THE LECTIN BINDING PROFILES OF CHICKEN PRIMORDIAL GERM CELLS

By

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(Under the Direction of Steven L. Stice)

ABSTRACT

The manipulation of PGCs has the potential to be a powerful method in creating an efficient system of transgenesis in birds. Here, we characterize nonadherent PGCs cultured long term *in vitro* that retain the ability to migrate to the gonad. To characterize, several lectins including STL, DBA, ConA, WGA, MAA, SBA, PNA, and RCA were used. Two lines of cPGCs and a line of CEFs were analyzed, one PGC line expressing a mixed population of the chemokine receptor CXCR4 and another negative for CXCR4. The cells were stained to determine if a lectin binding profile could predict migratory potential. Both lines of PGCs were positive for STL, ConA, WGA, MAA, and RCA while negative for DBA, SBA, and PNA. CEFs were positive for STL, ConA, WGA, and MAA and negative for DBA, SBA, PNA, and RCA. These results indicate that RCA could potentially be a marker for migration in PGCs.

INDEX WORDS: Primordial germ cells (PGCs), Avian, Chicken, Lectin, Glycans, Cell Surface

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B.S., University of Georgia, 2011

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

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December 2013

DEDICATION

To my dad who has encouraged me on this crazy journey every step of the way, sympathetically listened to countless desperate phone calls, then unsympathetically reminded me of how much harder it really was to get a PhD thirty years ago. Love you!

ACKNOWLEDGMENTS

I would like to acknowledge my major professor Dr. Steve Stice for the encouragement and support he provided along the way. I have grown so much as a scientist in the past two years and have felt so fortunate to have had the opportunity to learn from someone who has accomplished so much.

I would also like to express my gratitude to my fellow graduate students from the Stice and West lab. Xian, Amalia, Raymond, Jessica, Emily, and Holly all have kept me sane over the past two and a half years. From offering advice when another experiment went wrong to being a shoulder to (literally) cry on I wouldn't have made it without their support.

Also, to Erin who deserves a paragraph all her own. I'm not sure the lab would last longer than one week without all of the behind the scenes work she does for all of us. She always has a smile on her face as she answers yet another one of my questions and has listened sympathetically when something has gone wrong or I was at my limit at the end of a bad day. I'm so grateful to have had such a competent and kind lab coordinator working here during my time here.

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Chapter 1

Literature Review:

Better Understanding of Germ Cell Migration During Development Could Lead to More Efficient Systems for Generating Transgenic Animals

Introduction

Since the advent of the twentieth century, the chick embryo has been a popular model for the study of developmental biology. Before the avian embryo was established, sea urchins and frog embryos were the most studied developmental models due to their relative availability and the robust nature of the embryos [1]. The avian model became another attractive alternative for the study of development due to the fact that it is not only easily available but also robust This model also develops similarly to mammals but can develop *ex vivo*, facilitating embryo manipulations and clearer investigations into understanding cellular interactions during embryonic development [2]. As technology advanced throughout the twentieth century, so did the potential uses for the chick embryo. It has served as a screening platform for potential pharmaceuticals [3] teratogens [4], and as a model for gaining insight into metastatic cancers and tumorigenesis [5, 6]. Furthermore, the ability to manipulate genes or create transgenic chickens has long attracted researchers interested in increasing egg and meat production.

Early Chick Gonadal Development

Using the pre-primitive streak staging system of Eyal-Giladi and Kochav for chick embryonic development stages, the chick embryo begins to exist outside of the hen at Stage X, immediately after laying [7]. At Stage X, the chick embryo consists of a single layer of cells, the blastoderm, made up of two separate regions, the area pellucida and area opaca. The area pellucida will give rise to the embryo while the area opaca develops into the extra-embryonic ectoderm. [8]. It is as early as Stage X that primordial germ cells (PGCs) and their precursors begin to form in the central zone of the area pellucida which is located on the ventral surface of the epiblast [9].

