blood was isolated from the anterior splanchnopleure blood vessel of NH under a dissecting microscope (Nikon, SMZ 745T) using a fine glass micropipette created manually using a vertical pipette puller (David Kopf Instruments, model 700C). Isolated blood was cultured in 12-well tissue culture plates in cPGC culture conditions as described by Miyahara et al. (2014) with a few modifications. Briefly, 4-5µl whole blood containing cPGCs were seeded in 12-well tissue culture plates (VWR, Radnor, PA) with complete cPGCs culture media comprising 1X advanced DMEM (supplemented with high glucose, non-essential amino acids, sodium pyruvate and phenol red; ThermoFischer Scientific, Waltham, MA, USA), 10% (v/v) Fetal Bovine Serum (FBS; ThermoFischer Scientific), 2.5% Chicken serum (CS, ThermoFischer Scientific), 1X Nucleosides (EmbryoMax® ; Millipore, Billarica, MA, USA), 1X Antimycotic-Antibiotic solution (ThermoFischer Scientific), 2mM glutamine (GlutaMax; ThermoFischer Scientific), 0.55 mM β-mercaptoethanol (ThermoFischer Scientific) and growth factors including 10ng/ml human fibroblast growth factor (h-FGF2; ProSpec, Rehovot, Israel), 5ng/ml human Stem Cell Factor (h-SCF; ProSpec, Israel), 2ng/ml human Leukocyte Inhibitory Factor (h-LIF; ProSpec, Israel), and 2.5ng/ml human recombinant Insulin Growth Factor 1 (IGF1; ThermoFischer Sientific). The cell cultures were maintained in an incubator at 37^0C and 5% CO_2. The seeded cells containing blood cells and cPGCs were cultured for 3-4 days without changing the medium. Once the cells started attaching to the culture wells, the PGC media was changed every 2 days. The cellular morphology and colony

forming characteristics of cPGCs became apparent after 6-8 weeks of culture in feeder-less conditions. cPGCs were distinguished from remaining blood cells owing to their large nucleus (10-20μm) and greater refractive index due to numerous cytoplasmic lipid droplets. The cellular morphology and integrity was checked daily using an inverted microscope (Olympus, Phase contrast).

Sexing of the donor embryos

Sexing of the donor NH embryos was done by polymerase chain reaction (PCR). Bailes et al. (2007) protocol for DNA isolation was utilized to extract DNA for sexing by PCR. Approximately 4-5μl of blood was collected/embryo (HH stage 14) using glass micropipette and mixed with 200μl STM buffer (64 mM sucrose, 20 mM Tris Cl, pH 7.5, 10 mM MgCl2, and 0.5% Triton X-100). Cool temperature was maintained using ice throughout the extraction procedure until specified otherwise. The nuclei were pelleted by centrifuging blood-STM mixture @ 1000 g for 5 minutes at room temperature. The supernatant was discarded and pelleted nuclei were resuspended in 200μl TEN + pronase cocktail (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 μg/mL of pronase; Sigma-Aldrich, St. Louis, MO) by repeated trituration. The samples were incubated @37^{0}C for 1 h with shaking in a bacteriological incubator. Finally, the pronase was inactivated by incubation in a 65^{0}C water bath for 10 min. Prepared DNA (2 μl) was used as a template for sexing embryos using primers for W-chromosome *XhoI* repetitive sequence (Wxho) and 18S ribosomal sequence (Ribo) (Clinton et *al*., 2001). The primers for sexing PCR are as follows:

Wxho forward primer 5`-CCCAAATATAACACGCTTCACT-3`,

WCS reverse primer 5`- GAAATGAATTATTTTCTGGCGAC-3`;

Ribo forward primer 5`-AGCTCTTTCTCGATTCCGTG-3` and

Ribo reverse primer 5`-GGGTAGACACAAGCTGAGCC-3`.

The concentrations and volumes of reagents used for sexing reaction are as follow: 2µl DNA, 2.0 mM each primer pairs (Wxho and Ribo), 0.2 mM dNTPs, 4 U (0.2µM) Taq polymerase, 1x Taq buffer (50 mMTris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/ml BSA, 0.25mM $MgCl_2$). The reaction conditions used were 90°C for 2 min, 45 cycles of 90°C for 30 s, 55°C for 15 sec., 72°C for 1 min, followed by a final extension of 72°C for 3 min. The expected PCR sizes were 416 bp and 256 bp for Wxho & Ribo products, respectively. PCR products were resolved in 1.5% agarose gel. If two bands are evident the gender is female (carries W chromosome) while in males only 1 band is evident.

Periodic Acid-Schiff (PAS) staining

Colony-forming PGCs obtained after 6-8 weeks *in-vitro* culture were subjected to PAS staining. Chicken fibroblast cell cultures were used as a negative control. Cells were detached from 12-well culture plates using accutase (Sigma-Aldrich, Saint Louis, MO) cell dissociation agent. Dissociated cPGCs were seeded in a 6-well culture plate @ $1x10^5$ cells/well. Wells used for used for PAS staining were first fixed with 95% ethanol for 10 minutes and then rinsed with 1X PBS 3 times for 5 minutes. After rinsing, the cells were stained with 1 ml periodic acid solution (Sigma-Aldrich) per well for 5 minutes and later washed three times with PBS for 1 minute each. Subsequently, the cells were treated with equal volume of Schiff's reagent (Sigma-Aldrich) for 15 minutes. At the end of staining, cells were rinsed three times with PBS to avoid overstaining. Hematoxylin (Sigma-Aldrich) was used as a counterstain for nuclear staining. After one-minute

cells were rinsed again 2 times with PBS. Each step was conducted at room temperature and the stained cells were imaged using inverted microscope (Olympus, Phase contrast).

Immunohistochemistry analysis of donor cPGCs

The immunocytochemical analysis of cultured cPGCs was done with the help of Nhung T. Nguyen (M.S. graduate student), to analyze any deviations from the germ cell characteristics under feeder-less culture conditions. Chicken fibroblast cells were used as a negative control. The staining protocol was adapted from McDonald *et al*. (2010). In short, approximately 10^4 PGCs were seeded in four chambered glass slides (VWR) and once confluent, fixed in 4% (v/v) paraformaldehyde/PBS (Boston Bioproducts, Ashland, MA) for 10 minutes at room temperature followed by 1X PBS washing 3 times for 5 minutes each. The fixed cells were incubated with 500µl of 5% goat serum in 1X PBS for 30 minutes. Goat serum was aspirated and cells were treated with primary antibodies. Primary antibodies obtained from DSHB (University of Iowa) were: MC-480 (SSEA-1)-s (mouse anti-SSEA-1 monoclonal antibody), MC-631 (SSEA-3)-s (mouse anti-SSEA-3 monoclonal antibody), and MC-813-70 (SSEA-4)-s (mouse anti-SSEA-4 monoclonal antibody). Cells were first incubated with primary antibodies (200µl each) overnight at 4^0C. The treated cells were then rinsed with PBS thrice with 5 minutes incubation time at room temperature. The secondary antibody was FITC-labelled goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA), diluted in 1X PBS and 0.05% Tween 20. Slides were incubated with secondary antibody for 1h at room temperature in dark. The slides were then rinsed with sterile 1X PBS for 3 times, then incubated for 2 minutes at room temperature in DAPI (1µg/ml in PBS) as a counter-stain. Finally, the slides were washed with PBS, the chamber was removed from the slide, and a coverslip was placed on the slide using 30% glycerol

as a mounting medium. The stained cells were visualized using an inverted fluorescence microscope (Carl-Zeiss, Axio-imager 2). Controls for non-specific binding (staining without adding the primary antibody) and background (staining without adding both the primary and secondary antibodies) were included to validate the specificity of aforementioned antigens for the cPGCs.

RNA isolation and germ-line specific gene expression

Total RNA was isolated from cultured cells using RNA isolation kit (Biorad, CA, USA) as per manufacturer's instructions. The quality and quantity of RNA samples were analyzed using spectrophotometer (Nanodrop, ND-100) and only high quality RNAs were used for cDNA synthesis. RT-qPCR followed a 2 -step protocol to detect expression of germ-line specific genes. First strand synthesis reactions were in 20µl total volume comprised of 5µl of 5x SS buffer (RT buffer), 0.2µl of 20 mM dNTPs, 0.4µl of 30µM $CT_{23}V$, 1µl DTT, 0.2µl of (1-2 U) RNAsin (Promega), 0.5µl (100U) reverse transcriptase enzyme (Superscript III, Life Technologies), and 10-20µg total RNA. The reaction was prepared in 0.2 ml PCR tube, mixed thoroughly and then incubated at 42^0C for 20-30 minutes. The mixture was then moved to ice. The cDNA synthesized was used immediately for qPCR. The reaction mix for second step qPCR (20µl total reaction volume) was comprised of: 2µl 10x Taq polymerase buffer, 0.2µl 20mM dNTPs, 0.2µl 25mM $MgCl_2$, 0.4µl 50 µM forward and reverse primers, 2µl cDNA (~2-5ng), and 4 U Taq polymerase. The PCR cycling conditions were 90°C for 2 min, 35 cycles of 90°C for 30 s, 60°C for 15 sec., 72°C for 1 min, followed by a final extension of 72°C for 3 minutes. The primers sequences were:

Dazl: Forward: 5'-TGTGGACAGGAGCATACAAACA-3'

Reverse: 5’-AAGTGATGCGCCCTCCTCT-3’

Cvh: Forward: 5’-GGCGGGATTTAATGTCATGT-3’

Reverse: 5’- TGTGGTTCTTGCTGCTTTTG-3’

Stra8: Forward: 5’-CTGTGGTCTCCACGGCTATT-3’

Reverse: 5’-GAAACCAGCAGCAACATCAA-3’

Sycp 3: Forward: 5’-GAAGGTTTTTCAACAGGCAAG-3’

Reverse: 5’-TTGCGAAGTTCATTTTGTGC-3’

Sdf1: Forwards: 5’- TCATCACCTTGCCATTCTGG-3’

Reverse: 5’- GCTGTTGGTGGCATGGACTA-3’

β2M: Forward: 5’- TGTAGACGGCTTCGCTGC-3’ and

Reverse 5’- AGGAGTGTGTGCTAACCGTTAC-3’

The amplified PCR products were electrophoresed in 1.5% agarose gel. Liver cDNA was used as a negative control whereas juvenile testis and ovary cDNAs were used as positive controls.

Preparation of busulfan emulsion (BuDMF/O)

The busulfan emulsion (BuDMF/O) contained busulfan (1,4-butanediol dimethylsulfonate) from TCI (Tokyo Chemical Industry Co., Ltd., Tokyo), DMF (N, N-dimethylformamide) from Sigma-Aldrich, and extra virgin Sesame oil (Lorina, USA). The sustained-release BuDMF/O was prepared as per Nakamura et al. (2009) to deplete endogenous PGCs in the recipient embryos. Busulfan powder was dissolved in DMF and later 10-fold diluted with Ca^{2+} and Mg^{2+} free PBS (Phosphate Buffer Saline). An equal volume of sesame oil was later added to prepare BuDMF/O

emulsion with the final busulfan concentration of 1.5µg/µl. The injected amount of busulfan per embryo used in the current study was 75µg.

Embryo culture and busulfan treatment

The protocol for busulfan treatment used in this study followed that of Song et al. (2005). Unincubated, fertilized WL eggs were incubated (see above) for 24 h. WL embryos were laid flat (horizontally) for 2h in the incubator prior to the busulfan treatment. With the help of a rotary drill a small hole was drilled at the blunt end of each egg without damaging the inner shell membrane. Using a 27 ¼ - gauge needle (BD, USA) attached to a 1 ml tuberculin syringe (BD, USA), a total volume of 50µl (75µg of busulfan/embryo) of BuDMF/O was injected into the yolk of each WL embryo. After the treatment, the hole was sterilized using ethanol and then sealed using transparent tape. The treated eggs were then placed vertically into their racks and egg rotation was begun the following day.

Transfer of donor cPGCs

The characterized NH low line cPGCs cultured in feeder-less advanced DMEM medium were transferred into the vasculature of HH stage 14-15 (54 h of incubation) BuDMF/O treated WL embryos. The protocol for PGCs transplantation was adapted from Kim *et. al.* (2010). In short, a small window was created at the blunt end (where the hole was drilled for BuDMF/O treatment) with ethanol sterilized scissors and 2µl of donor PGCs containing approximately 1000-2000 cells was injected into the dorsal aorta. Donor PGCs solution was mixed with 0.04% trypan blue solution to track the route of PGCs suspension. After PGCs transfer, the egg window was wiped

with absolute ethanol and sealed twice with transparent tape. The sealed eggs were placed in the incubator with blunt end facing upwards and retained in the incubator with rocking until hatch.

Detection of donor derived sperm in the recipient semen

The presence of donor derived sperm in the recipient's ejaculates was measured by TaqMan assay. An A/G SNP distinguishing the high line and low line chicken was reported in the mitochondrial $tRNA^{Arg}$ gene (at 11177 bp). The low line roosters carry a G allele whereas the reference and high line genome carry an A allele (Froman and Kirby, 2005). The assay was evaluated through serial dilution of low line DNA in White Leghorn DNA and the diluted samples were tested for the SNP signal strength using the TaqMan assay. In this TaqMan assay the following probes and primers were used:

mt-tArg-Forward: 5`-GCTTCTTCCCCTTCCATGAGCCATCC-3`

mt-tArg-Reverse: 5`-AGAGATGAGGTGTGTTCGGTGGAATGC-3`

mt-tArgTmA: 5`-AGaCCCACCTATAACTTTCTTaTGTCTCC-3`

mt-tArgTmG: 5`- AGgCCCACCTATAACTTTCTTaTGTCTC-3`

with reaction conditions of 90^0C for 30 sec., 10 cycles of 90^0C for 15 sec., 63^0C for 30 sec., followed by 30 cycles of 90^0C for 15 sec., 63^0C for 30 sec., and plate read. The qPCR data were used to create a graph to estimate the proportion of low line sperm DNA in an unknown sample. DNA was isolated from the recipient semen and the TaqMan assay was conducted using mitochondrial primers and probes. The qPCR data was recorded and the proportion of low line donor sperms in the recipient semen was determined by comparing it with signals from the diluted samples of low line DNA in WL DNA.

RESULTS

PGCs proliferation in feeder-free media

A defined feeder free culture condition was used to culture cPGCs from 3-day old chicken embryonic blood. After two weeks of culture, PGCs start proliferating and outnumbering red blood cells. The presence of PGCs was confirmed by their morphological characteristics as mentioned by van de Lavoir *et al*., (2006). The cells were spherical in shape, larger in size (~10-20μm in diameter), contained cytoplasmic lipids (Meyer *et al*., 1964) and appeared to have cytoplasmic projections (Figure 4.1 A, F). From four weeks onwards, cPGCs started forming adherent colonies that were similar in morphology as cPGCs cultured in feeder-layer conditions (Figure 4.1C). The colonies were spherical in shape and their frequencies increased from 6 to 8 weeks of culture (Figure 4.1 E, G, and H). Chicken embryonic fibroblasts cells were cultured in the feeder-free condition and were used as negative control in immunocytochemical experiments (Figure 4.1 I). The PGCs colonies differ slightly based on the gender. Female PGCs took longer time to form colonies, and the colonies were smaller in size and scattered whereas male PGCs proliferated at faster pace and had bigger colonies (data not shown). These differences in the male and female PGCs growth pattern *in-vitro* have been described previously (Mayahira *et al*., 2014).

The PGC cell lines were maintained until they reached approximately 80% confluence after which they were either sub cultured or cryo-preserved. The seeded cells formed visible colonies as early as 2 weeks after culture while cryo-preserved cells when re-cultured took ~5-6 weeks to proliferate and form visible colonies.

PGCs sex determination

Avian females are heterogametic (ZW) whereas males are homogametic (Z) for the sex chromosomes. Wxho primers are for a female specific primer that amplifies the *Xho*I repetitive sequence from the chromosome W whereas the Ribo primers amplify a region of the 18S ribosomal gene present in both genders. The Wxho and Ribo primers were used for the sex determination of cultured PGCs. Embryonic blood used for PGCs culture was also used to isolate DNA for sex determination by PCR. The PCR products were then electrophoresed in 1.5% agarose gel. The result of PGCs sex determination by PCR is depicted in Figure 3. Lanes with bands at 416bp (Wxho) and 256bp (Ribo) represent females (lanes 1, 2, 5, 6, 7, 12, 13, 14, 15, 16 and 17) whereas lanes with just one band at 256bp (Ribo) represent males (lane 3, 4, 8, 9, 10, and 11). Female PGCs after reaching 80% confluency were discontinued for further sub-culture and were cryo-preserved in liquid N_2, whereas male PGCs cultures were continued for further experimentation. Male PGCs were used as donor PGCs in this study.

Characterization of cultured cPGCs

One of the characteristic features of chicken PGCs is high cytoplasmic refractive index. These cells contain high proportions of lipid droplets in their cytoplasm as compared to the somatic cells. This feature has been used to characterize cPGCs when grown on feeder layers/stromal cells (Jung *et al*.,2005; Lu *et al*., 2014) through PAS staining. PAS reagent reacts with the complex carbohydrates and stains the cells red. PAS staining is not just specific for chicken PGCs as it also stains positive for mammalian PGCs (mouse PGCs) and embryonic stem cells (ESCs). PAS is still used to characterize germ cells *in-vitro*. Single celled PGCs and PGCs colonies formed after 6 weeks of culture were tested for PAS staining. For PAS staining, PGCs

colonies were passaged and sub-cultured. Chicken embryonic fibroblasts (CEFs) were used as a negative control. Both single celled PGCs and PGCs colonies stained strongly with PAS (Figure 4.3 B, D) and appeared to have red to pinkish coloration based on the cytoplasmic lipid/carbohydrate content. CEFs stained negative and appeared dark blue due to the hematoxylin staining (Figure 4.3 F).

Cultured PGCs were further characterized for germ-cell specific cell surface antigens. As previously demonstrated (Montono *et al*., 2008, Lu *et. al*., 2014, Jung *et al*., 2005) PGCs stained strongly with anti-SSEA-1 antibodies (Figure 4.4a: A-D) but unlike previous studies they were only weakly stained with anti-SSEA-3 (Figure 4.4a: E-H) & anti-SSEA-4 antibodies (Figure 4.4a: I-L). As expected, no reactivity was detected in chicken embryonic fibroblast cells against any stem cell specific surface antigens (Figure 4.4b: B, F, & J). Both non-specific and background controls showed no reactivity for the tested antigens (data not shown).

Expression of germ-line specific genes in the cultured cPGCs

Chicken vasa homologue (Cvh), and deleted in azoospermia-like (Dazl) genes are exclusively expressed in the germ cells and considered as the most germ cell specific markers (Lavoir *et al*., 2006; Lavial *et al*., 2009). Sdf-1/ CXCR-4 expression is crucial for germ cell migration (Stebler *et al*., 2004) whereas Stra-8 and Sycp-3 genes are expressed in premeiotic and meiotic germ cells respectively (Oulad-Abdelghani et al., 1996; West et al., 2008). The expression of these five germ-line specific genes, Cvh, Dazl, Sdf-1, Sra-8 and Sycp-3, was evaluated in the cultured PGCs using RT-PCR (reverse-transcription polymerase chain reaction) to evaluate their germline competency. Chicken juvenile testis and ovary cDNA were used as positive controls whereas chicken juvenile liver was used as a negative control. Male and female PGCs were tested

separately for their relative expression. β2M was used as the internal control. Dazl, Cvh and Stra-8 were strongly expressed in both male and female PGCs (Figure 5). Although both Sdf-1 and Sycp-3 had a lower level of expression in male and female PGCs, their expression level differed in the positive controls. Both juvenile testis and ovary expressed Sdf-1 at higher levels than the PGCs, the expression of Sycp-3 was low in the testis and absent in the ovary (Figure 5). This suggest that Sycp gene is expressed equally in the germ cells irrespective of their gender prior to the process of differentiation but continues to be expressed only in the male germ cells after their differentiation and maturation. All genes were highly expressed in both PGCs, testis and ovaries (except Sycp-3) whereas no expression of these germ line related genes was observed in the liver. Among the studied genes, Cvh was highly expressed in the PGCs and hence confirmed their germ line specific attributes. These results also strengthened the previous reports on Cvh being a germ-line specific marker for chicken PGCs (Tsunekawa *et al*., 2000; Lavial *et al*., 2009).