PGCs are the predecessors of spermatozoa and ova and were first observed in chicken in 1914 by Swift [10]. They are characterized by their relatively large size (approximately 16 micra in diameter), especially when compared to neighboring cells. This is due in large part to an increase in cytoplasm and nuclei size. They are almost exclusively round or oval shaped, making them distinguishable from other cell types. In addition to large nuclei, PGCs contain prominent vacuoles containing lipids as well as cytoplasm rich in glycogen particles [11]. In chicken, these cells express various germ cell specific proteins and are often characterized by CVH, chicken vasa homologue, and CDH, chicken dead end homologue [12, 13]. Both are RNA processing proteins that are necessary for the survival of germ cells and specification [14, 15]. While the exact role CVH plays in chicken has yet to be elucidated, VASA plays an extremely important role in the development of PGCs in

other developmental models. First discovered in *Drosophila*, the VASA protein is a DEAD-box RNA helicase and regulates mRNAs, including *Nanos* [16]. In the mouse, mouse vasa homolog (MVH) is known to be essential for the processing of mRNA and piRNA in germ cells [15]. PGCs are also known to express many of the same pluripotency factors as embryonic stem cells (ESCs), including *Oct3/4, Nanog,* and *Sox2* [17].

After colonizing in the anterior area of the epiblast, the PGCs gradually move to the extra-embryonic hypoblast before migrating to the germinal crescent region [18] (Figure 1.1). In their 1979 textbook, *Primordial Germ Cells in the Chordates,* Nieuwkoop and Sutasurya originally hypothesized that PGCs originated in the hypoblast [19,20] but later studies using chimeras of quail hypoblasts and the chicken epiblast determined that avian PGCs are in fact of epiblast origins [21].

The germinal crescent is localized along the border between the area opaca and area pellucida and lies above the embryonic disc. The gathering of PGCs into the germinal crescent begins well before gonadal development and the movement from the germinal crescent to the gonads occurs in two phases. The first phase is a passive migration that relies on the circulating blood and vasculature system to invade the embryonic tissue [22]. Using Hamburger & Hamilton's embryonic staging system [23], PGCs begin to migrate from the germinal crescent in the hypoblast and begin appearing in the blood vessels of the extra-embryonic mesoderm during Stage 11 (40 hours post laying) [24]. The extra-embryonic vascular system transports the PGCs from the extra-embryonic tissue into the intra-embryonic system vasculature using the vitelline veins [25]. By Stage HH15, PGCs begin to leave the intra-

embryonic vasculature system and start settling near the germinal epithelium [26]. This begins the second, active phase of PGC migration to the gonadal anlage where they will colonize before they being penetrating the gonad at approximately day 2.5 of incubation [10].