Embryonic development and survival after donor cPGCs transplantation

Male cPGC cultures that fulfilled the criteria for germ line specificity and pluripotency as tested by immunocytochemistry, RT-PCR analysis and PAS tests (Table 1) were used for transplantation experiments. PGCs that conformed to all parameters were PGC ID# *11/30 2A2, 11/30 2B3, 12/6 2B3, 12/6 2B2, 12/3 1.5, 11/30 3.6, 11/30 2C1, & 12/3 2B4*. cPGCs were injected into the vasculature of 3-day old bulsufan treated WL embryos. Table 2 delineates the outcome of germline transplantation experiments. In brief, eight separate trials were conducted with variable numbers of recipient embryos. Nearly 80% of the treated embryos survived after the BuDMF/O treatment (except trial#5, Table 2) whereas the hatchability and survivability decreased sharply after the

PGCs injection. The hatchability percentages ranged from 11%-43% in these trials (Table 2). Except one trial (trail# 6), all hatched chicks survived. The number of injected PGCs per embryo were within the range of 1000-2000 cells in 2 to 3µl of PGCs solution. Even though only male PGCs were injected into the recipient embryos, the hatched chicks had representation from both sexes. Nearly 57% of hatched chicks were males whereas 43% were females. Out of 162 WL embryos used in this study, only 28 embryos hatched. Out of 28 hatchlings, female chicks were culled after sexing and only 13 male chicks were raised until they reached sexual maturity. The extremely low survivability (17%) of the treated embryos indicates that the effects of external manipulation dramatically impacted embryonic development. These results indicate that the dexterity of the researcher, and specific manipulation techniques are likely to influence the embryo survivability in transplantation experiments.

Detection of donor derived sperms in recipient semen

All hatched chicks were tagged with wing bands for identification. After 2 weeks of age, sex identification by PCR was done and female chickens were culled. The putative male chimeric hatchlings were maintained at the Poultry Research farm until sexual maturity (28 weeks). Semen was collected from these roosters and DNA was isolated according to Bailes et al (2007). The semen DNA samples were genotyped using TaqMan assays for the mitochondrial $tRNA^{Arg}$ A/G SNP which distinguishes WL and low line mitochondria (Froman and Kirby, 2005). The TaqMan assay was validated for the mitochondrial SNP on low line, high line, and WL DNA. Both high line and WL mitochondria carried an A nucleotide at 11177 position in the mitochondrial $tRNA^{Arg}$ gene whereas there was A-to-G transition in the low mobile chicken lines. Based on this observation, mitochondrial A/G SNP was chosen to detect the presence of donor derived genotype

in the recipient WL semen.

The sensitivity of A/G SNP marker to detect the donor derived sperm in the recipient semen was tested by conducting TaqMan analysis on a two-fold dilution series of low line DNA into WL DNA. Although BuDMF/O emulsion was used to deplete the endogenous PGCs, we expected survival of endogenous PGCs that would also populate the gonad and produce sperm (Song *et al*., 2005; Nakamura *et al*., 2009). The allelic discrimination among the diluted samples and the positive controls (undiluted high line and low line DNA) was plotted using delta Cq values (Figure 6). The G-allele was detected down to a 1:32 dilution after which the signal for G-allele was masked by the A allele (Figure 6). By increasing the number of PCR cycles, the G allele expression could have been detected down to 1:128 dilutions but for the purpose of our study, detection down to 1:32 dilution was judged as sufficiently sensitive. At 1:2 dilution, we expected to see equal Ct values for both alleles. Instead the A allele showed lower Ct value than the G allele (delta Cq -1.9). The higher melting temperature required to break GC hydrogen bonds could explain the faster amplification and hence lower Ct value for the A allele at 1:2 dilution. The ability of our TaqMan assay to detect the G allele signal at 1:32 dilution made it a suitable assay to identify the percentage of low line donor sperms in the recipient WL semen.

Signal for the A allele was detected when TaqMan analysis was conducted on the putative transgenic rooster's semen. The Ct value for the A allele varied among the transgenic roosters (n=15; each test sample ran in triplicates) but none of the samples showed any signal for the G allele. This result indicated that the low line donor PGCs did not populate the recipient embryo gonads effectively or the endogenous WL PGCs that survived the BuDMF/O-emulsion treatment predominantly participated in the process of spermatogenesis. Since the representation of donor derived sperms was determined to be less than 3% in the recipient semen, the effect of germ cells-

somatic cells interactions on the mobility phenotype could not be determined.

DISCUSSION

Characterization of chicken PGCs grown without feeder-layer

In this study the morphological and biochemical characteristics of cPGCs cultured without the support of feeder layers were demonstrated. The PGCs cultured from the embryonic blood of HH stage 14 embryos in a well defined medium have the distictive morpholgy with large nucleus and the characteristic clusters of cytoplasmic lipid vacuoles (Figure 3A) observed by others in feeder-layer growth. The morphological characteristics of single cell PGCs were not influenced by the absence of feeder-layer. The presence of high lipid content has been reported in chicken PGCs both in-vitro (Song *et al*., 2010) and in migratory PGCs *in ovo* (Fujimoto et al., 1976). Human PGCs also display high cytoplasmic refractive index due to the presence of numerous lipid vacuoles (De Felici *et al*., 2004) whereas in other mammalian species like mouse and pig, PGCs were reported to be devoid of lipid vacuoles (Spiegelman & Bennett, 1973; Bielanska-Osuchowska, 2006). The cultured cPGCs started proliferating after 6 weeks of culture and colonies started to appear after 6-8 weeks of culture. The colonies were spherical in shape, scattered through out the culture wells and appeared to communicate through cytoplasmic projections/pseudopodia (Figure 3D, E, F, arrowheads). The rate of PGC proliferation without feeder layer was lower than the reported growth of PGCs in feeder layers (Han *et al*., 2002; Song *et al*., 2014). The morphological characteristics of PGCs colonies cultured without feeder-layer support were very similar to chicken PGCs colonies reported in the earlier studies (Park and Han, 2000; Jung *et al*., 2005). The presence of high glycogen content in both single cell and colonies of cPGCs was observed after PAS staining. In contrast, the chicken embryonic

fibroblast cells (CEFs) were devoid of cytoplasmic glycogen as demonstrated by their negative PAS staining. This property of chicken PGCs was in accordance with the previous observations of high glycogen content in the migratory cPGCs (Jung et al., 2005; Fujimoto *et al*., 1976; Mozdziak *et al*., 2005). The presence of numerous lipid vacuoles and high glycogen content indicate the energy dynamics in chicken PGCs.

The presence of SSEA-1 (stage specific embryonic antigen-1) on the surface of human, murine and chicken undifferentiated embryonic stem cells have been demonstrated in multiple studies (Matsui *et al*., 1992; Shamblott et al., 1998; Mozdiak *et al*., 2005; Durcova-Hills and Surani, 2008) and hence is used as a marker for pluripotency. In this study, SSEA-1 was detected in the cultured cPGCs (Figure 4a) confirming their undifferentiated pluripotent characteristic. Unlike murine embryonic stem cells (ESCs) and embryonic carcinoma cells (ECs), chicken PGCs were positive for SSEA-3 (stage specific embryonic antigen-3) and SSEA-4 (stage specific embryonic antigen-4) as expected for chicken PGCs (Jung *et al*., 2005).

The expression of germ line specific genes was tested to further estimate the germ specific character of cultured cPGCs in feeder-less conditions. Vasa gene is one of the germ line specific genes that is reported to be indespensable for germ cells development in various species ranging from Drosophila to mouse (Hay et al., 1990; Gruidl et al., 1996; Ikenishi and Tanaka, 1997; Youngren *et al*., 2005). Studies have shown that VASA protein controls germ line development by regulating transcription of genes responsible for germ line determination such as Nanog and Oskar in Drosophila (Hay et al., 1990). Since its discovery in Drosophila, vasa homologues have been reported in many vertebrates and appears to be evolutionarily conserved in animal species (Fujiwara et al., 1994; Gruidl et al., 1996; Ikenishi and Tanaka, 1997; Tsunekawa *et al*., 2000). Cvh, Chicken vasa homologue, was also reported to have germ line specific expression and cells

expressing CVH protein were detected at the earliest stages of embryogenesis (Tsunekawa *et al*., 2000). In this study, both male and female PGCs expressed Cvh gene validating the germ line competency of cultured PGCs. Another germ cell-specific gene that is crucial for germ cells differentiation is Dazl. The expression of Dazl gene has been reported in both vertebrate and invertebrate species but with varying tissue specificity. Studies have found Dazl expression in both testis and ovaries in various species, except cattle and Drosophila where it had male specific expression, and nematode where it was exclusively expressed in the ovaries (Eberhart *et al*., 1996; Karashima et al., 2000; Liu et al., 2007). Dazl expression was not gender specific in chicken and DAZL protein was located in both testis and ovaries (Rengaraj *et al*., 2010). In our analyses, Dazl expression was detected in both male and female cPGCs. Furthermore, germ-line specific genes like Sdf-1, Sycp-3 and Stra-8 involved in PGCs development, migration, differentiation and survival were also tested in the current studies. While Sdf-1 (stromal cell derived factor), a chemokine was shown to be critical for the gonadal colonization of PGCs in mice, chicken and zebrafish (Ara *et al*., 2003; Stebler *et al*., 2004, Knaut *et al*., 2003) whereas Sycp-3 was reported as meiotic germ cell marker in humans and mice (Di Carlo *et al*., 2002; West et al., 2008). Stra-8 was reported to be expressed in pre-meiotic germ cells in mice (Oulad-Abdelghani et al., 1996) and was shown to be involved in early stages of spermatogenesis. In the current study, the cultured chicken PGCs expressed all germ line specific genes irrespective of their sex. Sycp-3 showed little to no expression in adult chicken ovaries whereas was expressed at same level in both male and female PGCs.

Our results showed that the chicken PGCs maintain their undifferentiated germ line culture characteristics when grown in feeder-less conditions with the support of defined culture media. A growth lag was observed while proliferating PGCs in feeder-free conditions, but the factors

behind this lag could not be determined. An in-depth study is required to understand the factors needed for the long-term culture of chicken PGCs without any feeder support. This will enhance the culture efficiency and give more control over the variations caused due to different types of feeder layers used in chicken PGCs culture.

Germline transmission of low-line donor New Hampshire PGCs in busulfan-treated recipient high-line White Leghorn embryos and assessment of donor derived sperms in the sexually mature chimeric chicken

This experiment was aimed to reveal the effect of reproductive tract features on sperm mobility phenotype. If the donor germ cells with low line genotype yielded sperms with low mobility phenotype in the recipient's semen, then the germ cell genotype would be solely responsible for the phenotypic variation but if the donor sperm would display a high mobility phenotype, then the contributions of the somatic components of the reproductive tract would be determined to influence this critical sperm phenotype. To achieve the stated goal, a germ line chicken model was adopted. Injection of BuDMF/O emulsion in 1- day old embryo has been demonstrated to partially deplete the migratory PGCs in developing chicken (Song et al., 2005). The injection of 75µg BuDMF/O into embryos has been reported to kill 98% of the gonadal PGCs (Song *et al*., 2005). At HH stage 14-15 (52-54 h of incubation), when the population of migratory PGCs reach at its peak (Bernardo et al., 2012) in the vascular system, male donor PGCs which were maintained in the feeder-less culture conditions were transplanted into the dorsal aorta of recipient embryos. Male mouse PGCs were shown to differentiate and enter into the process of gametogenesis in opposite-sex recipients, whereas female PGCs were reported to be incompetent in generating functional gametes in male embryos (Ford *et al*., 1975; Palmer and Burgyoni, 1991). The inefficiency of *in-vitro* female PGCs to undergo spermatogenesis in male recipient

embryos was also reported in chicken (Tagami *et al*., 2007; Sang *et al*., 2010). Hence, male PGCs were selected as donor PGCs in this study. Approximately 82% of the BuDMF/O treated embryos survived to the time for PGCs injection, yet the hatchability percentage declined sharply and varied significantly between trials ranging from 11% to 43%. The previous studies on the germline generation of transgenic chicken using cultured PGCs have demonstrated the difficulty in the survivability of manipulated embryos where the maximum hatchability reached up to 38% using BuDMF/O emulsion as PGCs depleting medium (Song *et al*., 2005). The male hatchlings we generated were reared till they reach sexual maturity after which their semen was tested for the presence of donor derived sperms using TaqMan assay. Out of 15 males, not a single test subject showed any presence of the low line mitochondrial signal ($tRNA^{Arg}$ G SNP) in their semen, indicating either the proportion of donor PGCs participating in the process of gametogenesis was very low (less than 3%) or the BuDMF/O emulsion treatment to deplete endogenous PGCs was not efficient. Previous reports on PGCs transfer using BuDMF/O emulsion in chicken has shown the higher efficiency of this emulsion medium in depleting endogenous germ cells but have also reported the lower germline transmission of donor PGCs (Song et al., 2005). In a different study, where γ irradiation was used to deplete germ cells in recipient chicken embryos, the germ line transmission of donor PGCs was as high as 70% (Liu *et al*., 2012). Studies have reported the importance of dose rate and injection time of BuDMF/O emulsion in depleting the endogenous germ cells (Song *et al*., 2005; Kim *et al*., 2010) as low dose rate might not deplete the endogenous PGCs efficiently and higher dose may disrupt embryonic developmental (Song *et al*., 2005; Tagami *et al*., 2011). Also, BuDMF/O injection prior to 24 h of incubation wouldn't kill the migratory PGCs while after 24 h it could hinder the survival and proliferation of donor PGCs. The tight window of BuDMF/O injection and

developmental defects might explain the poor representation of donor derived sperms in the recipient's semen in our study. Research on the generation of transgenic chicken through PGCs transplantation have reported lower rates of germ-line transmission of donor PGCs and sex bias with regard to male PGCs being more efficient in transmission than females (Naito *et al*., 1999; Lavoir *et al*., 2006; Kim *et al*., 2012). Owing to the low migration rate of injected donor PGCs into recipient's gonads, it may be necessary to increase the sample size to determine the percentage of donor- derived sperms in the recipient semen. In this study, the hatchability percentage was less than 50% and total number of recipient males tested for the presence of donor-derived sperms were very less (n=15). Due to the low sample size, the ability to detect the presence of donor genotype in the recipient semen was reduced drastically. Since, the representation of donor derived sperms was less than 3% in the recipient semen, it was impractical to perform the sperm mobility assay to determine the effect of somatic cells-germ cells interactions on the mobility phenotype.

SUMMARY AND CONCLUSION

The aim of this study was to i) establish cPGCs culture-system in feeder-less culture medium using defined culture medium and ii) to deduce the effect of germ cell-somatic cell interactions on the phenotype of the differentiated germ cells. In this study, with respect to sperm mobility. The qualitative and quantitative data generated from this study depicted that the cPGCs maintained their morphological and germ line specific characteristics in feeder-free culture medium supplemented with growth factors. The rate of colony formation lagged slightly as per the reports on the cPGCs culture in feeder layers but it could be attributed to the source of PGCs isolation since most of the studies using feeder layer isolated PGCs from the embryonic gonads rather than blood. Furthermore, to fulfill our second objective *in-vitro* cPGCs isolated from the blood of low sperm mobile line embryos were injected into BuDMF/O emulsion treated high line WL embryos. The semen of the recipient roosters was tested for the presence of donor derived sperm genotype using TaqMan assay. The low hatchability percentage and the extremely low prevalence (< 3%) of donor genotype in the recipient semen projected the inefficacy of BuDMF/O emulsion injection in reducing endogenous PGCs and its use in chicken transgenesis experiments. Due to inability to detect donor derived genotype in the recipient semen, this study couldn't answer the second question about the effect of cellular communications between the low line PGCs and high line somatic cells (Sertoli's cells) in sperm mobility phenotype. It was unclear from our data as to whether the absence of donor derived sperms in the recipient semen was due to a) the inefficacy of busulfan in inducing sterility in recipient embryos; b) the donor PGCs were unable to colonize recipient gonads; c) the donor PGCs populated the gonad but did not produce sperm; or d) the donor PGCs failed to survive injection. Further research, will need to explore each of these potential issues to improve chicken embryo germ line transmission of

donor PGCs. To explore these issues it would be important to develop donor PGCs genetically modified with reporter genes via gene editing tools. Genetic modification might either use CRISPR/Cas or the PiggyBac transposon system (McDonald *et al*., 2012). PGCs with reporter genes could answer questions about percent colonization of embryonic gonads by the donor PGCs.

FIGURES

Figure 4.1. Chicken primordial germ cells (cPGCs) morphology at different time points. PGCs start appearing as early as 6 days of primary culture (A) and start outnumbering RBCs 2-3 weeks onwards (B). PGCs were characterized by their large size, spherical shape and large cytoplasmic lipid contents (A; arrowhead). PGCs colonies started to appear after 6 weeks of primary culture (C). The colonies strated to expand in size and were scattered in the feeder-less culture conditions (D, and E; arrowheads). The colonies appeared to communicate through cytoplasmic projections (F; arrowhead). PGCs colonies kept on increasing in size and the isolated PGCs started diminishing at 8 weeks of culture (G, H). Chicken embryonic fibroblast (CEFs) were used as a negative control in staining experiments (I, arrowhead). Scale bar =25µm (except C where scale bar =50µm).

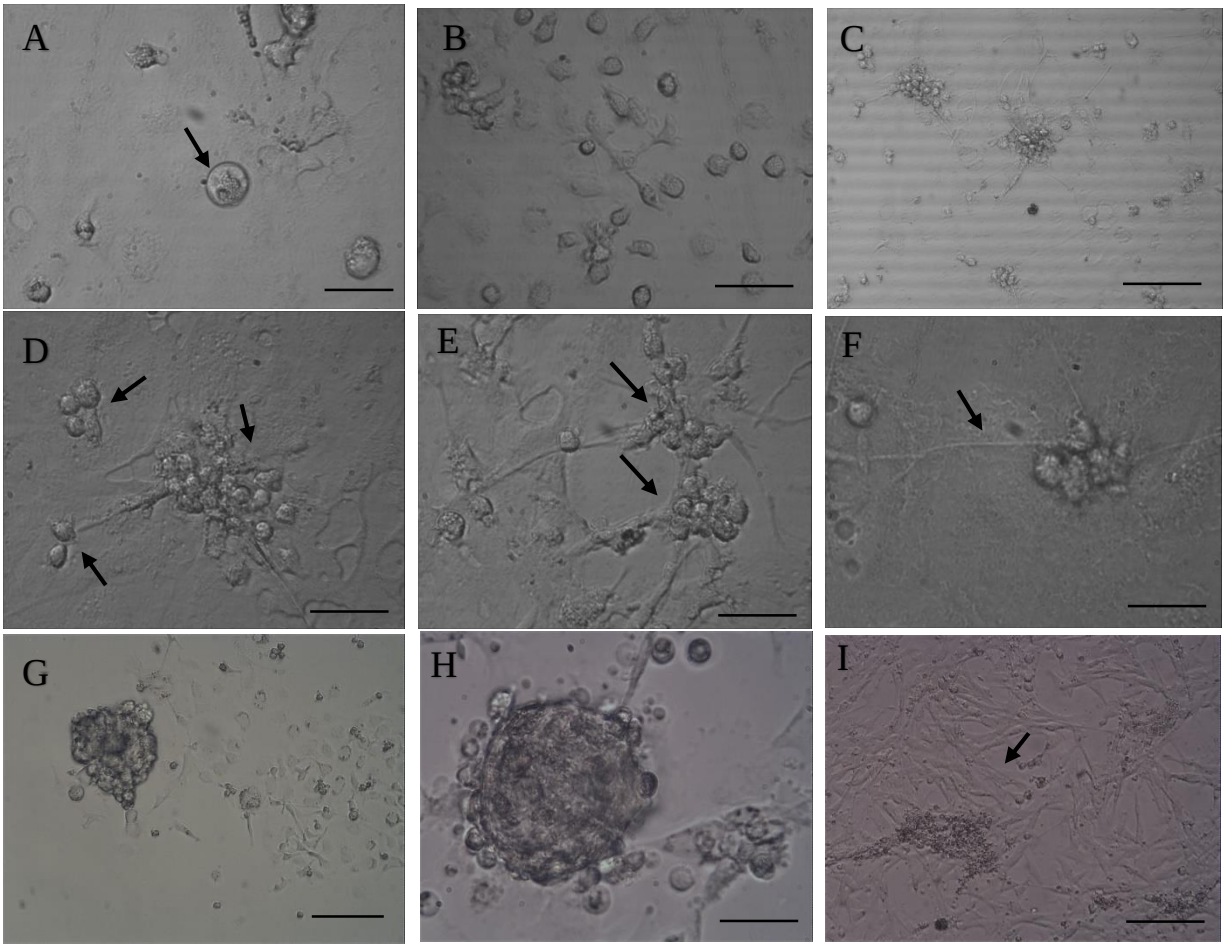


Figure 4.2: Determination of PGCs sex using PCR. Blood was extracted from three-day old chicken embryos for DNA isolation and sex determination. Wxho and Ribo primers were used for DNA amplification. Female DNAs amplified two regions (Lanes 1,2,5,6,7,12,13,14,15,16 and 17) whereas male being homogametic for sex chromosome amplified just one region (3,4,8,9,10, and 11). Lane M, Ma, F, and Nc represents marker, male DNA, female DNA, and negative control respectively.

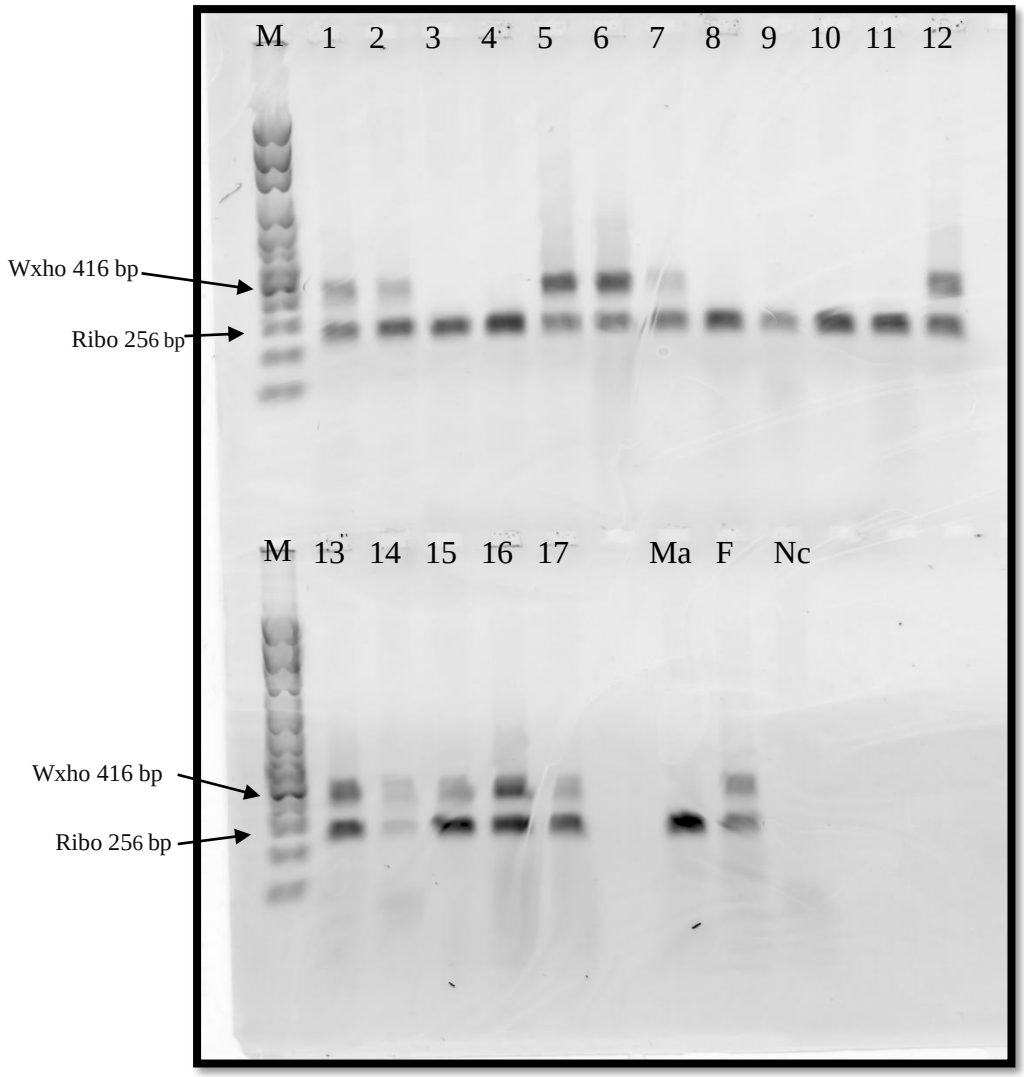


Figure 4.3: PAS staining of cPGCs and CEFs. cPGCs were positive for the PAS stain. Both isolated PGCs and PGCs colonies reacted strongly with PAS stain and were stained in deep magenta (B, D; arrowhead). CEFs were negative for the stain but reacted with hematoxylin, a nuclear stain and turned blue (F; arrowhead). Panel A, C, D depicts the unstained pictures of PGCs colonies, single cell PGCs and CEFs respectively. Scale bar=25µm (C, D) & Scale bar=50µm (A, B, E, and F)

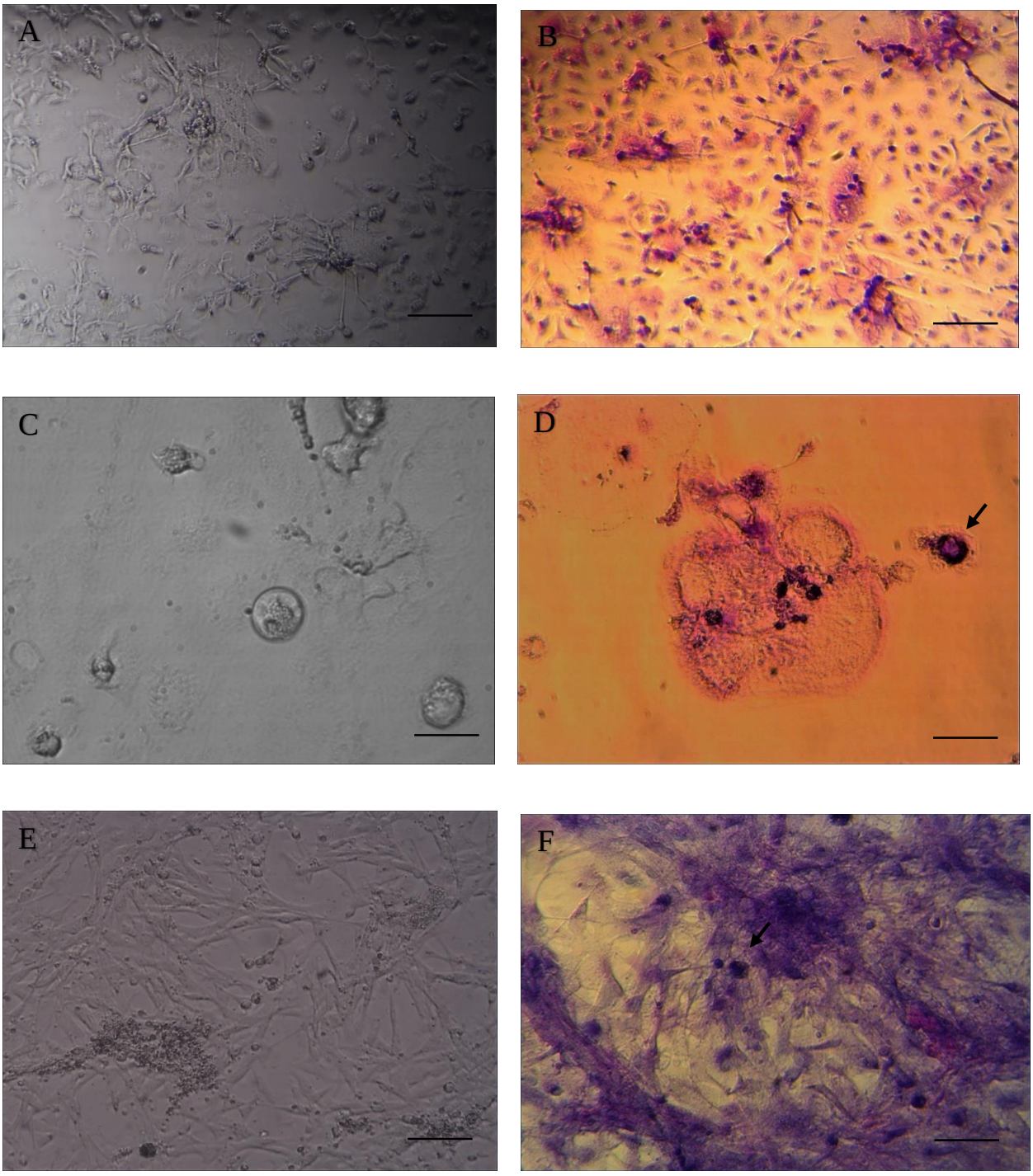


Figure 4.4a: PGCs characterization using stage specific cell surface antigens. Anti-SSEA-1(A-D), anti-SSEA-3 (E-H) & anti-SSEA-4 (I-L) antibodies were used to detect the presence of respective cell surface antigens. First Column (A, E, I) was phase contrast image of double immunostaining of anti-SSEA-1(B), anti-SSEA-3 (F), and anti-SSEA-4 (J) with FITC conjugated goat anti-mouse IgG, and DAPI staining (third column; C, G, K). Fourth column represent the merged images from second and third column. PGCs stained strongly for SSEA-1 antigen (B; arrowhead) whereas were weakly positive for SSEA-3 (F; arrowhead) & SSEA-4 (J; arrowhead) antigens. Scale bar=50µm.

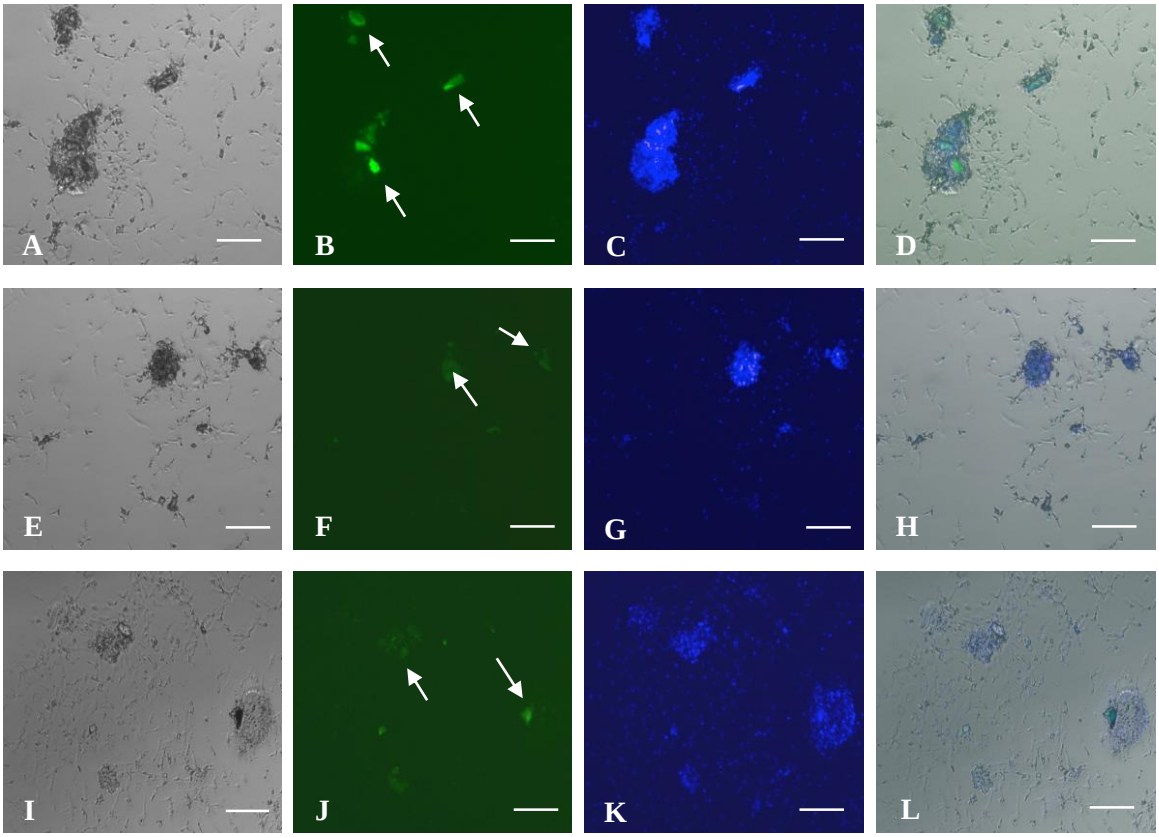


Figure 4.4b: Detection of stem cell specific markers in CEFs. Anti-SSEA-1(A-D), anti-SSEA-3 (E-H) & anti-SSEA-4 (I-L) antibodies were used to detect the presence of cell surface antigens. No reactivity was detected in CEFs against SSEA-1 (B), SSEA-3 (F) and SSEA-4 (J) antigens. CEFs images were captured as phase contrast (panel A, E, and I), immunofluorescence for SSEA antigens (panel B, F, & J), DAPI fluorescence for nucleus (panel C, G, & K), and fluorescent SSEA-DAPI merged panels (D, H, & L). Scale bar=50µm

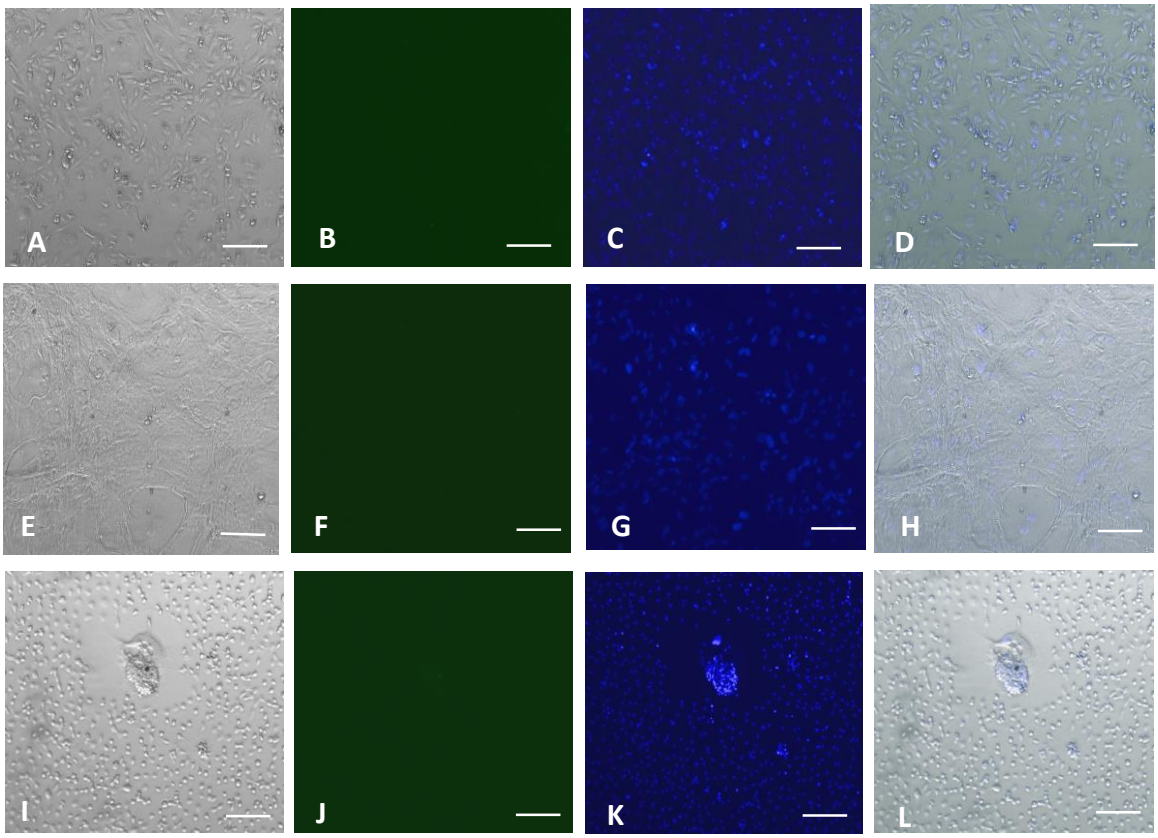


Figure 4.5: Germ line-related gene expression of male and female PGCs**.** Dazl, Sdf-1, Stra-8 were expressed strongly in both male, female PGCs as well as in chicken juvenile testis and ovaries. Cvh and Sycp-3 were expressed specifically in the PGCs. There was slight amplification of Cvh gene in both testis and ovaries but Sycp-3 did not amplify in the ovaries at all. Expression of all genes was negative for liver (negative control) and NTC (Non-template control).

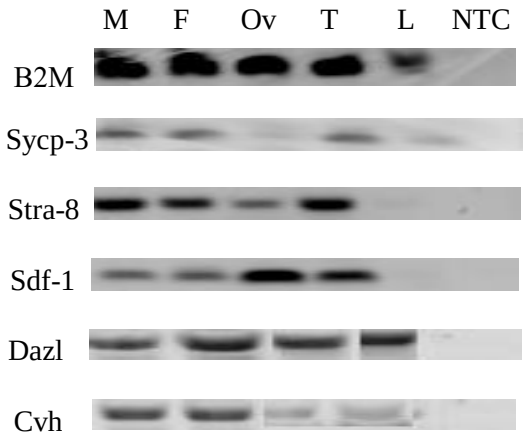
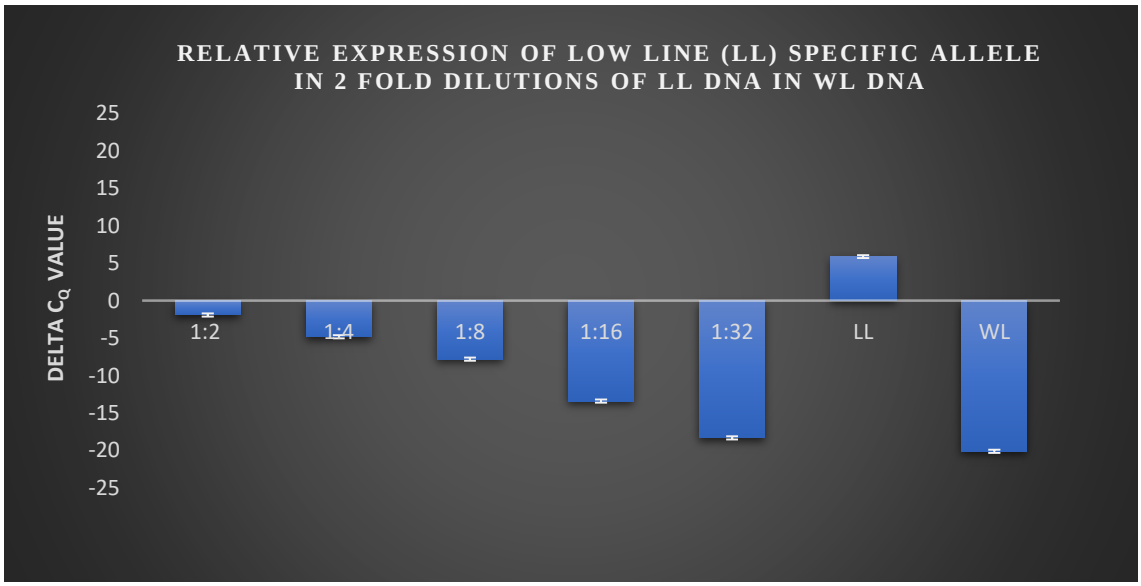


Figure 4.6: Allelic discrimination of mitochondrial tRNA^{Arginine} A/G SNP in the diluted low line semen samples. Serial dilutions (1:2-1:128) of low line semen DNA in high line DNA was tested for the relative expression of G allele as detected by Hex dye in the TaqMan assay. The X axis represents relative expression of low line (LL) specific G allele in two-fold dilution series of LL DNA in White Leghorn (WL) DNA. There was a proportionate decrease in the expression of G allele in the successive dilutions as expected. The undiluted LL and WL DNA exclusively expressed G allele and A allele respectively.



TABLES

Table 4.1: Parameters used to define cPGCs isolated from3 days old embryonic blood and cultured in feeder-less defined DMEM media. Male PGCs confirming to all parameters were used for transgenic experiments. These parameters include PGCs growth rate, morphological characteristics, presence of embryonic stage specific antigens, expression of germ line specific genes, and PAS staining results. Serial number # 7, 8, 9, 13, 15, 17, and 20 were used as donor PGCs in germ line transplant experiments.

S. No.	cPGCs ID	Sex	cPGCs growth after 6 weeks of culture	Morphology	SSEA reactivity	Germ line Specificity
1	11/30 1.1	Female	Scattered growth	Large round cells but few in number	Positive	++*
2	11/30 3.5	Female	No colonies	Few cells with pseudopodia	Weakly positive	+
3	11/30 1.3	Female	Multiple colonies	Multiple spherical shaped colonies	Positive	++
4	12/6 2B1	Female	No growth	Cells couldn't survive	Negtaive	-
5	11/30 3.2	Male	1-2 colonies	Large cells with high refractive index	Weakly positive	+
6	11/30 3.1	Female	Scattered growth	Cells with high refractive index but few colonies	Positive	++
7	11/30 2A2	Male	Large colonies	Spherical shaped colonies with cellular projections	Positive	++
8	11/30 2B3	Male	Multiple colonies	Well rounded spherical colonies	Positive	++
9	12/6 2B2	Male	Multiple colonies	Well rounded spherical colonies	Positive	++
10	12/6 2A3	Male	Scattered growth	Large cells but fewer and smaller colonies	Negative	+
11	12/6 2C1	Male	No growth	Cells could not survive	-	-
12	12/6 3C1	Male	Few colonies	Cells with high refractive index but few colonies	Positive	++
13	12/3 1.5	Male	Multiple colonies	Spherical shaped colonies with cellular projections	Positive	++
14	12/4 1B1	Female	Scattered growth	Cells with higher nuclear/cytoplasmic ratio but few colonies	Positive	++
15	11/30 3.6	Male	Multiple colonies	Multiple spherical shaped colonies	Positive	++
16	11/30 3.3	Male	No colonies	Few scattered cells without any colonies	Negative	-
17	11/30 2C1	Male	Multiple colonies	Multiple psherical shaped colonies with cellular projections	Positive	++
18	11/30 1.4	Male	Scattered growth	Large round cells but few in number	Positive	++
19	12/3 3A1	Female	No colonies	Few cells with pseudopodia without colonies	Positive	++
20	12/3 2B4	Male	Multiple colonies	Multiple spherical shaped colonies	Positive	++

***:** Double positives signify higher expression.

Table 4.2: Hatchability and survivability percentage of manipulated embryos after BuDMF/O treatment.