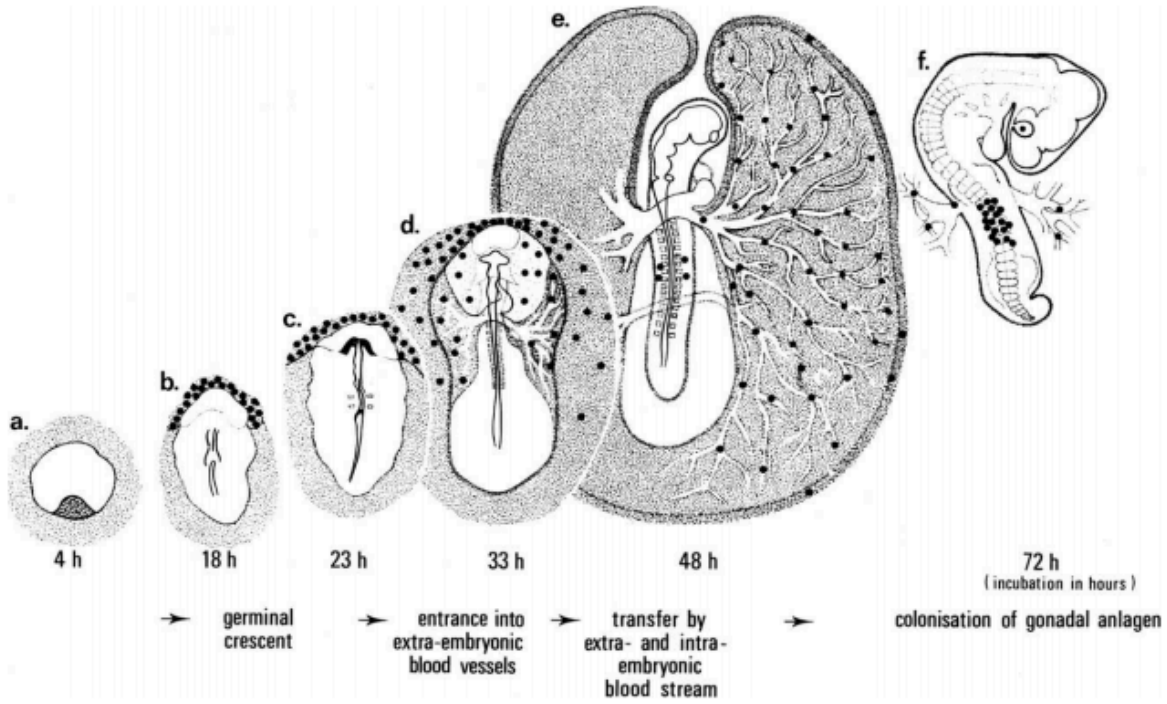

Figure 1.1 Migration of PGCs in the chick embryo [27]
The journey of the PGC as it begins. From unidentifiable at 4 h before the formation of the primitive streak to aggregation along the anterior area of the germinal crescent. Then integration into extra-embryonic blood vessels and circulation of the PGCs through extra-embryonic vasculature to intra-embryonic vasculature before colonizing outside of the gonadal ridge.

PGC migration is less understood in the chick embryo than in other developmental biology models (i.e. mouse, xenopus, zebrafish) but is considered to be reliant upon transmembrane receptors that receive external chemoattractant signals. These signals are then translated to cytoskeletal changes by effector molecules such as phospholipids and small GTPases [28]. Results from electron

microscopy studies have proven that communication from surrounding somatic cells is necessary for a successful migration to the gonadal anlage [26].

There is evidence that migration is regulated by a structurally similar family of chemoattractant cytokines, called chemokines, specifically the stromal cell-derived factor-1a (SDF-1a) and its receptor CXCR4 [29]. SDF-1 not only provides PGCs with directional cues, guiding the cells to their directional targets, but it also serves a regulatory role in controlling PGC proliferation and differentiation[29]. mRNA of SDF-1a is expressed in locations where PGCs often migrate and in tissues where it is thought that they migrate towards once they leave the vasculature in chick embryos [29]. This suggests that SDF-1a is necessary for the execution of the final steps of migration for chick PGCs [30]. *Ara et al.* furthered these observations by creating SDF-$1^{-/-}$ and $CXCR4^{-/-}$mutant mice then studying the involvement of SDF-1 in the colonization of the gonads during development [31]. Their results found that, while some PGCs can still migrate to the genital ridge, this migration was seriously delayed in SDF-$1^{-/-}$ mice and the number of PGCs within the gonad was diminished compared to the control. These results suggest that SDF-1 serves an important role in promoting PGC migration and the homing of PGCs to the gonadal anlage.

Also, transmission electron microscopy studies of the chick, mouse, and xenopus embryo have found the presence of another glycoprotein, fibronectin, in the presence of the tissues where PGCs migrate before they begin their migration [32-34]. The role of fibronectin in the regulation of PGC migration is not yet fully

understood but it is believed to have an adhesion effect on PGCs [35] that increases the rate of migration.