S. no.	Recipient embryos	Survivability % after busulfan treatment	Injected number of donor cPGCs per µl	Hatchability % of manipulated recipient embryos	Chimera wing band #	Chimera sex
1	29	83	1200	29	#9282 #9283 #9284 #9285 #9286 #9287	Male Male Female Male Female Female
2	28	82	1000	17.23	# 9289 #9290 #9291 #9299	Male Male Female Female
3	26	81	1500	43	#9211 #9212 #9213 #9214 #9215 #9255 #9251 #9210 #9245	Male Male Male Female Female Female Female Male Male
4	9	80	1000	11	NA	NA
5	22	50*	1200	18	#9269 #9253	Male Male
6	20	80	1000	25	# 9204 #9205 #9206 #9207	Female** Female** Female** Male**
7	10	81	1200	12.5	#9278	Male
8	18	72	1000	15.38	#9277 #9280	Male Male

Unexpected low survivability after busulfan treatment*
Hatched chickens did not survive till sexual maturity**

REFERENCES

Ando, Y., & Fujimoto, T. (1983). Ultrastructural Evidence that Chick Primordial Germ Cells Leave the Blood-Vascular System Prior to Migrating to the Gonadal Anlagen. *Development, growth & differentiation*, *25*(4), 345-352.

Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y. & Nagasawa, T. (2003). Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proceedings of the National Academy of Sciences*, *100*(9), 5319-5323.

Bailes, S. M., Devers, J. J., Kirby, J. D., & Rhoads, D. D. (2007). An inexpensive, simple protocol for DNA isolation from blood for high-throughput genotyping by polymerase chain reaction or restriction endonuclease digestion. *Poultry Science*, *86*(1), 102-106.

Benesova, B., Mucksova, J., Kalina, J., & Trefil, P. (2014). Restoration of spermatogenesis in infertile male chickens after transplantation of cryopreserved testicular cells. *British Poultry Science*, *55*(6), 837-845.

Bernardo, A. D. M., Sprenkels, K., Rodrigues, G., Noce, T., & Lopes, S. M. C. D. S. (2012). Chicken primordial germ cells use the anterior vitelline veins to enter the embryonic circulation. *Biology open*, BIO20122592.

Bielanska-Osuchowska, Z. (2006). Oogenesis in pig ovaries during the prenatal period: ultrastructure and morphometry. *Reproductive Biology*, *6*(2), 161-193.

Braat, A. K., Speksnijder, J. E., & Zivkovic, D. (2004). Germ line development in fishes. *International journal of developmental biology*, *43*(7), 745-760.

Brehm, R., Zeiler, M., Rüttinger, C., Herde, K., Kibschull, M., Winterhager, E., Willecke, K., Guillou, F., Lecureuil, C., Steger, K., Konrad, L., Biermann, K., Failing, K., & Bergmann, M. (2007). A Sertoli cell-specific knockout of connexin43 prevents initiation of spermatogenesis. *The American journal of pathology*, *171*(1), 19-31.

Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences*, *91*(24), 11303-11307.

Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences*, *91*(24), 11298-11302.

Bruzzone, R., White, T. W., & Goodenough, D. A. (1996). The cellular internet: On-line with connexins. *Bioessays*, *18*(9), 709-718.

Chang, I. K., Yoshiki, A., Kusakabe, M., Tajima, A., Chikamune, T., Naito, M., & Ohno, T. (1995). Germ line chimera produced by transfer of cultured chick primordial germ cells. *Cell biology international*, *19*(7), 569-576.

Chang, K., Qian, J., Jiang, M., Liu, Y. H., Wu, M. C., Chen, C. D., Lai, C.K., Lo, H.L., Hsiao, C.T., Brown, L. & Bolen, J. (2002). Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. *BMC biotechnology*, *2*(1), 5.

Cheng, C. Y., & Mruk, D. D. (2002). Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. *Physiological reviews*, *82*(4), 825-874.

Clinton, M., Haines, L., Belloir, B., & McBride, D. (2001). Sexing chick embryos: a rapid and simple protocol. *British Poultry Science*, *42*(1), 134-138.

De Felici, M., Scaldaferri, M. L., Lobascio, M., Iona, S., Nazzicone, V., Klinger, F. G., & Farini, D. (2004). Experimental approaches to the study of primordial germ cell lineage and proliferation. *Human reproduction update*, *10*(3), 197-206.

Decrouy, X., Gasc, J. M., Pointis, G., & Segretain, D. (2004). Functional characterization of Cx43 based gap junctions during spermatogenesis. *Journal of cellular physiology*, *200*(1), 146-154.

Di Carlo, A. D., Travia, G., & De Felici, M. (2002). The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. *International Journal of Developmental Biology*, *44*(2), 241-244.

Durcova-Hills, G., & Surani, A. (2008). Reprogramming primordial germ cells (PGC) to embryonic germ (EG) cells. *Current protocols in stem cell biology*, 1A-3.

Eberhart, C. G., Maines, J. Z., & Wasserman, S. A. (1996). Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature*, *381*(6585), 783.

Ephrussi, A., & Lehmann, R. (1992). Induction of germ cell formation by oskar. *Nature*, *358*(6385), 387-392.

Extavour, C. G., & Akam, M. (2003). Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development*, *130*(24), 5869-5884.

Eyal-Giladi, H., Ginsburg, M., & Farbarov, A. (1981). Avian primordial germ cells are of epiblastic origin. *Development*, *65*(1), 139-147.

Ford, C. E., Evans, E. P., Burtenshaw, M. D., Clegg, H. M., Tuffrey, M., & Barnes, R. D. (1975). A functional 'sex-reversed 'oocyte in the mouse. *Proceedings of the Royal Society of London B: Biological Sciences*, *190*(1099), 187-197.

Froman, D. P., & Kirby, J. D. (2005). Sperm mobility: phenotype in roosters (Gallus domesticus) determined by mitochondrial function. *Biology of Reproduction*, *72*(3), 562-567.

Fujimoto, T., Ninomiya, T., & Ukeshima, A. (1976). Observations of the primordial germ cells in blood samples from the chick embryo. *Developmental biology*, *49*(1), 278-282.

Fujiwara, Y., Komiya, T., Kawabata, H., Sato, M., Fujimoto, H., Furusawa, M., & Noce, T. (1994). Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. *Proceedings of the National Academy of Sciences*, *91*(25), 12258-12262.

Gilleron, J., Carette, D., Durand, P., Pointis, G., & Segretain, D. (2009). Connexin 43 a potential regulator of cell proliferation and apoptosis within the seminiferous epithelium. *The international journal of biochemistry & cell biology*, *41*(6), 1381-1390.

Giraldo, P., & Montoliu, L. (2001). Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic research*, *10*(2), 83-103.

Godet, M., Sabido, O., Gilleron, J., & Durand, P. (2008). Meiotic progression of rat spermatocytes requires mitogen-activated protein kinases of Sertoli cells and close contacts between the germ cells and the Sertoli cells. *Developmental biology*, *315*(1), 173-188.

Gruidl, M. E., Smith, P. A., Kuznicki, K. A., McCrone, J. S., Kirchner, J., Roussell, D. L., Stome, S., & Bennett, K. L. (1996). Multiple potential germ-line helicases are components of the germ-line-specific P granules of Caenorhabditis elegans. *Proceedings of the National Academy of Sciences*, *93*(24), 13837-13842.

Han, J. Y., Park, T. S., Hong, Y. H., Jeong, D. K., Kim, J. N., Kim, K. D., & Lim, J. M. (2002). Production of germline chimeras by transfer of chicken gonadal primordial germ cells maintained in vitro for an extended period. *Theriogenology*, *58*(8), 1531-1539.

Hay, B., Jan, L. Y., & Jan, Y. N. (1990). Localization of vasa, a component of Drosophila polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development*, *109*(2), 425-433.

Herrid, M., Olejnik, J., Jackson, M., Suchowerska, N., Stockwell, S., Davey, R., Hutton, K., Hope, S., & Hill, J. R. (2009). Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. *Biology of Reproduction*, *81*(5), 898-905.

Hird, S. N., Paulsen, J. E., & Strome, S. (1996). Segregation of germ granules in living Caenorhabditis elegans embryos: cell-type-specific mechanisms for cytoplasmic localization. *Development*, *122*(4), 1303-1312.

Honaramooz, A., Megee, S. O., & Dobrinski, I. (2002). Germ cell transplantation in pigs. *Biology of Reproduction*, *66*(1), 21-28.

Howarth, B. (1995). Physiology of reproduction: the male. *Poultry production. Elsevier, Amsterdam*, 243-270.

Huleihel, M., & Lunenfeld, E. (2004). Regulation of spermatogenesis by paracrine/autocrine testicular factors. *Asian journal of andrology*, *6*(3), 259-268.

Ikeneshi, K., Nakazato, S., & Okuda, T. (1986). Direct evidence for the presence of germ cell determinant in vegetal pole cytoplasm of Xenopus laevis and in a subcellular fraction of it. *Development, growth & differentiation*, *28*(6), 563-568.

Ikenishi, K., & Tanaka, T. S. (1997). Involvement of the protein of Xenopus vasa homolog (Xenopus vasa-like gene 1, XVLG1) in the differentiation of primordial germ cells. *Development, growth & differentiation*, *39*(5), 625-633.

Ivarie, R. (2003). Avian transgenesis: progress towards the promise. *TRENDS in Biotechnology*, *21*(1), 14-19.

Ivarie, R. (2006). Competitive bioreactor hens on the horizon. *TRENDS in Biotechnology*, *24*(3), 99-101.

Jung, J. G., Lee, Y. M., Park, T. S., Park, S. H., Lim, J. M., & Han, J. Y. (2007). Identification, culture, and characterization of germline stem cell-like cells in chicken testes. *Biology of Reproduction*, *76*(1), 173-182.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., & Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biology of Reproduction*, *69*(2), 612-616.

Karashima, T., Sugimoto, A., & Yamamoto, M. (2000). Caenorhabditis elegans homologue of the human azoospermia factor DAZ is required for oogenesis but not for spermatogenesis. *Development*, *127*(5), 1069-1079.

Kim, J. N., Park, T. S., Park, S. H., Park, K. J., Kim, T. M., Lee, S. K., Lim, J.M. & Han, J. Y. (2010). Migration and proliferation of intact and genetically modified primordial germ cells and the generation of a transgenic chicken. *Biology of Reproduction*, *82*(2), 257-262.

Knaut, H., Werz, C., Geisler, R., & Nusslein-Volhard, C. (2003). A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature*, *421*(6920), 279.

Krimpenfort, P., Rademakers, A., Eyestone, W., van der Schans, A., van den Broek, S., Kooiman, P., Kootwijk, E., Platenburg, G., Pieper, F., Strijker,R. & de Boer, H. (1991). Generation of transgenic dairy cattle using 'in vitro'embryo production. *Nature biotechnology*, *9*(9), 844-847.

Lacerda, S. M. S. N., Batlouni, S. R., Silva, S. B. G., Homem, C. S. P., & França, L. R. (2006). Germ cells transplantation in fish: the Nile-tilapia model. *Animal Reproduction*, *3*(2), 146-159.

Lacerda, S. M., Costa, G. M., Campos-Junior, P. H., Segatelli, T. M., Yazawa, R., Takeuchi, Y., Morita, T., Yoshizaki, G., & França, L. R. (2013). Germ cell transplantation as a potential biotechnological approach to fish reproduction. *Fish physiology and biochemistry*, 1-9.

Lavial, F., Acloque, H., Bachelard, E., Nieto, M. A., Samarut, J., & Pain, B. (2009). Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate. *Developmental biology*, *330*(1), 73-82.

Lillico, S. G., McGrew, M. J., Sherman, A., & Sang, H. M. (2005). Transgenic chickens as bioreactors for protein-based drugs. *Drug discovery today*, *10*(3), 191-196.

Liu, W. S., Wang, A., Uno, Y., Galitz, D., Beattie, C. W., & De León, F. P. (2007). Genomic structure and transcript variants of the bovine DAZL gene. *Cytogenetic and genome research*, *116*(1-2), 65-71.

Lu, Y., West, F. D., Jordan, B. J., Jordan, E. T., West, R. C., Yu, P., & Beckstead, R. B. (2014). Induced pluripotency in chicken embryonic fibroblast results in a germ cell fate. *Stem cells and development*, *23*(15), 1755-1764.

Macdonald, J., Glover, J. D., Taylor, L., Sang, H. M., & McGrew, M. J. (2010). Characterisation and germline transmission of cultured avian primordial germ cells. *PLoS One*, *5*(11), e15518.

Macdonald, J., Taylor, L., Sherman, A., Kawakami, K., Takahashi, Y., Sang, H. M., & McGrew, M. J. (2012). Efficient genetic modification and germ-line transmission of primordial germ cells using piggyBac and Tol2 transposons. *Proceedings of the National Academy of Sciences*, *109*(23), E1466-E1472.

Majhi, S. K., Hattori, R. S., Yokota, M., Watanabe, S., & Strüssmann, C. A. (2009). Germ cell transplantation using sexually competent fish: an approach for rapid propagation of endangered and valuable germlines. *PloS one*, *4*(7), e6132.

Matsui, Y., Zsebo, K., & Hogan, B. L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell*, *70*(5), 841-847.

McGrew, M. J., Sherman, A., Ellard, F. M., Lillico, S. G., Gilhooley, H. J., Kingsman, A. J., Mitrophanous, K.A., & Sang, H. (2004). Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO reports*, *5*(7), 728-733.

McLachlan, R. I., O'donnell, L., Meachem, S. J., Stanton, P. G., De Kretser, D. M., Pratis, K., & Robertson, D. M. (2002). Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Progress in Hormone Research*, *57*(1), 149-179.

Meyer, D. B. (1964). The migration of primordial germ cells in the chick embryo. *Developmental biology, 10*(1), 154-190.

Miyahara, D., Mori, T., Makino, R., Nakamura, Y., Oishi, I., Ono, T., Nirasawa, K., Tagami, T., & Kagami, H. (2014). Culture conditions for maintain propagation, long-term survival and germline transmission of chicken primordial germ cell-like cells. *The Journal of Poultry Science*, *51*(1), 87-95.

Mozdziak, P. E., & Petitte, J. N. (2004). Status of transgenic chicken models for developmental biology. *Developmental dynamics, 229*(3), 414-421.

Mozdziak, P. E., Wysocki, R., Angerman-Stewart, J., Pardue, S. L., & Petitte, J. N. (2006). Production of chick germline chimeras from fluorescence-activated cell-sorted gonocytes. *Poultry science*, *85*(10), 1764-1768.

Mruk, D., Zhu, L. J., Silvestrini, B., Lee, W. M., & Cheng, C. (1997). Interactions of proteases and protease inhibitors in Sertoli-germ cell cocultures preceding the formation of specialized Sertoli-germ cell junctions in vitro. *Journal of andrology, 18*(6), 612-622.

Naito, M., Harumi, T., & Kuwana, T. (2010). Long term in vitro culture of chicken primordial germ cells isolated from embryonic blood and incorporation into germline of recipient embryo. *The Journal of Poultry Science, 47*(1), 57-64.

Naito, M., Tajima, A., Yasuda, Y., & Kuwana, T. (1994). Production of germline chimeric chickens, with high transmission rate of donor-derived gametes, produced by transfer of primordial germ cells. *Molecular reproduction and development*, *39*(2), 153-161.

Nakamura, Y., Usui, F., Atsumi, Y., Otomo, A., Teshima, A., Ono, T., Takeda, K., Nirasawa, K., Kagami, H., & Tagami, T. (2009). Effects of busulfan sustained-release emulsion on depletion and repopulation of primordial germ cells in early chicken embryos. *The Journal of Poultry Science, 46*(2), 127-135.

Nakamura, Y., Yamamoto, Y., Usui, F., Mushika, T., Ono, T., Setioko, A. R., Takeda, K., Nirasawa, K., Kagami, H. & Tagami, T. (2007). Migration and proliferation of primordial germ cells in the early chicken embryo. *Poultry Science*, *86*(10), 2182-2193.

Oishi, I., Yoshii, K., Miyahara, D., Kagami, H., & Tagami, T. (2016). Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Scientific reports*, *6*, 23980.

Oulad-Abdelghani, M., Bouillet, P., Décimo, D., Gansmuller, A., Heyberger, S., Dollé, P., Bronner, S., Lutz, Y. & Chambon, P. (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *The Journal of cell biology*, *135*(2), 469-477.

Palmer, S. J., & Burgoyne, P. S. (1991). In situ analysis of fetal, prepuberal and adult XX—XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. *Development*, *112*(1), 265-268.

Park, T. S., & Han, J. Y. (2000). Derivation and characterization of pluripotent embryonic germ cells in chicken. *Molecular reproduction and development*, *56*(4), 475-482.

Park, T. S., & Han, J. Y. (2012). piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. *Proceedings of the National Academy of Sciences*, *109*(24), 9337-9341.

Park, T. S., Hong, Y. H., Kwon, S. C., Lim, J. M., & Han, J. Y. (2003). Birth of germline chimeras by transfer of chicken embryonic germ (EG) cells into recipient embryos. *Molecular reproduction and development*, *65*(4), 389-395.

Park, T. S., Jeong, D. K., Kim, J. N., Song, G. H., Hong, Y. H., Lim, J. M., & Han, J. Y. (2003). Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos. *Biology of Reproduction*, *68*(5), 1657-1662.

Pointis, G., & Segretain, D. (2005). Role of connexin-based gap junction channels in testis. *Trends in Endocrinology & Metabolism*, *16*(7), 300-306.

Rashidi, H., & Sottile, V. (2009). The chick embryo: hatching a model for contemporary biomedical research. *Bioessays*, *31*(4), 459-465.

Risley, M. S., Tan, I. P., & Farrell, J. (2002). Gap junctions with varied permeability properties establish cell-type specific communication pathways in the rat seminiferous epithelium. *Biology of Reproduction*, *67*(3), 945-952.

Salter, D. W., Smith, E. J., Hughes, S. H., Wright, S. E., & Crittenden, L. B. (1987). Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology*, *157*(1), 236-240.

Schusser, B., Collarini, E. J., Yi, H., Izquierdo, S. M., Fesler, J., Pedersen, D., Klasing, K. C., Kaspers, B., Harriman, W. D., van de Lavoir, M. C., & Etches, R. J. (2013). Immunoglobulin knockout chickens via efficient homologous recombination in primordial germ cells. *Proceedings of the National Academy of Sciences*, *110*(50), 20170-20175.

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P.D., Huggins, G.R. & Gearhart, J. D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proceedings of the National Academy of Sciences*, *95*(23), 13726-13731.

Smith, C. A., & Sinclair, A. H. (2001). Sex determination in the chicken embryo. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *290*(7), 691-699.

Song, Y., D'Costa, S., Pardue, S. L., & Petitte, J. N. (2005). Production of germline chimeric chickens following the administration of a busulfan emulsion. *Molecular reproduction and development*, *70*(4), 438-444.

Song, Y., D'Costa, S., Pardue, S. L., & Petitte, J. N. (2005). Production of germline chimeric chickens following the administration of a busulfan emulsion. *Molecular reproduction and development*, *70*(4), 438-444.

Song, Y., Duraisamy, S., Ali, J., Kizhakkayil, J., Jacob, V. D., Mohammed, M. A., Eltigani, M. A., Amisetty, S., Shukla, M. K., Etches, R. J., & de Lavoir, M. C. V. (2014). Characteristics of long-term cultures of avian primordial germ cells and gonocytes. *Biology of Reproduction*, *90*(1), 15-1.

Spiegelman, M., & Bennett, D. (1973). A light-and electron-microscopic study of primordial germ cells in the early mouse embryo. *Development*, *30*(1), 97-118.

Sridharan, S., Simon, L., Meling, D. D., Cyr, D. G., Gutstein, D. E., Fishman, G. I., Guillou, F., & Cooke, P. S. (2007). Proliferation of adult sertoli cells following conditional knockout of the Gap junctional protein GJA1 (connexin 43) in mice. *Biology of Reproduction*, *76*(5), 804-812.

Stebler, J., Spieler, D., Slanchev, K., Molyneaux, K. A., Richter, U., Cojocaru, V., Tarabykin, V., Wylie, C., Kessel, M., & Raz, E. (2004). Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Developmental biology*, *272*(2), 351-361.

Tagami, T., & Nakamura, Y. (2011). Improving rates of germline transmission in chimeric chickens using busulfan solubilised sustained-release emulsion. *Avian Biology Research*, *4*(2), 74-77.

Tagami, T., Kagami, H., Matsubara, Y., Harumi, T., Naito, M., Takeda, K., Hanada, H., & Nirasawa, K. (2007). Differentiation of female primordial germ cells in the male testes of chicken (Gallus gallus domesticus). *Molecular reproduction and development*, *74*(1), 68-75.