There is an important need for more information as to how PGCs migrate to the genital ridge. However, little is also known on how PGCs actually penetrate the developing gonad. A current hypothesis is that the gonadal anlage attracts PGCs using cell signaling factor [36]. *In vitro* studies have determined that isolated PGCs reinjected into the embryo will migrate to the gonadal anlage instead of other embryonic tissues and that this attractive effect is strongest at Stage 13, suggesting a temporal release of a cell signaling factor during the PGC migration phase [37]. Furthermore, when mouse dorsal mesentery tissue containing PGCs was transplanted into the coelomic cavity of chick embryos, migration of the mouse PGCs towards the chick gonadal anlage was observed. This suggests that the mechanism of attracting PGCs towards the anlage is conserved across species [38].

Chapter 2:

Methods to Generate Transgenic Animals

Established Methods of Generating Avian Chimeras

The use of transgenic technology to create transgenic birds has been a tantalizing idea for poultry scientists and developmental biologists alike. There are many gains that could be achieved through implementing transgenic technology in creating avian chimeras, however, and many sectors of private industry are interested in furthering the research. The potential uses for transgenic chickens are varied and numerous, ranging from serving as bioreactors for protein-based drugs [39] to the generation of monoclonal antibodies [40]. There are a number of methods established to create avian chimeras but no method has yet to stand out as more efficient. So far attempts to achieve transgenesis have concentrated on three specific approaches: direct DNA injection into the blastocyst, culture of chick ESCs or PGCs, and the use of viral vectors to deliver DNA [41]. All methods have successfully generated transgenic birds but at a less than desirable efficiency. However, as transgenic technology in other species evolves, new methods have begun to emerge.

Currently there are significant limitations preventing transgenic technology from becoming popular in many avian labs, primarily due to the technical difficulties and low efficiency in generating transgenic birds. The inability to establish a stable line of avian pluripotent cells has stagnated research attempting to manipulate the

avian genome [42]. While the chick embryo has definite advantages over the mammalian system, embryos are easy to manipulate, are relatively low cost to obtain, and initial transgenic specimen can be bred rapidly with gestation lasting only twenty-one days [43]. There are also a number of limitations caused by the chick embryo preventing progress. For example, it is technically difficult to utilize transgenic technology requiring embryos near the time of fertilization due to the 24-hour incubation period of the embryo within the hen. After the four-hour journey of the ovum from the infundibulum to the shell gland, the developing egg requires another approximately 20 hours to form the eggshell. During this time the ovum undergoes many cleavage divisions, with the embryo containing between 50,000 and 60,000 cells upon laying [44]. Another technical difficulty involves poor hatchability of genetically manipulated eggs. Generally a window is cut into the eggshell then sealed with an adhesive after manipulation [45]. However, this method led to very low rates of hatchability. Improvements involving washing the shell membrane with phosphate buffered saline (PBS) before cutting into the membrane then sealing with Duco's cement have increased hatchability but optimization of the protocol is still needed [46]. These complications significantly impede delivering transgenes to the avian embryo compared to a mammalian embryo. Despite these complications, many labs have been successful in creating transgenic birds using a number of different methods and, as transgenic technology continues to grow, so will the prospects for avian transgenesis.

Introduction of Replication-Defective Vectors into the Blastocyst

The technology to create transgenic chick embryos has actually been prevalent since the late 1980's when the Salter lab injected virus near the blastodermal embryo in newly laid eggs [47]. The retroviral transgenic technology had already been established in the mouse model, making it an attractive option for other species [48]. Additionally, there are several avian retroviruses that have been thoroughly studied therefore the background knowledge needed to modify the virus for transduction was established. *Salter et al.* furthered the technology by using their established method to generate transgenic birds with replication-competent vectors derived from avian leucosis virus (ALV) in 1993. *Bosselman et al.* and *Thoraval et al.* were able to generate transgenic birds using the replication defective vectors, reticuloendotheliosis virus (REV) and ALV, respectively [49, 50].

Retroviruses come from the family *Retroviridae*, a family of enveloped, single-stranded RNA viruses that implement a DNA intermediate to replicate [51]. The replication cycle of a retrovirus begins as the virus attaches to the cell surface using an interaction between its envelope surface protein and a specific transmembrane protein located on the cell surface. Once connected, the virus releases its core into the host cytoplasm where the RNA genome becomes uncoated and reverse transcribed into double strand DNA. The transcribed viral DNA then enters the nucleus and integrates into the host chromosome. With the exception of lentivirus, the *Retroviridae* family requires a disassembly of the nuclear membrane before retroviral DNA can enter the host nucleus. After transcription, viral proteins are translated from unspliced or spliced transcripts and the viral proteins, coupled

with a portion of full-length transcripts, are transported to the cell surface where virus particles are assembled and released from the host cell to infect other cells [51].

There are two types of retroviral vectors: replication-competent and replication-deficient. Replication-competent vectors can carry out all the functions of the normal virus life cycle while also encoding an additional transcript of the transgene of interest. This means that the virus can continue to replicate and spread to new cells after the initial infection [52]. This is advantageous as widespread infection can be achieved with a minimal number of original cells. However, this vector type also has a limited level of technical control due to a lack of control over the exact time point and location of the viral infection occurring. Furthermore, this type of vector is limited in the ability of cargo load is able to carry with a maximum insert size at approximately 2.0-2.4 kb [52]. For these reasons, replication-defective vectors are often the vector of choice for infection. Another concern in using replication-competent vectors is the possibility that the proliferation of the virus could potentially activate harmful genes [53]. Replication-defective vectors are only capable of infecting its initial host cell and the host cell's subsequent progeny [54] due to the replacement of the essential viral genes such as gag, pol, and env with transgenes. Since viral particle formation requires the proteins encoded by gag, pol, and env, the virus can only infect its original host [55]. This creates a more spatially and temporally defined transduction system for expression of exogenous genes. Replication-defective vectors also can accommodate much larger insert than

replication-competent vectors, allowing for a reporter system to be implemented to test efficacy of transduction [56].

Currently, the list of transgenes inserted into the chick genome by retrovirus is limited to: GFP, β-galactosidase, β-lactamase, interferon-α2b, interferon-β, erythropoietin, granulocyte-colony stimulating factor, parathormone, and single-chain antibody fused to Fc [57-64] and the efficiency varied considerably with the highest frequency producing a substantial 45% transgenic birds [58]. One factor that is considerably holding back efficient transfection is gene silencing due primarily to DNA methylation. DNA methylation is a normal process for the differentiation of cells and functional development of organ systems and has been studied extensively in mammalian models [65, 66]. However, information on methylation patterns of the chicken are just now emerging and suggests that methylation patterns do change dramatically as the chick matures, similar to what is observed in the mouse model [67]. Integrated transgenes are not excluded from risk of DNA methylation and this can significantly reduce the expression of the transgene of interest. Varying the time of transfection has abated this problem. *Kamirhira et al.* found that infection at day 2.5 can circumvent the DNA methylation that might silence gene expression [62]. However, this does not account for gene silencing that might occur as the chick approaches sexual maturity.

Genetic Modification of Embryonic Stem Cells

Chick embryonic stem cells (cESCs) were first isolated and *in vitro* cultured in 1996 [68]. Chick Stage X embryos were isolated and dissociated into a single cell

culture. These cells expressed alkaline phosphatase activity, contained a high telomerase activity, and, upon the removal of LIF, cells seemed to spontaneously from embryoid body-like structures containing cells representative of all three germ layers. Furthermore, these cells gave rise to chimeric chicks upon injection into recipient embryos.