Tsunekawa, N., Naito, M., Sakai, Y., Nishida, T., & Noce, T. (2000). Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. *Development*, *127*(12), 2741-2750.

Ukeshima, A., Yoshinaga, K., & Fujimoto, T. (1991). Scanning and transmission electron microscopic observations of chick primordial germ cells with special reference to the extravasation in their migration course. *Journal of electron microscopy*, *40*(2), 124-128.

Van de Lavoir, M. C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L.T., Gessaro, T.M., Swanberg, S.E. & Delany, M. E. (2006). Germline transmission of genetically modified primordial germ cells. *Nature*, *441*(7094), 766-769.

Vergara, M. N., & Canto-Soler, M. V. (2012). Rediscovering the chick embryo as a model to study retinal development. *Neural development*, *7*(1), 22.

Watanabe, M., Kinutani, M., Naito, M., Ochi, O., & Takashima, Y. (1992). Distribution analysis of transferred donor cells in avian blastodermal chimeras. *Development*, *114*(2), 331-338.

West, F. D., Machacek, D. W., Boyd, N. L., Pandiyan, K., Robbins, K. R., & Stice, S. L. (2008). Enrichment and Differentiation of Human Germ-Like Cells Mediated by Feeder Cells and Basic Fibroblast Growth Factor Signaling. *Stem cells*, *26*(11), 2768-2776.

Youngren, K. K., Coveney, D., Peng, X., Bhattacharya, C., Schmidt, L. S., Nickerson, M. L., Lamb, B.T., Deng, J.M., Behringer, R.R., Capel, B., & Rubin, E. M. (2005). The Ter mutation in the Dead-end gene causes germ cell loss and testicular germ cell tumours. *Nature*, *435*(7040), 360.

Zhu, L., Van de Lavoir, M. C., Albanese, J., Beenhouwer, D. O., Cardarelli, P. M., Cuison, S., Deng, D.F., Deshpande, S., Diamond, J.H., Green, L. & Halk, E. L. (2005). Production of human monoclonal antibody in eggs of chimeric chickens. *Nature biotechnology*, *23*(9), 1159-1169.

CHAPTER 5

Identification of Gender specific chicken PGCs markers based on RNAseq and RT-qPCR

ABSTRACT

This study utilized the transcriptomic profiles of male and female chicken primordial germ cells (cPGCs) to identify differentially expressed genes and gender specific PGCs markers. RNA was isolated from five male and five female PGCs cell lines and subjected to high throughput RNA-sequencing (RNAseq). There were nearly 50 genes that were differentially expressed between the genders ($p<0.05$), out of which 24 genes were consistently different between at least three male and female PGC lines. HMGCA (3-hydroxyl-3-methylglutaryl CoA reductase), LOC100859602 (zinc finger SWIM domain containing transcription factor), GCL (germ cell-less spermatogenesis associated), SLC1A1 (solute carrier family 1 member 1), and LOC427134 (ubiquitin conjugating enzyme, UBE2R2L) were selected as putative gender specific markers. We did not find significant difference in the expression of both HMGCA and LOC100859602 (SWIM) between the sexes in the qPCR analysis. GCL was specifically expressed in male PGCs ($p<0.0002$) and was not expressed in either juvenile testis or ovaries, making it a specific marker for male PGCs. UBE2R2L expression was exclusive for female PGCs and juvenile ovary, hence can be used as a marker for female PGCs. SLC1A1 was exclusively expressed in female PGCs ($p<0.00001$) and its expression was significantly higher in the juvenile ovary ($p<0.0004$) making it a potential gender specific marker. Except for LOC100859602 (SWIM), the expression pattern of HMGCA, GCL1, SLC1A1 and LOC427134 (UBE2R2L) was consistent with the RNAseq results. The present study provides novel gender specific germ cell markers in the broiler chicken. These results will help in elucidating the genetic programming of gender specific germ line development in broilers.

INTRODUCTION

Germ cells are the specialized cells responsible for transferring genetic information to the next generation. Primordial germ cells (PGCs) are the undifferentiated pluripotent germ cells that are the precursors of oocytes and spermatozoa. Like all vertebrate species avian PGCs are segregated early in embryogenesis and retain their undifferentiated characteristics throughout embryonic development. In birds, PGCs are first detected at stage X in the ventral surface of the area pellucida as a cluster of 30-40 alkaline phosphatase positive cells (Eyal-Giladi and Kochav, 1976). Like mammals, avian PGCs originate from the epiblast layer (Eyal-Giladi et al., 1981) but unlike mammals, avian PGCs migrate to the genital ridges via the circulatory system (Nieuwkoop and Sutasurya, 1979; Kuwana, 1993). In mammals, PGCs migrate through the embryonic tissues to reach the genital ridges (Pillai and Chuma, 2012). Chicken germ cell lineages are reported to originate from pre-formed cytoplasmic determinants in the oocytes early in embryogenesis which is in stark contrast with mouse germ cells which appear to be induced by external stimuli later in embryonic development (Johnson *et al*, 2003a; Johnson *et al*., 2003b).

Once PGCs reach the genital ridge, they proliferate and differentiate according to their sex chromosome constitution. In both mammals and avian, after a few rounds of mitosis, male germ cells become quiescent until sexual maturity, whereas female germ cells enter meiosis and pass through leptotene, zygotene, and pachytene stages of prophase I then arrest at the diplotene stage at the time of birth/hatch (McLaren, 2003; Petitte, 2006). Studies in mice have shown that the germ cells, irrespective of their sex, are destined to differentiate into oocytes and hence the pathway of oogenesis is cell-autonomous in mammals (Upadhyay and Zamboni, 1982; McLaren 1995; Chuma and Nakatsuji, 2000). Interestingly, the fate of spermatogenesis and differentiation of germ cells into spermatogonial stem cells (SSCs) is an induced response and is tightly

controlled by Sertoli cells (McLaren and Southee, 1997; Adams and McLaren, 2002). It has been hypothesized that Sertoli cells secrete an uncharacterized signal that blocks the germ cells in entering meiosis. Even though intensive studies have been conducting on understanding the key mechanisms and regulatory pathways of germ cell differentiation and gametogenesis in mammals yet relatively little is known. To date, the mechanism of gametogenesis and factors influencing germ cell differentiation in avian species is still obscure.

Germline chimera generation in chickens is a focus in both life science research and pharmaceutical industries. Not only are chickens considered a powerful model system to understand human development and disease, they have also been used as bioreactors for commercial protein production in the pharmaceutical industries (Zhu *et al*., 2005; Park *et al*., 2015; Cao *et al*., 2015; Johnson, 2006). Extensive research has been done on selecting the best way to create transgenic chickens. Blastodermal cells, PGCs, and gonadal stem cells have been tested to identify the best approach for the generation of germ line chimeras. PGCs are considered the best vehicle for production of transgenic chickens because of: a) ease in embryo manipulation, b) a higher efficiency than blastodermal cells in generating chimeras, and c) the potential of PGCs to differentiate into either oocytes or spermatozoa irrespective of their sex chromosome constitution (Pain et al., 1996; Han *et al*., 2015; Lavoir et al., 2006). Owing to the lower proportion of PGCs in the embryonic blood at HH stage 14-15, long term in-vitro culture of PGCs becomes indispensable to use them effectively for transgenic experiments. The cultural characteristics, germ line specific gene expression, pluripotent gene expression, and determination of PGC specific markers in-vitro have been extensively studied to develop a robust and reliable culture system (Wentworth *et al*., 1989; Naito *et al*., 1999; Park *et al*., 2003; Li *et al*., 2005; McDonald *et al*., 2010; Naito *et al*., 2010; Jung *et al*., 2005). Even though PGC

specific markers are well characterized in chicken, there is still a need to identify gender specific PGCs markers. Although PGCs are bi-potent to differentiate into either gamete, germ line transmission of donor germ cells in mixed sex chimeras is missing or extremely low (Lavoir et al., 2006; Liu et al., 2012). There has been extensive research and progress in the field of chicken PGC characterization and characterization of PGC expression of marker genes under culture conditions. However, there are no reports on sex specific PGC gene expression in avian species. In mammals, PGCs are sexually bipotent and their differentiation is controlled by gonadal somatic cells. In the absence of gonadal somatic cells, PGCs differentiate into oocytes irrespective of their chromosomal constitution (Adams and McLaren, 2002). In avian, germ cells lineages are maternally determined and sexual differentiation of PGCs is influenced by both somatic gonadal cells and PGCs themselves (Tagami et al., 2007). This poses the question as to what genes and genetic pathways are involved in this intrinsic mechanism. In order to understand male and female gamete biology it is important to look at their precursor cells, i.e. PGCs. The detection of global gene expression profiles for male and female PGCs may help in deciphering the intrinsic mechanisms that differentiate PGCs later in embryonic development and will also help in refining the culture conditions for cPGCs *in-vitro*.

In the current study, we have utilized next generation, high throughput RNA-sequencing (RNAseq) to screen for differentially expressed genes and pathways between male and female PGCs. The RNAseq method has been used widely in various systems for studying transcriptomes and identifying intrinsic mechanisms of cell proliferation, differentiation, cell-cell interactions and development (Sultan *et al*., 2008; Trapnell *et al*., 2010; Qi *et al*., 2011; Lian *et al*., 2015). Based on our analyses of the RNAseq data, we selected five differentially expressed genes and validated the transcriptomic data using reverse transcriptase quantitative PCR (RT-qPCR). In this

study, we have identified genes that are exclusively expressed in a gender-specific manner and hence can act as gender based PGC markers.

MATERIALS & METHODS

Experimental specimens

All procedures were approved (#15002) by the UofA (University of Arkansas) Institutional Animal Care and Use Committee (IACUC) and experimentation guidelines were followed throughout the study. Fertilized broiler eggs for PGC isolation were provided by the Cobb-Vantress hatchery in Springdale (AR). Fertile eggs for the PGCs isolation were incubated (NatureForm® hatchery systems) at 99^0 F and 55-60% relative humidity for 3 days (50-54 h of incubation). For optimum embryonic development, the racks were tilted at opposing 45^0 angles every 30 minutes.

Once embryos reached at HH stage 14, blood was extracted from the dorsal aorta under a dissecting microscope (Nikon, SMZ 745T) using a fine glass micropipette created manually using a vertical pipette puller (David Kopf Instruments, model 700C). Using 12-well culture plates (VWR, Radnor, PA), 4-5µl whole blood containing PGCs was cultured in feeder-less culture conditions as described by Miyahara *et al*. (2014) with few modifications. Complete culture media supplemented with growth factors was used to culture the PGCs. The complete culture media was comprised of 1X DMEM (supplemented with high glucose, non-essential amino acids, sodium pyruvate and phenol red; Life technologies, Carlsbad, CA, USA), 10% (v/v) Fetal Bovine Serum (FBS; Life technologies), 2.5% Chicken serum (CS; Life technologies), 1X Nucleosides (EmbryoMax®; Millipore, Billarica, MA, USA), 1X Antimycotic-Antibiotic

solution (Life technologies), 2mM glutamine (GlutaMax; ThermoFischer Scientific, Waltham, MA, USA), 0.55 mM β-mercaptoethanol (Life technologies) and growth factors. Growth factors were: 10ng/ml human fibroblast growth factor (h-FGF2; ProSpec, Israel), 5ng/ml human Stem Cell Factor (h-SCF; ProSpec, Israel), 2ng/ml human Leukocyte Inhibitory Factor (h-LIF; ProSpec, Israel), and 2.5ng/ml human recombinant Insulin Growth Factor 1 (IGF1; Life Technologies). The cellular morphology and colony forming characteristics became apparent after 6-8 weeks of culture in feeder-less conditions. Chicken PGCs (cPGCs) were distinguished from remaining blood cells owing to their large nucleus (10-20μm) and greater refractive index due to numerous cytoplasmic lipid droplets. The cellular morphology and integrity was checked daily using an inverted microscope (Olympus, Phase contrast).

Sexing of the cPGC colonies

Embryonic blood used for PGC culture was also used to determine sex of the respective PGCs. Bailes et al. (2007) protocol for DNA isolation was utilized to extract DNA for sexing by polymerase chain reaction (PCR). Briefly, 4-5μl of blood was collected from each embryo (HH stage 14) using a glass micropipette and mixed with 200μl STM buffer (64 mM sucrose, 20 mM Tris Cl, pH 7.5, 10 mM MgCl2, and 0.5% Triton X-100). Samples were kept on ice throughout the extraction procedure until specified otherwise. The nuclei were pelleted by centrifuging blood-STM mixture at 1000 g for 5 minutes at room temperature. The supernatant was discarded and pelleted nuclei were resuspended in 200μl TEN + pronase cocktail (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 μg/mL of pronase; Sigma-Aldrich, St. Louis, MO) by repeated trituration. The samples were incubated at 37^0C for 1 h with shaking in a bacteriological incubator. Finally, pronase was inactivated by incubation at 65^0C water bath for 10 min. PCR

primers used for sexing PGCs were for the W-chromosome *XhoI* repetitive sequence (Wxho) and 18S ribosomal sequence (Ribo) (Clinton et *al*., 2001). The primer-pairs are listed in Table 5.1.

The 20µl PCR for sexing was comprised of 2 µl DNA, 1 mM each for all four primers (Wxho and Ribo), 0.2 mM dNTPs, 4 U Taq polymerase, 1x Taq buffer (50 mM Tris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/ml BSA, 0.25 mM $MgCl_2$). The reaction conditions used were 90°C for 2 min, 45 cycles of 90°C for 30 s, 55°C for 15 sec., 72°C for 1 min, followed by a final extension of 72°C for 3 min.

Characterization of cultured cPGCs

Colony-forming PGCs were further characterized for their germ cell specific and pluripotent characteristics using PAS staining, immunocytochemistry and quantitative PCR. cPGCs obtained after 6-8 weeks of culture were subjected to PAS staining. Chicken fibroblast cell cultures were used as a negative control. Cells were detached from 12-well culture plates using accutase (Sigma-Aldrich) cell dissociation agent. Dissociated cPGCs were seeded in a 6-well culture plate @ $1x10^5$ cells/well. Wells used for used for PAS staining were first fixed with 95% ethanol for 10 minutes and then rinsed with 1X PBS 3 times for 5 minutes. After rinsing, the cells were stained with 1 ml periodic acid solution (Sigma-Aldrich) per well for 5 minutes and later washed three times with PBS for 1 minute each. Subsequently, the cells were treated with equal volume of Schiff's reagent (Sigma-Aldrich) for 15 minutes. At the end of staining, cells were rinsed three times with PBS to avoid overstaining. Hematoxylin (Sigma-Aldrich) was used as a counterstain for nuclear staining. After one-minute cells were rinsed again 2 times with PBS.

Each step was conducted at room temperature and the stained cells were imaged using inverted microscope (Olympus, Phase contrast).

The immunocytochemical analysis of cultured cPGCs was done with the help of Nhung T. Nguyen (M.S. graduate student), to analyze any deviations from the germ cell characteristics under feeder-less culture conditions. Primary chicken embryonic fibroblast (CEF) cells were used as a negative control. The staining protocol was adapted from McDonald *et al*. (2010). In short, ~ 10,000 PGCs were seeded in four chambered glass slides (VWR) and once confluent, fixed in 4% (v/v) paraformaldehyde/PBS (Boston Bioproducts, Ashland, MA) for 10 minutes at room temperature followed by 1X PBS washing 3 times for 5 minutes each. The fixed cells were incubated with 500µl of 5% goat serum for 30 minutes. Goat serum was aspirated and cells were treated with primary antibodies. Primary antibodies obtained from DSHB (University of Iowa) were: MC-480 (SSEA-1)-s (mouse anti-SSEA-1 monoclonal antibody), MC-631 (SSEA-3)-s (mouse anti-SSEA-3 monoclonal antibody), and MC-813-70 (SSEA-4)-s (mouse anti-SSEA-4 monoclonal antibody). Cells were first incubated with primary antibodies (200µl each) overnight at 4^0C. The treated cells were then rinsed with PBS thrice with 5 minutes incubation time at room temperature. The secondary antibody was FITC-labelled goat anti-mouse IgG (Santa Cruz Biotechnology Inc.), diluted in 1X PBS and 0.05% Tween 20. Slides were incubated with secondary antibody for 1h at room temperature in dark. The slides were then rinsed with sterile 1X PBS for 3 times, then incubated for 2 minutes at room temperature in DAPI (1µg/ml in PBS) as a counter-stain. Finally, the slides were washed with PBS, the chamber was removed from the slide, and a coverslip was placed on the slide using 30% glycerol as a mounting medium. The stained cells were visualized using an inverted fluorescence microscope (Carl-Zeiss, Axio-imager 2).

The germ-line specificity of cultured cPGCs was determined by RT-endpoint PCR. Total RNA was isolated from cultured cells using an RNA isolation kit (Biorad, CA, USA) as per the manufacturer's instructions. The quality and quantity of RNA samples were analyzed using spectrophotometer (Nanodrop, ND-100) and only high quality RNAs were used for cDNA synthesis. RT-PCR followed a 2-step protocol to detect expression of germ-line specific genes. First strand synthesis reactions were in 20µl total volume comprised of 5 µl of 5x SS buffer (Promega, Madison, WI), 0.2 µl of 20 mM dNTPs, 0.4 µl of 30 µM $CT_{23}V$, 1µl DTT (), 0.2µl of (1-2 U) RNAsin (Promega), 0.5µl (100U) reverse transcriptase enzyme (Superscript III, Life Technologies), 10-20µg total RNA. The reaction was prepared in 0.2 ml PCR tube, mixed thoroughly and then incubated at 42^0C for 20-30 minutes. The mixture was then moved to ice. The cDNA synthesized was used immediately for PCR. The reaction mix for second step PCR (20µl total reaction volume) was comprised of: 1x Taq polymerase buffer, 1 mM dNTPs, 0.25 mM $MgCl_2$, 1 µM forward and reverse primers, 2 µl cDNA (~2-5ng), 4 U Taq polymerase. The PCR cycling conditions were 90°C for 2 min, 35 cycles of 90°C for 30 s, 60°C for 15 sec., 72°C for 1 min, followed by a final extension of 72°C for 3 minutes. The primer-pair sequences as well as product sizes are listed in Table 5.1.

The amplified PCR products were resolved in 1.5% agarose gels. Liver cDNA was used as a negative control whereas juvenile testis and ovary cDNAs were used as positive controls.

RNA Sequencing

Total RNAs were extracted from male and female PGCs using RNA isolation kit (Biorad, CA, USA). The RNA integrity and quantity was tested by using Bioanalyzer by detecting 18S & 28S RNA. The RNAseq library preparation, alignment, and downstream pathway analysis was done

by Nhung Thi Nguyen (Transcriptomics of chicken primordial germ cells. MS Thesis, University of Arkansas, 2015). In brief, RNAseq libraries were prepared separately for 4 male and 5 female PGC cell lines. Libraries were barcoded, pooled together, and sequenced on an Illumina HiSeq 2500 at the University of Delaware Sequencing facility. The quality control of raw RNAseq output in Fastq format was performed by FASTQC Tool (Version 0.11.4, Babraham Bioinformatics). Chicken genome (Galgal4) was used to map the sequences and DNAstar-NGen (Madison, WI) sequencing tools or fRNAkenstein program (http://geco.iplantc.org/frnakenstein) were used to generate Fragments Per Kilobase per Million (FPKM) values. Pathrings program (http://raven.anr.udel.edu/~sunliang/PathRings/) was used for placing genes in each functional pathway.

Primer Design

Based on the RNAseq results, target genes were selected which were significantly different in at least three 3 female vs male chicken PGC lines. The reference gene was ribosomal protein S14 (RPS14). The primer-sets used for RT-qPCR were designed using Primer-BLAST (NCBI tool) and each primer set was chosen so that the primers flanked at least one intron to minimize amplification from any trace contaminating genomic DNA. The primer sets used are listed in Table 5.1.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-qPCR)

Two step RT-qPCR was used for detecting expression analysis of the target genes. Protocol of total RNA extraction and first strand cDNA synthesis was as described above.

For qPCR, all genes were run in triplicate and a cocktail was made with all reaction components except primers and cDNA. The cocktail components for 20 µl qPCR were as follows: 1x Taq buffer (50 mM Tris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/ml BSA, 0.25 mM $MgCl_2$), 0.2 mM dNTPs, 0.25 mM $MgCl_2$, 1x EvaGreen dye, 4U Taq Polymerase, and nuclease free water to make up the final volume of 20 µl. The components were mixed properly and the cocktail was aliquoted to PCR tubes. For each gene, nearly 6 µl of cDNA was added. After aliquoting the cDNA PCR mix in the 96 well qPCR plate, 6 µl 10 mM forward and reverse primers were added per target gene. The mix was mixed by trituration and aliquoted in the respective wells using multichannel pipettors. The plate was sealed using transparent film and the amplification protocol was run in the FAM/SYBR channel in CFX96 real time PCR detection machine (BioRad, Hercules, CA) The reaction conditions used were: 45 ^oC for 20 min., 90 ^oC for 30 sec., 10 cycles of 90 ^oC for 15 sec., 60 ^oC for 15 sec., and 72 ^oC for 60 sec. followed by 30 cycles of 90 ^oC for 15 sec., 90 ^oC for 15 sec., 72 ^oC for 60 sec. and plate read in the end. A melting-curve analysis was conducted for each sample to eradicate any false positive amplification during the analysis. A high-resolution melt curve was deployed with following conditions: 72 ^oC for 180 sec., 90 ^oC for 15 sec., and 65 ^oC for 180 sec. The reference RPS 14gene was used to determine the Ct/Cq values for the target genes.

Statistics

Significant differences in the data sets generated by RT-qPCR for ZZ and ZW PGCs were compared with a two-tailed Student t-test with heterogeneous variance. Data sets having p value < 0.05 were considered as significant.

RESULTS

Pproliferation of cPGCs in feeder-free media

cPGC lines that showcased all defined cultural parameters including morphological characteristics, germ line specificity, and pluripotency under culture conditions were used for RNAseq analysis. The cultural characteristics exhibited by cPGCs in feeder-less conditions are demonstrated in chapter 4 (Results section). Briefly, both male and female PGC lines start developing colonies after 6 weeks of culture in feeder-less culture conditions. PGC colonies became apparent earlier in male PGC lines as compared to females. Differences in the male and female PGCs growth pattern *in-vitro* has been described previously (Mayahira *et al*., 2014). The chicken PGC morphology as well as the characteristics of the PGC cluster were in accordance with the literature (Lavoir *et al*., 2006). Both male and female PGC lines depicted these characteristics (Chapter 4, Results section I, Figure 4.1).

Periodic Acid Stain (PAS) was used to further validate the chicken PGC characteristics under culture. Chicken PGC contains glycogen reserves in the form of cytoplasmic granules which reacts strongly with PAS reagent (Jung *et al*.,2005; Lu *et al*., 2014). As expected, irrespective of the gender PGC colonies stained strongly with PAS (Chapter 4, Results section III, Figure 4.3).

Cultured PGCs were further characterized for germ-cell specific cell surface antigens using immunocytochemistry. cPGCs stained strongly for anti-SSEA-1 antibodies (Chapter 4, results section, Figure 4.4a) but unlike previous studies they were only weakly stained for anti-SSEA-3 (Chapter 4, results section, Figure 4.4a) & anti-SSEA-4 antibodies (Chapter 4, results section, Figure 4.4a). As expected, no reactivity was detected in CEF cells against any stem cell specific surface antigens (Chapter 4, results section, Figure 4.4b).

Sexing of chicken PGC cell lines

Avian females are homozygous (ZZ) for the sex chromosomes whereas males are heterozygous (ZW). The primers used for sex determination exploited this feature to identify the gender of cultured chicken PGCs. Wxho is a female specific primer that amplifies the *Xho*I repetitive sequence from the chromosome W whereas the Ribo primers amplify a region of the 18S ribosomal gene present in both genders. Embryonic blood used for PGCs culture was also used to isolate DNA for sex determination by PCR. The PCR products were then electrophoresed in 1.5% agarose gel. The result of PGCs sex determination by PCR is depicted in Figure 5.4. Lanes with bands at 416bp (Wxho) and 256bp (Ribo) represent females (lanes 2, 3, 6, 7, 8, 13, 14, 15, 16, 17, and 18) whereas lanes with just one band at 256bp (Ribo) represent males (lane 4, 5, 9, 10, 11, and 12).

Expression of germ-line specific genes in the cultured PGCs

The germ line specific genes selected to further characterize cultured chicken PGCs were chicken vasa homologue (Cvh), deleted in azoospermia-like (Dazl), Sdf-1/CXCR-4, Stra-8 and Sycp-3.

Chicken vasa homologue (Cvh), and deleted in azoospermia-like (Dazl) genes are exclusively expressed in the germ cells and considered as germ cell specific markers. Sdf-1/ CXCR-4 expression is crucial for germ cell migration whereas Stra-8 and Sycp-3 genes are expressed in premeiotic and meiotic germ cell respectively (Stebler *et al*., 2004; Oulad-Abdelghani et al., 1996; West et al., 2008). RT-PCR (reverse-transcription polymerase chain reaction) was used to detect the germ line specific signals and showed expression of all five genes in the both male and

female PGCs. Chicken juvenile testis and ovary cDNA were used as positive controls whereas chicken juvenile liver was used as a negative control. Results showed that Dazl, Cvh and Stra-8 were strongly expressed in both male and female PGCs (Figure 5.5). Although both Sdf-1 and Sycp-3 had a lower level of expression in the PGCs, their expression level differed in the positive controls. Sdf-1 was expressed in both ovary and testis whereas Sycp-3 was only expressed in testis at lower levels (Figure 5.5). This suggest that Sycp-3 gene is expressed equally in the germs cells irrespective of their gender prior to the process of differentiation but continues to be expressed only in the male germ cells after their differentiation and maturation. All genes were highly expressed in both PGCs, testes and ovaries (except Sycp-3) whereas no expression of these germ line related genes was observed in the liver. Among the studied genes, Cvh was highly expressed in the PGCs and hence confirmed their germ line specific attributes. These results also strengthened the previous reports on Cvh being a germ-line specific marker for chicken PGCs (Tsunekawa *et al*., 2000; Lavial *et al*., 2009).

RNAseq data analysis

RNAseq library construction and sequencing was under the supervision of Dr. Carl Schmidt (University of Delaware). Initial analyses were by Nhung Thi Nguyen. Detailed results of RNAseq work and pathway analysis can be found in her thesis (Transcriptomics of chicken primordial germ cells, 2015). In short, five female and four male chicken PGC cell lines were chosen for RNAseq library preparation. The number of expressed genes detected in the male and female groups with RPKM value greater than 0.1 were 13,695. Student T-test was performed to detect the significantly upregulated/downregulated genes. Based on the statistical analysis, 87 genes appeared to be differentially expressed out of which 13 genes were significantly

upregulated in female and 8 genes were significantly upregulated in male PGCs. It has been observed that PGCs of both sexes exhibited similar expression pattern in both gender, yet there were 87 genes that were expressed differentialy between the lines (Tables 8 & 9, Nhung T. Nyugen, Transcriptomics of chicken PGCs).

Uniquely represented pathways between the two genders include female PGCs upregulated in pathways associated with cell cycle, disease and protein-metabolism whereas males were predominant in pathways associated with immune system, neural system, developmental biology and extracellular matrix organization. Nearly 24 genes were observed which were significantly differentially expressed in at least three of the five female vs the male samples (Table 5.2). Out of these 24 genes, five genes were selected for further validation by RT-qPCR. The genes selected were LOC100859602 (SWIM), HMGCR, GCL, SLCL1 and LOC427134 (UBE2R2L). These genes were selected due to their fold difference in their FPKM values as well as their germ cell related roles.

Real time quantitative PCR

The relative expression of selected genes for male and female PGCs was detected through RT-qPCR (Figure 5.6). The results of RT-qPCR were consistent with the results from the RNAseq with the exception of LOC100859602 (SWIM domain) gene. There was no gender specific significant difference in the expression level of HMGCR and SWIM ($p>0.05$) in chicken PGCs but HMGCR gene was expressed at significantly higher level in juvenile testis than in juvenile ovary ($p=0.04$) (Figure5.6). The expression of GCL was exclusively expressed in the male PGCs and was not expressed in either juvenile testis or ovary. On the other hand, UBE2R2L gene was expressed exclusively in females. Neither male PGCs nor juvenile testis showed any expression

of the gene. Since UBE2R2L is a W-specific gene this is not surprising. Another gene that was exclusively expressed in female PGCs was SLCL1. The SLCL1 gene expression was also found to be expressed at significantly higher levels than male PGCs (p=0.0004).

DISCUSSION

Characterization of chicken PGCs

The main aim of this study was to determine the gender based variations at the PGC level in chicken. To achieve this goal, a two pronged approach was used by first analysing the whole genome transcriptomics of cultured male and female chicken PGCs and then validating the results by using RT-qPCR. It was important to examine the cultural characteristics of male and female PGCs grown in feeder less culture conditions before conducting RNA-seq analysis.

The PGCs used in this study were isolated from the blood of HH stage 14 embryos (50-54 h of incubation), a stage where PGCs are highest in number in the embryonic blood. The aim was to isolate PGCs early in the embryonic development, before their migration to the germinal crescent so as to identify gender specific variations in the gene expression and identify gender specific chicken PGC markers at an early stage. The PGCs were cultured in a well defined medium without support of any feeder layer. The PGCs cultured in this study exhibited their characteristic morphology of comparatiely larger size (10-20 µm in diameter) and higher number of cytoplasmic lipid vacuoles (Figure 5.1A). High lipid content has been reported as one of the major characteristics of the chicken PGCs (Fujimoto *et al*., 1976; Song *et al*., 2014). The colonies were spherical in shape and cells appeared to communicate via cytoplasmic projections (Figure 5.1C, D, E, arrowheads). The characteristics of chicken PGC colonies are well

documented (Park and Han, 2000; Jung *et al*., 2005) and the colonies of both male and female PGCs in this study showed the same characteristics.

Furthermore, the presence of glycogen content and stage specific embryonic antigens were tested for the cultured PGCs in this study. As predicted, the PGCs tested postive for both PAS reagent (reacts with complex carbohydrates) and SSEA-1(stage specific embryonic antigen-1), SSEA-3 (stage specific embryonic antigen-3) and SSEA-4 (stage specific embryonic antigen-4) antigens (Figure 5.2; Figure 5.3a& 5.3b). The presence of embryonic antigens SSEA-1, SSEA-3 and SSEA-4 have been reported in the undifferentiated chciken embryonic stem cells. These atigens represents pluripotency in the respective cells (Matsui *et al*., 1992; Shamblott et al., 1998; Mozdiak *et al*., 2005; Durcova-Hills and Surani, 2008). The expression of germ line specific genes was also tested to further estimate the germ specific character of cultured PGCs. Cultured chicken PGCs expressed all germ line specific genes that were chosen for the current study irrespective of their sex. These genes includes chicken vasa homologue (Cvh), Dazl, Sdf-1, Stra-8, and Sycp-3. Sycp-3 showed little to no expression in adult chicken ovaries whereas was expressed at same level in both male and female PGCs (Figure 5.4).

RNAseq analysis and RT-qPCR

This study analyzed transcriptomes of male and female PGCs isolated from the HH stage 14 chicken embryos. The aim was to identify novel transcripts that can act as gender specific markers for chicken PGCs. Also, specific gene expression based on the sex chromosome constitution of the PGCs at an early embryonic stage would help in understanding the germ cell biology and gametogenesis in general. Certain pathways that were found to be upregulated in female PGCs were associated with signal transduction, cell proliferation, PGC migration and embryogenesis

whereas pathways that were upregulated in male PGCs were involved with cell division and cell division (Nhung T. Nguyen, 2015). Genes that were selected for further analysis were involved in the process of homeostasis, protein metabolism, and germ cell biology. Furthermore, only those genes that were significantly different in at least three independent biological samples were included in the study to reduce false positives generated due to individual variation.

HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) is an enzyme that is associated with a rate limiting step in cholesterol biosynthesis (Brown *et al*., 1979; Goldstein and Brown, 1990). Besides cholesterol synthesis, HMGCR also plays a crucial role in developmental biology. In Drosophila, HMGCR was reported to be expressed in the somatic gonads and was indispensable for the PGCs migration to the future gonads within the mesoderm (Brand *et al*., 1993; Van Doren *et al*., 1998; Santos and Lehmann, 2004). Interestingly in Drosophila, HMGCR expression was not reported in PGCs but was expressed in the mesoderm that guides PGCs towards its target tissue (Van Doren *et al*., 1998). Mutations in the HMGCR gene resulted in the disruption of PGC migration and depletion of germ cells in the developing gonads in both zebrafish and Drosophila (Santos and Lehmann, 2004; Thorpe *et al*., 2004). Another study in zebrafish has shown the mevalonate dependent prenylation reaction that is mediated by Geranylgeranyl Transferase 1 (GGT1), was crucial for PGC migration (Thorpe *et al*., 2004). Synthesis of mevalonate is a rate limiting step and is regulated by HMGCR. It has been hypothesized that vertebrate PGCs might get attracted towards the lipid intermediates synthesized by HMGCR (Molyneaux and Wylie, 2004). It was interesting to find HMGCR expression in the chicken PGCs as there has been no reports on the expression of HMGCR in PGCs so far. We found the expression of HMGCR gene in both male and female chicken PGCs but there was no significant difference between them. The HMGCR expression in chicken juvenile testis was significantly higher than the juvenile ovary

($p<0.05$). PGC migration in vertebrates has been reported to be cell autonomous and controlled by somatic gonadal cells (Molyneaux and Wylie, 2004; Santos and Lehmann, 2004; Thorpe *et al*., 2004). In chicken, the details of PGCs for migration from the extraembryonic region to the blood vascular system and from there to the germinal crescent are still obscure. Proposed hypotheses include either passive, active, or both passive and active migration of chicken PGCs towards their target tissues (Nakamura *et al*., 2007). Detection of HMGCR expression in chicken PGCs indicates a possible role in the passive migration of PGCs to their destination. To date there is no data on the HMGCR expression from the chicken gonadal somatic cells. The expression of HMGCR gene in PGCs is suggestive of either a self-migratory role or the combined effect of PGCs and the chicken gonadal somatic cells in directing the PGCs towards the germinal crescent. Our finding on the expression of HMGCR expression in chicken PGCs is highly encouraging but needs to be further examined for its role in the PGCs migration and germ cell biology in chicken.

GCL (Germ cell-less) gene encodes a transcriptional repressor protein and is highly conserved from *Caenorhabditis elegans* to humans. In *Drosophila*, the GCL gene is critical for PGCs development (Jongens *et al*., 1994; Robertson et al., 1999). The GCL protein helps in the formation of pole cells that ultimately give rise to PGCs which are maternally derived in *Drosophila*. In zebrafish, the GCL homologue is expressed in adult testes and ovaries and was supposed to be involved in PGCs formation (Li *et al*., 2006). In mammals, PGCs are not maternally derived, but are induced from pluripotent epiblast cells. The homologue of GCL was later identified in mouse as mGCL (mouse germ cell-less) that had 49% similarity with the *Drosophila* GCL gene and was localized to the nuclear envelope (de la Luna et al., 1999; Kimura et al., 1999; Leatherman *et al*., 2000). The mGCL gene was reported to be expressed in all embryonic stages and in the adult tissues at low levels but was strongly expressed in male germ cells. Its participation in

spermatogenesis was demonstrated by Maekawa et. al. (2004) where they showed mGCL appearance in the nuclei of mid-pachytene spermatocytes through the spermatid stage. The presence of abnormal sperms in mGCL negative mice clearly indicates its importance in male gametogenesis. Similarly, in humans the GCL orthologue was identified as hGCL and its absence was shown to be associated with defective sperm motility and severe testicular impairment (Kleiman *et al*., 2003). It has been proposed that the hGCL gene is involved in the process of spermatogenesis in later stages (Kleiman *et al*., 2003) unlike in *Drosophila* where GCL expression was involved in pole plasm formation in the oocytes before fertilization. The chicken GCL homologue (cGCL) has not been placed in any chromosome yet and is only partially annotated. There is insufficient information on its definitive role in PGCs development or in spermatogenesis. Our RNAseq analyses on male and female chicken PGCs identified significant differences in the levels of cGCL mRNA between genders. Furthermore, the RT-qPCR analysis revealed gender specific expression of this gene in chicken PGCs. The complete absence of cGCL expression in chicken juvenile testis raises important questions on the stage specific expression of cGCL gene in chicken. Moreover, the expression of cGCL in male PGCs signifies its probable role in male germ cell biology in chicken and makes it a novel marker for gender specific PGCs identification in chicken. The exact role of cGCL in male PGCs differentiation in chicken needs further investigation.

Another gene that projected out from our RNAseq analysis was LOC427134 gene that is homologous to UBE2R2L (Ubiquitin conjugating enzyme E2 R2 like). This gene is mapped to chromosome W, female specific sex chromosome in avian. As expected UBE2R2L was specifically expressed in the female PGCs and juvenile ovaries. Ubiquitin conjugating enzymes are involved in protein ubiquitnylation that led to the degradation of the target protein

(Hochstrasser, 1996). The ubiquitin pathway is very important in cell cycle progression (Peters *et al*., 1998), endocytosis, and inflammatory responses (Chen *et al*., 1995; Palombella *et al.*, 1994). The presence of UBE2R2L like mRNAs in chicken PGCs is note-worthy and since it is W chromosome specific gene, can be used as a female PGC specific marker that can be used to study gender specific changes in chicken PGCs differentiation in ovo.

Solute carrier family 1 member 1 (SLC1A1), is a member of neuronal/epithelial high affinity glutamate transporters that are usually present in the cell membrane of neurons and glial cells. These transporters regulate extracellular glutamate levels and helps in preventing glutamate toxicity. Thus, they are highly important in maintaining homeostasis in the central nervous system (CNS), (Otori *et al.*, 1994; Derouiche & Rauen, 1995; Rauen, 2000). In our study, we found the expression of SLC1A1-1 mRNA in the chicken PGCs. On further analysis, the SLC1A1 expression was significantly higher in the female PGCs and juvenile ovaries as compared to male PGCs and juvenile testis respectively (Figure 5.6). This is an interesting observation since expression of glutamate transporters have been reported only in the neurons, glial cells and the retinal cells so far. The presence of SLC1A1 expression in female PGCs and later in the ovaries suggest a unique role of these transporters in gender specific differentiation of germ cells in chicken. Since it is expressed only in female PGCs, SLC1A1 may be used as a marker to identify chicken PGCs based on its gender.

The last gene that we tested in our study was an uncharacterized gene LOC100859602, that shares homology with a zinc finger SWIM-type containing 6 transcription factor. Studies in mice have shown the involvement of this gene in transcriptional control of a variety of genes related to emotional neural activity. Like SLC1A1, this transcriptional factor is heavily implicated in brain function. In this case, the transcription factor has a role in regulating transcription of G-protein

coupled signal transduction pathways in the brain. Genes containing zinc factor SWIM domain are involved in axonal growth and Wnt signaling pathways (Kai et al., 2004). The identification of LOC100859602 gene in the transcriptome of chicken PGCs is interesting. Furthermore, this poorly characterized gene has been mapped to the Z chromosome. RT-qPCR analysis revealed expression of this gene in both male and female PGCs and in juvenile testis and ovaries. There was no significant differential expression of this gene between the sexes. The mere expression of this transcription factor in chicken PGCs is quite interesting and suggests a possible role in transcriptional regulation of certain genes. Future research on the function of this gene in the germ cells, may reveal its specific role in the germ cell differentiation and proliferation.

SUMMARY AND CONCLUSION

The information on genes regulating the process of germ cell migration, differentiation, proliferation and survival in chicken is still very weak. There is little to no information on the gender specific gene expression in chicken PGCs. We tried to decipher the differential gene expression between male and female chicken PGCs cultured in feeder less conditions. The PGCs used in this study were isolated from 3-day old chicken embryos at HH stage 14 (50-54 h of incubation), a stage where PGCs are in the migratory phase and are highest in number in the blood vascular system. Our approach was to identify the novel transcripts that has sexually dimorphic expression in chicken PGCs using high throughput RNAseq technology. We were able to identify nearly 24 genes in RNAseq analysis that were significantly different between the male and female PGCs. The five genes were selected based on their location in the sex chromosomes, the fold differences in the RPKM value between the sexes as well as their respective functions in the germ cell biology. Interestingly, our study identified genes that were reported to be involved in PGCs migration, gametogenesis, transcription, and glutamate transporteration. HMGCR gene was identified in the chicken PGCs for the first time and was found to be significantly different between the juvenile testis and ovaries. The chicken homologue of GCL was detected and its germ line specific expression in the male PGCs was encouraging since GCL gene was implicated with male fertility and sperm motility. The direct involvement of GCL gene in spermatogenesis, demands to look deeper into the role of this gene in the male germ cell biology. It also makes it a novel marker to identify male chicken PGCs at an early age. UBE2R2L maps to the chromosome W and hence was expressed only in the female samples. This makes it a candidate gene to act as female PGCs specific marker. The other two genes, SLC1A1 and SWIM were also being expressed in the PGCs with SLC1A1 being highly

expressed in the female PGCs whereas we found no significant difference in swim expression. The genes discovered in this study were not reported before in context to chicken PGCs. The identification of these genes in PGCs and also in sex specific manner, is very encouraging as it will help in identifying model mechanism that will help in identifying novel mechanism that regulate differentiation and gametogenesis in chicken PGCs.

FIGURES

Figure 5.1: Sex determination of chicken PGCs using Wxho and Ribo primers. Male PGC lines were amplified by just Ribo primers whereas female lines were amplified by both Wxho and Ribo primers. Lane 1 represent marker, lanes 2, 3, 6, 7, 8, 13, 14, 15, 16, 17, 18 were females while lanes 4, 5, 9, 10, 11, and 12 were males. Lane 20 & 21 represents male and female DNA as positive controls. Ma represent male positive control, Fe represent female positive control and NTC represents non-template negative control.

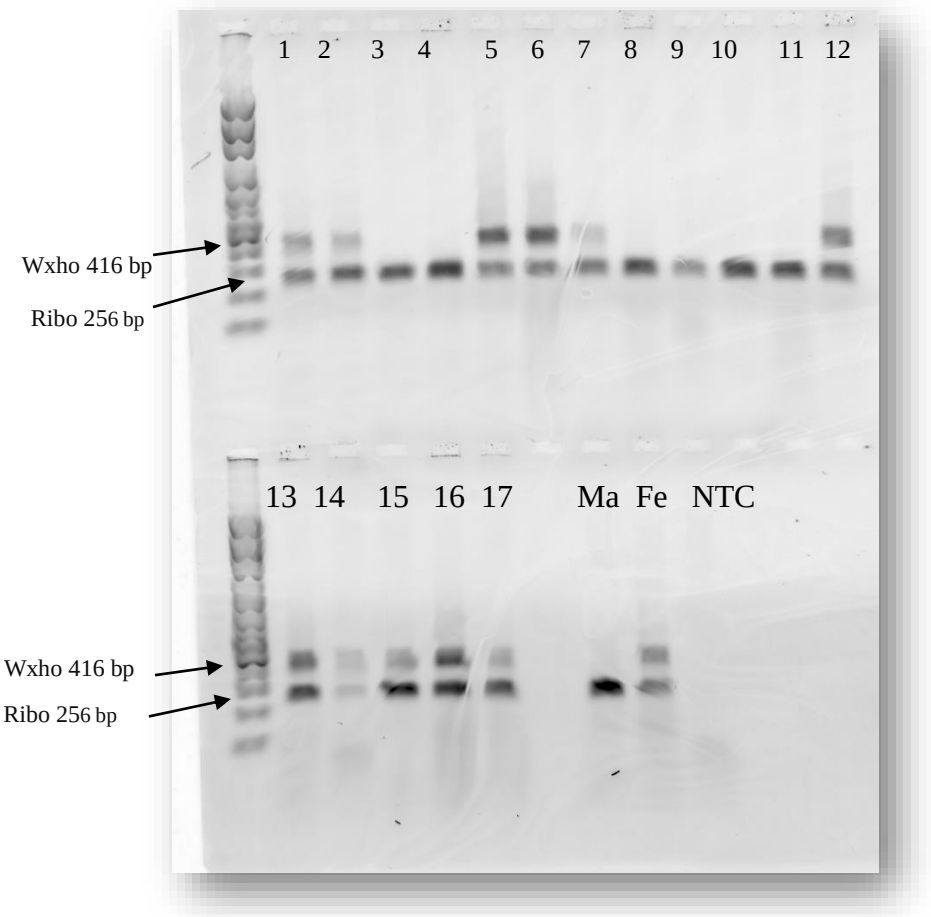


Figure 5.2: Germ line-related gene expression of male and female PGCs**.** Dazl, Sdf-1, Stra-8 were expressed strongly in both male, female PGCs as well as in chicken juvenile testis and ovaries. Cvh and Sycp-3 were expressed specifically in the PGCs. There was slight amplification of Cvh gene in both testis and ovaries but Sycp-3 did not amplify in the ovaries at all. Expression of all genes was negative for liver (negative control) and NTC (Non-template control).

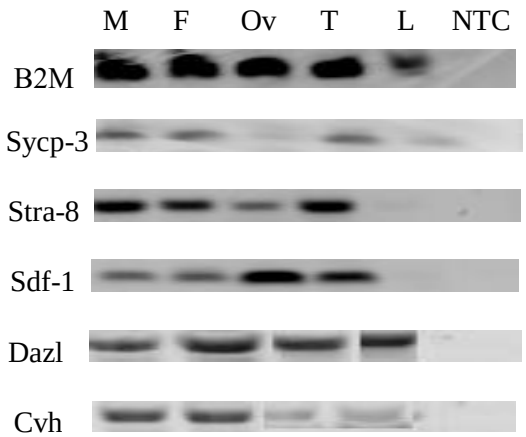
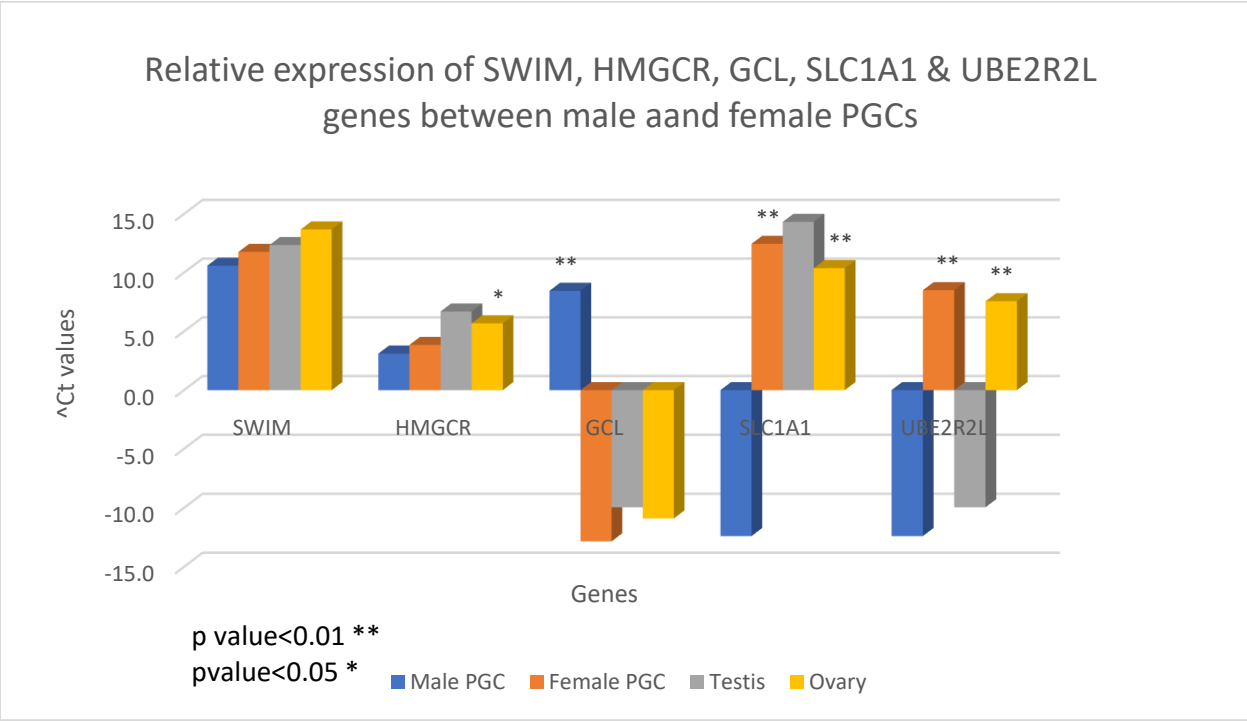


Figure 5.3: The RT-qPCR analysis of SWIM, HMGCR, GCL, SLCL1 and UBE2R2L genes on male and female chicken PGCs. Chicken juvenile testis and juvenile ovary was used as positive controls. Unpaired student t test was used to analyze the data.



TABLES

Table 5.1: Sequences of primer pairs used in the PCR. Top five sequences were used for qRT-PCR, sequence number 6 & 7 were used for cPGCs sex determination and sequences from 8 onwards were used to test germ line specificity of cultured cPGCs. All primer sequences were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

Serial No.	Gene	Primers	Product length	Chromosome
1	HMGCR	**Forward**: CAGTCATTCCAGCCAAGGTT **Reverse**: GCTGCCTTCTTAGTGCAGGT	535 bp	Z
2	GMCL1	**Forward**: CACAAGCTGATGCCTGGTT **Reverse**: TGTTCCGCTTGAAGATGATG	395 bp	Z
3	SWIM1	**Forward**: GGAGGCTGGAAACAGTGTTAG **Reverse**: GCTTCGGTTGGAGTGAAWA	299 bp	Z
4	UBE2R2L	**Forward**: AATGAGCCCAACACRTTCTC **Reverse**: CAATCAGCATCCTCTTCTTCC	287 bp	W
5	SLC1A1	**Forward**: GATGGCACAGCTCTCTACGA **Reverse**: GCTTCTCCACTATCCCAGTACC	292 bp	Z
6	RPS 14	**Forward:** GACYGGYGGCAYGAAGGYGAAGG **Reverse:** CACGGCGACCACCCYTYCYG	299 bp	13
6	WXHO1	**Forward:** CCCAAATATAACACGCTTCACT **Reverse**: GAAATGAATTATTTTCTGGCGAC	415 bp	W
7	RIBO	**Forward:** AGCTCTTTCTCGATTCCGTG **Reverse:** GGGTAGACACAAGCTGAGCC	256 bp	13
8	DAZL	**Forward:** TGTGGACAGGAGCATACAAACA **Reverse:** AAGTGATGCGCCCTCCTCT	114 bp	2
9	CVH	**Forward:** GGCGGGATTTAATGTCATGT **Reverse**: TGTGGTTCTTGCTGCTTTTG	127 bp	Z
10	STRA-8	**Forward:** CTGTGGTCTCCACGGCTATT **Reverse**: GAAACCAGCAGCAACATCAA	245 bp	1
11	SYCP-3	**Forward:** GAAGGTTTTTCAACAGGCAAG **Reverse**: TTGCGAAGTTCATTTTGTGC	144 bp	1
12	SDF-1	**Forward:** TCATCACCTTGCCATTCTGG **Reverse**: GCTGTTGGTGGCATGGACTA	170 bp	6
13	β2M	**Forward:** TGTAGACGGCTTCGCTGC **Reverse**: AGGAGTGTGTGCTAACCGTTAC	225 bp	10

Table 5.2: Differentially expressed genes in broiler female vs male significant in at least three independent samples. Genes were selected based on the cuff differences in the FPKM value, their function and chromosomal location.

Gene	cuffdiff_female_FPKM	cuffdiff_male_FPKM	cuffdiff_log2
DENND4C	8.59745	10.2237	0.249937
PIGY	15.3373	16.6283	0.116598
FOXN1	0.0106907	0.0756565	2.8231
LOC100859602	2.74104	0	0
LOC101749207	0.325418	0	0
GNE	36.3764	72.4802	0.994583
LOC100857280	0.0139395	0.067311	2.27167
LOC427353	0.0411904	0	0
KIAA0284	0.479938	1.50444	1.64831
SCNN1D	0.0863319	0.0915238	0.0842533
LOC101749077	21.5091	188.211	3.12933
PPTC7	16.062	17.9983	0.164203
LRRC58	2.46303	5.52924	1.16665
LOC427134	4.5484	0	0
MAP1B	0.33718	2.66265	2.98127
C2ORF18	61.3006	30.9555	-0.985704
LOC101750795	16.943	146.327	3.11043
GOLM1	3.15686	9.82919	1.63858
HMGCR	24.2183	41.9746	0.793419
LOC101748860	0.0232573	0.21088	3.18067
LOC101750188	0.0089244	0.109103	3.61179
LOC770556	0.0160854	0.477938	4.893
SLC1A1	4.37284	0.04377	-6.64249
GMCL1	14.1911	9.06415	-0.646744
STK11	21.9525	24.091	0.134109

REFERENCES

Adams, I. R., & McLaren, A. (2002). Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development*, *129*(5), 1155-1164.

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*(2), 401-415.

Brown, M. S., Goldstein, J. L., & Dietschy, J. M. (1979). Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat. Comparison with the rate of cholesterol synthesis in different physiological states. *Journal of Biological Chemistry*, *254*(12), 5144-5149.

Cao, D., Wu, H., Li, Q., Sun, Y., Liu, T., Fei, J., Zhou, Y., Wu. S., Hu, X., & Li, N. (2015). Expression of recombinant human lysozyme in egg whites of transgenic hens. *PloS one*, *10*(2), e0118626.

Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., & Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes & development*, *9*(13), 1586-1597.

Derouiche, A., & Rauen, T. (1995). Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: Evidence for coupling of GLAST and GS in transmitter clearance. *Journal of neuroscience research*, *42*(1), 131-143.

de la Luna, S., Allen, K. E., Mason, S. L., & La Thangue, N. B. (1999). Integration of a growth-suppressing BTB/POZ domain protein with the DP component of the E2F transcription factor. *The EMBO journal*, *18*(1), 212-228.

Eyal-Giladi, H., & Kochav, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick: I. General morphology. *Developmental biology*, *49*(2), 321-337.

Eyal-Giladi, H., Ginsburg, M., & Farbarov, A. (1981). Avian primordial germ cells are of epiblastic origin. *Development*, *65*(1), 139-147.

Fargeix, N., Didier, E., & Didier, P. (1981). Early sequential development in avian gonads. An ultrastructural study using selective glycogen labeling in the germ cells. *Reproduction Nutrition Development*, *21*(3), 479-496.

Fujimoto, T., Ninomiya, T., & Ukeshima, A. (1976). Observations of the primordial germ cells in blood samples from the chick embryo. *Developmental biology*, *49*(1), 278-282.

Golsdtein, J.L., & Brown, M.S. (1990). Regulation of the mevalonate pathway. *Nature*, 343 (6257), 425.

Gruidl, M. E., Smith, P. A., Kuznicki, K. A., McCrone, J. S., Kirchner, J., Roussell, D. L., Strome, S., & Bennett, K. L. (1996). Multiple potential germ-line helicases are components of the germ-line-specific P granules of Caenorhabditis elegans. *Proceedings of the National Academy of Sciences*, *93*(24), 13837-13842.

Hay, B., Jan, L. Y., & Jan, Y. N. (1990). Localization of vasa, a component of Drosophila polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development*, *109*(2), 425-433.

Han, J. Y., Lee, H. C., & Park, T. S. (2015). Germline-competent stem cell in avian species and its application. *Asian journal of andrology*, *17*(3), 421.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. *Annual review of genetics*, *30*(1), 405-439.

Jongens, T. A., Ackerman, L. D., Swedlow, J. R., Jan, L. Y., & Jan, Y. N. (1994). Germ cell-less encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of Drosophila. *Genes & Development*, *8*(18), 2123-2136.

Johnson, A. D., Crother, B., White, M. E., Patient, R., Bachvarova, R. F., Drum, M., & Masi, T. (2003). Regulative germ cell specification in axolotl embryos: a primitive trait conserved in the mammalian lineage. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *358*(1436), 1371-1379.

Johnson, A. D., Drum, M., Bachvarova, R. F., Masi, T., White, M. E., & Crother, B. I. (2003). Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution. *Evolution & Development*, *5*(4), 414-431.

Johnson, P. A. (2006). Use of genetic strains of chickens in studies of ovarian cancer. *Poultry Science*, *85*(2), 246-250.

Jung, J. G., Kim, D. K., Park, T. S., Lee, S. D., Lim, J. M., & Han, J. Y. (2005). Development of novel markers for the characterization of chicken primordial germ cells. *Stem Cells*, *23*(5), 689-698.

Kai, N., Iwase, K., Imai, K., Nakahira, E., Soma, M., Ohtsuka, S., Ygi, T., Kobayashi, K., Koga, H., Takiguchi, M., & Yuasa, S. (2006). Altered gene expression in the subdivisions of the amygdala of Fyn-deficient mice as revealed by laser capture microdissection and mKIAA cDNA array analysis. *Brain research*, *1073*, 60-70.

Kimura, T., Yomogida, K., Iwai, N., Kato, Y., & Nakano, T. (1999). Molecular cloning and genomic organization of mouse homologue of Drosophila germ cell-less and its expression in germ lineage cells. *Biochemical and biophysical research communications*, *262*(1), 223-230.

Kleiman, S. E., Yogev, L., Gal-Yam, E. N., Hauser, R., Gamzu, R., Botchan, A., Paz, G.,

Yavetz, H., Maymon, B.B.S., Schreiber, L., & Barzilai, S. (2003). Reduced Human Germ Cell-Less (HGCL) Expression in Azoospermic Men With Severe Germinal Cell Impairment. *Journal of andrology*, *24*(5), 670-675.

Kuwana, T. (1993). Migration of avian primordial germ cells toward the gonadal anlage. *Development, growth & differentiation*, *35*(3), 237-243.

Lavial, F., Acloque, H., Bachelard, E., Nieto, M. A., Samarut, J., & Pain, B. (2009). Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate. *Developmental biology*, *330*(1), 73-82.

Leatherman, J. L., Kaestner, K. H., & Jongens, T. A. (2000). Identification of a mouse germ cell-less homologue with conserved activity in Drosophila. *Mechanisms of development*, *92*(2), 145-153.

Li, B. C., Chen, G. H., Qin, J., Wu, X. S., Wu, S. L., & Cai, Z. T. (2005). Suitable Stages for Isolation and Culture PGCs from Chicken Embryos. *International Journal of Poultry Science*, *4*(11), 885-890.

Li, W., Deng, F., Wang, H., Zhen, Y., Xiang, F., Sui, Y., & Li, J. (2006). Germ cell-less expression in zebrafish embryos. *Development, growth & differentiation*, *48*(5), 333-338.

Lu, Y., West, F. D., Jordan, B. J., Jordan, E. T., West, R. C., Yu, P., & Beckstead, R. B. (2014). Induced pluripotency in chicken embryonic fibroblast results in a germ cell fate. *Stem cells and development*, *23*(15), 1755-1764.

Lian, C., Zuo, Q., Li, D., Zhang, L., Ahmed, M. F., Xiao, T., Tang, B., Wang, Y., Jin, K., Zhang, Y., & Li, B. (2015). Basing RNA-seq explored the regulatory mechanism of the carbohydrate metabolism pathways during chicken male germ cell differentiation. *In Vitro Cellular & Developmental Biology-Animal*, *51*(7), 690-696.

Macdonald, J., Glover, J. D., Taylor, L., Sang, H. M., & McGrew, M. J. (2010). Characterization and germline transmission of cultured avian primordial germ cells. *PLoS One*, *5*(11), e15518.

Matsui, Y., Zsebo, K., & Hogan, B. L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell*, *70*(5), 841-847.

McLaren, A. (2003). Primordial germ cells in the mouse. *Developmental biology*, *262*(1), 1-15.

McLaren, A., & Southee, D. (1997). Entry of mouse embryonic germ cells into meiosis. *Developmental biology*, *187*(1), 107-113.

Meyer, D. B. (1964). The migration of primordial germ cells in the chick embryo. *Developmental biology*, *10*(1), 154-190.

Mise, N., Fuchikami, T., Sugimoto, M., Kobayakawa, S., Ike, F., Ogawa, T., Tada, T., Kanava, S., Noce, T., & Abe, K. (2008). Differences and similarities in the developmental status of embryo-derived stem cells and primordial germ cells revealed by global expression profiling. *Genes to Cells*, *13*(8), 863-877.

Miyahara, D., Mori, T., Makino, R., Nakamura, Y., Oishi, I., Ono, T., Nirasawa, K., Tagami, T., & Kagami, H. (2014). Culture conditions for maintain propagation, long-term survival and germline transmission of chicken primordial germ cell-like cells. *The Journal of Poultry Science*, *51*(1), 87-95

Molyneaux, K., & Wylie, C. (2004). Primordial germ cell migration. *International Journal of Developmental Biology*, *48*(5-6), 537-543.

Naito, M., Harumi, T., & Kuwana, T. (2010). Long term in vitro culture of chicken primordial germ cells isolated from embryonic blood and incorporation into germline of recipient embryo. *The Journal of Poultry Science*, *47*(1), 57-64.

Naito, M., Matsubara, Y., Harumi, T., Tagami, T., Kagami, H., Sakurai, M., & Kuwana, T. (1999). Differentiation of donor primordial germ cells into functional gametes in the gonads of mixed-sex germline chimaeric chickens produced by transfer of primordial germ cells isolated from embryonic blood. *Journal of Reproduction and Fertility*, *117*(2), 291-298.

Niewkoop, P. D., & Sutasurya, L. A. (1979). Primordial germ cells in the chordates: Embryogenesis and Phylogenesis. *Cambridge University Press, pp118-123*.

Otori, Y., Shimada, S., Tanaka, K., Ishimoto, I., Tano, Y., & Tohyama, M. (1994). Marked increase in glutamate-aspartate transporter (GLAST/GluT-1) mRNA following transient retinal ischemia. *Molecular brain research*, *27*(2), 310-314.

Oulad-Abdelghani, M., Bouillet, P., Décimo, D., Gansmuller, A., Heyberger, S., Dollé, P., Bronner, S., Lutz, Y. & Chambon, P. (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *The Journal of cell biology*, *135*(2), 469-477.

Pain, B., Clark, M. E., Shen, M., Nakazawa, H., Sakurai, M., Samarut, J., & Etches, R. J. (1996). Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. *Development*, *122*(8), 2339-2348.

Palombella, V. J., Rando, O. J., Goldberg, A. L., & Maniatis, T. (1994). The ubiquitin proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell*, *78*(5), 773-785.

Park, T. S., & Han, J. Y. (2000). Derivation and characterization of pluripotent embryonic germ cells in chicken. *Molecular reproduction and development*, *56*(4), 475-482.

Park, T. S., Jeong, D. K., Kim, J. N., Song, G. H., Hong, Y. H., Lim, J. M., & Han, J. Y. (2003). Improved germline transmission in chicken chimeras produced by transplantation of gonadal primordial germ cells into recipient embryos. *Biology of Reproduction*, *68*(5), 1657-1662.

Park, T. S., Lee, H. G., Moon, J. K., Lee, H. J., Yoon, J. W., Yun, B. N. R., & Han, B. K. (2015). Deposition of bioactive human epidermal growth factor in the egg white of transgenic hens using an oviduct-specific mini synthetic promoter. *The FASEB Journal*, *29*(6), 2386-2396.

Petitte, J. N. (2006). Avian germplasm preservation: embryonic stem cells or primordial germ cells? *Poultry Science*, *85*(2), 237-242.

Pillai, R. S., & Chuma, S. (2012). piRNAs and their involvement in male germline development in mice. *Development, growth & differentiation*, *54*(1), 78-92.

Qi, Y. X., Liu, Y. B., & Rong, W. H. (2011). RNA-Seq and its applications: a new technology for transcriptomics. *Yi chuan= Hereditas*, *33*(11), 1191-1202.

Rauen, T. (2000). Diversity of glutamate transporter expression and function in the mammalian retina. *Amino Acids*, *19*(1), 53-62.

Robertson, S. E., Dockendorff, T. C., Leatherman, J. L., Faulkner, D. L., & Jongens, T. A. (1999). germ cell-less is required only during the establishment of the germ cell lineage of Drosophila and has activities which are dependent and independent of its localization to the nuclear envelope. *Developmental biology*, *215*(2), 288-297.

Santos, A. C., & Lehmann, R. (2004). Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Developmental cell*, *6*(2), 283-293.

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P.D., Huggins, G.R., & Gearhart, J. D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proceedings of the National Academy of Sciences*, *95*(23), 13726-13731.

Sultan, M., Schulz, M. H., Richard, H., Magen, A., Klingenhoff, A., Scherf, M., Seifert, M., Borodina, T., Soldatory, A., Parkhomchuk, D., & Schmidt, D. (2008). A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science*, 321(5891), 956-960.

Stebler, J., Spieler, D., Slanchev, K., Molyneaux, K. A., Richter, U., Cojocaru, V., Tarabykin, V., Wylie, C., Kessel, M., & Raz, E. (2004). Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Developmental biology*, *272*(2), 351-361.

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., Van Baren, M. J., Salzberg, S.L., Wold, B.J., & Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology*, *28*(5), 511-515.

Song, Y., Duraisamy, S., Ali, J., Kizhakkayil, J., Jacob, V. D., Mohammed, M. A., Eltiganj, M.A., Amisetty, S., Shukla, M.K., Etches, R.J. & de Lavoir, M. C. V. (2014). Characteristics of long-term cultures of avian primordial germ cells and gonocytes. *Biology of Reproduction*, *90*(1), 15-1.

Thorpe, J. L., Doitsidou, M., Ho, S. Y., Raz, E., & Farber, S. A. (2004). Germ cell migration in zebrafish is dependent on HMGCoA reductase activity and prenylation. *Developmental cell*, *6*(2), 295-302.

van de Lavoir, M. C., Mather-Love, C., Leighton, P., Diamond, J. H., Heyer, B. S., Roberts, R., Zhu, L., Winters-Digiacinto, P., Kerchner, A., Gessaro, T., & Swanberg, S. (2006). High-grade transgenic somatic chimeras from chicken embryonic stem cells. *Mechanisms of development*, *123*(1), 31-41.

Van Doren, M., Broihier, H. T., Moore, L. A., & Lehmann, R. (1998). HMG-CoA reductase guides migrating primordial germ cells. *Nature*, *396*(6710), 466.

Wentworth, B. C., Tsai, H., Hallett, J. H., Gonzales, D. S., & Rajcic-Spasojevic, G. (1989). Manipulation of avian primordial germ cells and gonadal differentiation. *Poultry Science*, *68*(7), 999-1010.

West, F. D., Machacek, D. W., Boyd, N. L., Pandiyan, K., Robbins, K. R., & Stice, S. L. (2008). Enrichment and Differentiation of Human Germ-Like Cells Mediated by Feeder Cells and Basic Fibroblast Growth Factor Signaling. *Stem cells*, *26*(11), 2768-2776.

Zamboni, L., & Upadhyay, S. (1983). Germ cell differentiation in mouse adrenal glands. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *228*(2), 173-193.

Zhu, L., Van de Lavoir, M. C., Albanese, J., Beenhouwer, D. O., Cardarelli, P. M., Cuison, S., Deshpande, D.S., Deng, D.F., Diamond, D.H., Green, L., & Halk, E. L. (2005). Production of human monoclonal antibody in eggs of chimeric chickens. *Nature biotechnology*, *23*(9), 1159.

CHAPTER 6

Discussion

The success of the broiler breeding industry is heavily dependent on male fertility (McGary *et al*., 2002). For successful copulation and fertilization, male broiler breeder must not only exhibit physiological and behavioral maturity to induce female sexual response but also possess high quality semen (Pollock, 1999; Kirby *et al*., 1998). The increasing demand for the chicken products both domestically and globally has led to intensive selection on production traits. Major focus has been on heritable growth performance traits such as increased feed conversion ratio, rapid gain in body weight, higher breast yield has generated high quality broilers with respect to meat production (Haverstein *et al*., 2003) but has negatively impacted reproduction traits leading to reduced fertility (McDaniel and Craig, 1959; Siegel, 1962). In the broiler breeding industry, the main emphasis has been given to semen traits to assess the fertilizing potential of males owing to the unreliability of selection based on bird physical attributes (Barabato, 1999; McGary *et al*., 2003). One of the variable that has been given weightage to determine semen quality is sperm mobility. Unlike reproductive traits, sperm mobility has a high heritability index (h^2=0.30) and has been reported to be the direct determinant of fertility in chicken (Froman *et al*., 2002; Froman and Rhoads 2013). High heritability allowed breeders to select chicken lines based on mobility phenotype, to identify the genes and gene networks influencing mobility trait in chicken (Froman *et al*., 1999). Studies conducted on the chicken lines differing in their mobility phenotype identified poor energy dynamics, defective mitochondria, and inability of motile sperm to effectively transit through sperm storage tubules (SSTs) in hen as some causes of poor mobility (Froman et al., 2011). Unlike mammalian species, where energy for sperm motility is mainly provided by glycolysis (Turner, 2003; Miki et al., 2004; Mukai and Okuno, 2004), avian sperm cells require both glycolysis and oxidative phosphorylation for optimum motility due to their internal long-term mode of fertilization (Jamieson, 2007; Blesbois, 2012; Nguyen, 2012).

The impact of mitochondrial function on sperm mobility has been documented in previous studies (Froman and Kirby, 2005). Higher proportions of immobile sperm and the presence of aberrant mitochondria in low mobile chicken lines suggested the involvement of genetic components in influencing the mobility trait. Previously, genome wide association studies (GWAS) conducted on the parental high and low line have identified multiple loci scattered all over the genome that showed significant association with the mobility phenotype (Froman and Rhoads, 2013). This study further highlighted the presence of quantitative trait loci (QTLs) for the mobility phenotype.

The influence of gonadal somatic cells in the survival and proliferation of germ cells is well known (Griswold, 1998; Mruk and Cheng, 2004). But the effect of Sertoli cells-germ cells interactions in influencing the phenotype of developing spermatozoa has not been probed. There are gaps in our knowledge regarding development biology of germ cells. Identification of gender specific gene expression in PGCs prior to their migration into the future gonads would contribute to understanding the mechanisms by which PGCs determine their target destination. Moreover, these genes could act as novel markers to identify PGCs based on their gender. The information would also help in probing genes involved directly or indirectly in gametogenesis. The collective information could throw some light on genes influencing the phenotype of male germ cells later in development.

We started our study with three objectives: to identify QTLs associated with low mobility phenotype in chicken by GWAS using moderate density 60k SNP chip assay, to demonstrate whether Sertoli cell-germ cell interactions can influence the mobility phenotype of developing spermatozoa by generating transgenic chicken and to identify gender specific PGCs markers using RNAseq and RT-qPCR analyses. To fulfill our first objective, we began analyzing high

and low mobile subpopulations within parental low line, double reciprocal cross between high line and low line (F_2), and progeny of F_2 population (F_3) by conducting GWAS using medium density 60k SNPchip. GWAS was utilized to analyze genomic regions and single nucleotide polymorphisms (SNPs) associated with the low mobility phenotypes. Identification of two regions on the chromosome Z at 32 Mbp and 63 Mbp indicated some association with the low mobility phenotype. TaqMan assays developed for the selected SNPs in these regions failed to show association of any genotype with the low mobility phenotype. Moreover, we observed shifts in the positions of significant associated loci on chromosome Z in the selected generations. This shifting of regions suggests the phenomenon of epistasis where multiple minor genes contributing to the phenotype (Carlborg and Haley, 2004).

To address our second objective, we used chicken primordial germ cells as a tool for germ line chimera generation. The logic was if PGCs of low line genotype could show high mobility phenotype after traversing through the reproductive tract of high mobile chickens, that would mean that the mobility trait is influenced not only by the genotype of the sperm, but also its interaction with the surrounding environment. Conversely, if the low line PGCs retained the low mobile phenotype then the mobility trait is influenced just by the genotype of the spermatozoa. Isolated low line PGCs were injected into busulfan treated White Leghorn embryos and male chicks were raised until sexual maturity. Semen of the recipient males was tested for the presence of donor derived sperms. In the current study, we could not detect donor genotype in the recipient's semen. Due to the absence of donor derived sperms in the recipient's semen, we didn't perform sperm mobility assays on the recipient's semen to detect changes in the phenotype of donor derived sperms. Absence of donor derived sperms could be either due to inefficient depletion of endogenous PGCs that didn't allow donor PGCs to repopulate the

recipient gonads or insufficient number of injected PGCs to begin with. Although cultured PGCs used in the study exhibited the germ line specificity and pluripotent characteristics, the absence of donor derived sperm in the recipient semen depicts the complexity of the experiment and dexterity required in transgenic studies.

To address our third objective, we conducted high throughput RNAseq analyses on male and female cPGCs. Based on our RNAseq data we selected HMGCR, SWIM, GCL, SLC1A1 and UBE2R2L genes for further analyzing their relative expression pattern in PGCs using qPCR. HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) is a critical enzyme involved in cholesterol biosynthesis (Brown *et al*., 1979; Goldstein and Brown, 1990). With respect to development biology, HMGCR was reported in *Drosophila* and zebrafish to be indispensable for PGCs migration to future gonads (Brand *et al*., 1993; Van Doren *et al*., 1998; Santos and Lehmann, 2004; Thorpe *et al*., 2004). In vertebrates PGC migration has been hypothesized to be influenced by lipid intermediates synthesized by HMGCR (Molyneaux and Wylie, 2004). In our study, we found HMGCR expression in both male and female PGCs. The molecular mechanisms controlling PGCs migration in chicken are not clear (Nakamura *et al*., 2007). Presence of HMGCR in the transcriptomes of both male and female PGCs signifies its probable involvement in passive migration of PGCs towards their respective gonads. The other gene that has been reported to be crucial for PGCs development and evolutionarily conserved in many species is GCL (Germ cell less) (Robertson et al., 1999; Li *et al*., 2006; Maekawa *et al.* 2004; Leatherman *et al*., 2000). While in invertebrates, expression of GCL has been reported at the earliest stages of PGC development, in vertebrates GCL expression has been documented only in males and later in the process of spermatogenesis (Kleiman *et al*., 2003). In chicken, there is not enough information on the definitive role of GCL in PGCs development or in spermatogenesis. Our

study found cGCL expression only in male PGCs. The male specific expression of cGCL signifies its probable role in the early development of male germ cells in chicken and makes it a novel marker for gender specific PGCs identification in chicken. Similarly, UBE2R2L (Ubiquitin conjugating enzyme E2 R2 like) was expressed only in female PGCs. Since, this gene is located in the female-specific sex chromosome W, UBE2R2L can be used as a marker to differentiate PGCs based on their gender at an early developmental stage. Solute carrier family 1 member 1 (SLC1A1), is a member of neuronal/epithelial high affinity glutamate transporters that are usually present in the cell membrane of neurons and glial cells (Otori *et al.,* 1994; Derouiche & Rauen, 1995; Rauen, 2000). We found SLC1A1 expression in the chicken PGCs and the expression was significantly higher in females as compared to males. This was an interesting observation since expression of glutamate transporters have been reported only in neurons, glial, and retinal cells, so far. The presence of SLC1A1 expression in female PGCs and later in the ovaries suggest a unique role of these transporters in gender specific differentiation of germ cells in chicken. SLC1A1 can be used as a marker to differentiate female PGCs from male in chicken. The last gene that we tested was zinc finger SWIM-type containing 6 transcription factor. Even though it showed significant difference in RNA-Seq study but we couldn't find any significant difference in the gender specific expression.

Our research was designed to identify genes and gene networks affecting mobility trait in chicken and to identify genes influencing male germ cells biology at PGCs level. Our findings suggest the probable involvement of multiple genes and loci in influencing the low mobility phenotype in chicken and revealed the inadequacy of medium density SNPchip assays in identifying QTLs involving minor genes. We also identified important genes that have been previously reported to play crucial roles in PGC survival, migration, and gametogenesis. These

findings further our knowledge of germ cells migration and development in chicken. This information can be used in developing novel markers to identify gender-specific PGCs which will be useful in transgenic studies involving transfer of male PGCs. In future, the combined approach of next generation sequencing technology and improved transgenic techniques will answer the questions pertaining to the mobility phenotype in chicken.

REFERENCES

Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y., & Nagasawa, T. (2003). Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proceedings of the National Academy of Sciences*, *100*(9), 5319-5323.

Barbato, G. F. (1999). Genetic relationships between selection for growth and reproductive effectiveness. *Poultry Science*, *78*(3), 444-452.

Blesbois, E. (2012). Biological features of the avian male gamete and their application to biotechnology of conservation. *The Journal of Poultry Science*, *49*(3), 141-149.

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *118*(2), 401-415.

Brown, M. S., Goldstein, J. L., & Dietschy, J. M. (1979). Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat: Comparison with the rate of cholesterol synthesis in different physiological states. *Journal of Biological Chemistry*, *254*(12), 5144-5149.

Carlborg, Ö., & Haley, C. S. (2004). Epistasis: too often neglected in complex trait studies? *Nature reviews. Genetics*, *5*(8), 618.

Cheng, C. Y., & Mruk, D. D. (2002). Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. *Physiological reviews*, *82*(4), 825-874.

Choi, M., Scholl, U. I., Ji, W., Liu, T., Tikhonova, I. R., Zumbo, P., Navir, A., Bakkaloglu, A., Ozen, S., Sanjad, S., & Nelson-Williams, C. (2009). Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences*, *106*(45), 19096-19101.

Derouiche, A., & Rauen, T. (1995). Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: Evidence for coupling of GLAST and GS in transmitter clearance. *Journal of neuroscience research*, *42*(1), 131-143.

Farini, D., Scaldaferri, M. L., Iona, S., La Sala, G., & De Felici, M. (2005). Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells. *Developmental biology*, *285*(1), 49-56.

Froman, D. P., & Feltmann, A. J. (1998). Sperm mobility: a quantitative trait of the domestic fowl (Gallus domesticus). *Biology of Reproduction*, *58*(2), 379-384.

Golsdtein, J.L., & Brown, M.S. (1990). Regulation of the mevalonate pathway. *Nature*,

343(6257), 425.

Griswold, M. D. (1998, August). The central role of Sertoli cells in spermatogenesis. *Seminars in cell & developmental biology*, 9(4), 411-416.

Havenstein, G. B., Ferket, P. R., & Qureshi, M. A. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poultry Science*, *82*(10), 1500-1508.

Jamieson, B. G. M. (2007). *Reproductive biology and phylogeny of birds*. Science Publishers.

Kirby, J. D., Tressler, C. J., & Kirby, Y. K. (1998). Evaluation of the duration of sperm fertilizing ability in five lines of commercial broiler breeder and Delaware cross males. *Poultry Science*, *77*(11), 1688-1694.

Kleiman, S. E., Yogev, L., Gal-Yam, E. N., Hauser, R., Gamzu, R., Botchan, A., Paz, G., Yavetz, H., Maymon, B.B.S., Schreiber, L., & Barzilai, S. (2003). Reduced Human Germ Cell-Less (HGCL) Expression in Azoospermic Men With Severe Germinal Cell Impairment. *Journal of andrology*, *24*(5), 670-675.

Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., McCarthy, M. I., Ramos, E. M., Cardon, L. R., Chakravarti, A., & Cho, J. H. (2009). Finding the missing heritability of complex diseases. *Nature*, *461*(7265), 747.

McDaniel, G. R., & Craig, J. V. (1962). Predicting Male Fertilizing Capacity in High and Low Fertility Strains of Chickens. *Poultry Science*, *41*(3), 866-869.

McGary, S., Estevez, I., & Russek-Cohen, E. (2003). Reproductive and aggressive behavior in male broiler breeders with varying fertility levels. *Applied Animal Behaviour Science*, *82*(1), 29-44.

McGary, S., Estevez, I., Bakst, M. R., & Pollock, D. L. (2002). Phenotypic traits as reliable indicators of fertility in male broiler breeders. *Poultry Science*, *81*(1), 102-111.

Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M., & O'Brien, D. A. (2004). Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16501-16506.

Molyneaux, K., & Wylie, C. (2004). Primordial germ cell migration. *International Journal of Developmental Biology*, *48*(5-6), 537-543.

Mruk, D. D., & Cheng, C. Y. (2004). Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocrine reviews*, *25*(5), 747-806.

Mukai, C., & Okuno, M. (2004). Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biology of Reproduction*, *71*(2), 540-547.

Nakamura, Y., Yamamoto, Y., Usui, F., Mushika, T., Ono, T., Setioko, A. R., Takeda, K., Nirasawa, K., Kagami, H. & Tagami, T. (2007). Migration and proliferation of primordial germ cells in the early chicken embryo. *Poultry Science*, *86*(10), 2182-2193.

Nguyen, T. M. D., Alves, S., Grasseau, I., Métayer-Coustard, S., Praud, C., Froment, P., & Blesbois, E. (2014). Central role of 5′-AMP-activated protein kinase in chicken sperm functions. *Biology of Reproduction*, *91*(5), 121-1.

Okutsu, T., Yano, A., Nagasawa, K., Shikina, S., Kobayashi, T., Takeuchi, Y., & Yoshizaki, G. (2006). Manipulation of fish germ cell: visualization, cryopreservation and transplantation. *Journal of Reproduction and Development*, *52*(6), 685-693.

Otori, Y., Shimada, S., Tanaka, K., Ishimoto, I., Tano, Y., & Tohyama, M. (1994). Marked increase in glutamate-aspartate transporter (GLAST/GluT-1) mRNA following transient retinal ischemia. *Molecular brain research*, *27*(2), 310-314.

Park, T. S., & Han, J. Y. (2012). piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens. *Proceedings of the National Academy of Sciences*, *109*(24), 9337-9341.

Petitte, J. N., Clark, M. E., Liu, G., Gibbins, A. V., & Etches, R. J. (1990). Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. *Development*, *108*(1), 185-189.

Pollock, D. L. (1999). A geneticist's perspective from within a broiler primary breeder company. *Poultry Science*, *78*(3), 414-418.

Rauen, T. (2000). Diversity of glutamate transporter expression and function in the mammalian retina. *Amino Acids*, *19*(1), 53-62.

Sang, H. (2004). Prospects for transgenesis in the chick. *Mechanisms of development*, *121*(9), 1179-1186.

Santos, A. C., & Lehmann, R. (2004). Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Developmental cell*, *6*(2), 283-293.

Siegel, P. B. (1962). A double selection experiment for body weight and breast angle at eight weeks of age in chickens. *Genetics*, *47*(10), 1313.

Tajima, A., Naito, M., Yasuda, Y., & Kuwana, T. (1993). Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus).

Theriogenology, 40(3), 509-519.

Thorpe, J. L., Doitsidou, M., Ho, S. Y., Raz, E., & Farber, S. A. (2004). Germ cell migration in zebrafish is dependent on HMGCoA reductase activity and prenylation. *Developmental cell*, *6*(2), 295-302.

Turner, R. M. (2003). Tales from the tail: what do we really know about sperm motility? *Journal of andrology*, *24*(6), 790-803.

Van de Lavoir, M. C., Diamond, J. H., Leighton, P. A., Mather-Love, C., Heyer, B. S., Bradshaw, R., Kerchner, A., Hooi, L.T., Gessaro, T. M., Swanberg, S. E., & Delany, M. E. (2006). Germline transmission of genetically modified primordial germ cells. *Nature*, *441*(7094), 766.

Van Doren, M., Broihier, H. T., Moore, L. A., & Lehmann, R. (1998). HMG-CoA reductase guides migrating primordial germ cells. *Nature*, *396*(6710), 466.

CHAPTER 7

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Douglas Rhoads

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: May 8, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **May 6, 2014**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13043 - "Primordial Germ Cell Transfer for Sperm Mobility Phenotype"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **05-06-2014** you may request an extension [via the Modification Request form] up to 05-02-2016. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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