Early blastodermal cells have proven capable of transfection by a variety of systems. Most notably, through the use of liposomes [69], electroporation [70], and viral vectors [47] but efficiency of integration remains a complication. And, in 2005, a cES cell line was used to generate a chick chimera [71]. A line of male cESCs was transfected to drive tissue-specific (specifically oviduct tissue) expression of human antibody (mAb) and injected into Stage X embryos. Sixty-nine percent of surviving embryos were chimeric. Unfortunately, there were no chimeric offspring produced from the original chimeras. To date, germ-line transmission of a transgene has yet to be observed when using cESCs.

Genetic Modification of Primordial Germ Cells

Due to the unique nature of the migration and development of PGCs in avian species, it is relatively easy to isolate and culture germ cells. For these reasons, achieving transgenesis by modifying PGCs is an appealing option (Figure 2.1). PGCs can be isolated from the bloodstream using a number of different strategies. The first established method of isolating PGCs used density-gradient centrifugation using either Ficol or Nycodenz. To collect cPGCs, blood samples were collected from the vitelline artery, heart, and terminal sinus from 2 day old chicks and PGCs were

separated from blood cells using either Ficoll density centrifugation or Nycodenz density centrifugation. Once isolated, these cells were injected into Stage 15 quail embryos. The quail embryos not only survived the injections but also allowed for the integration of the cPGCs into the gonad [72, 73]. Another strategy uses the cell surface marker, SSEA-1, for cell sorting, utilizing either an anti-SSEA-1 antibody for fluorescence activated cell sorting (FACS) [74] or magnet activated cell sorting (MACS) [75].

To generate genetically modified PGCs, viral methods are often utilized with retroviral and lentiviral reprogramming being the most popular. *Vick et al.* was the first lab to generate a transgenic animal using this method [76]. Using replication-defective vectors from avian leucosis virus or spleen necrosis virus, germinal crescent cells were infected. These cells were injected into recipient embryos and generated chimeric birds. *Motono et al.* infected chick embryos with a replication-defective lentiviral plasmid containing EGFP driven by a chicken β-actin promoter. After transfection, PGCs were FACS sorted and approximately 30% of PGCs expressed EGFP [77]. Once isolated, the EGFP-expressing PGCs were injected into the bloodstream of either 2.5 or 5.5 day old chick embryos. Ten percent of the injected PGCs migrated to the gonads and 3-6.6% of progeny chickens expressed EGFP.

Recently, the piggyBac transposon system was used to genetically modify chick PGCs. PiggyBac transgenesis has been established as an efficient vector capable of carrying relatively large transgenes in mice [78] and is now being used across many species in the attempt to knock in genes of interest. To produce chick

germline chimeras, a piggyBac GFP plasmid was inserted into cPGCs and GFP-expressing PGCs were transplanted into recipient embryonic gonads. As the six recipient roosters entered into sexual maturity they were mated with wild type hens. All six roosters produced hybrid progeny derived from the donor PGCs and germline transmission of the donor PGCs ranged from 90.4-98.9% [79]. This is an exciting finding as the transposon system appears to yield transgenic birds at an impressive efficiency and is considered safer than a viral vector system.

While viral transfection enables an efficient and consistent introduction of transgenes, there are some considerable concerns that prevent viral transfection from being used in the production of transgenic birds. The risk of introduced viral vectors replicating with wild-type viruses cannot be completely eliminated. Also, viral vectors can carry only a limited cargo of transgene size.

Obviously, there is still much to uncover in understanding how PGCs communicate between each other and the environment around them. Better understanding how PGCs migrate *in vivo* could greatly enhance how we culture and manipulate these cells for the purpose of transgenesis. There are considerable opportunities that remain to potentially increase the efficacy of germline chimerism. Many of these opportunities lie in better understanding the signaling pathways involved in the migration of chick PGCs from the germinal crescent to the genital ridge. One obvious benefit would be to uncover factors that could increase longevity of PGCs in an *in vitro* system. Very few labs have been able to culture PGCs *in vitro* for a prolonged period of time while also maintaining the plasticity that is necessary for these cells to migrate and integrate into the gonad[27]. Furthermore, elucidating

the factors that influence cell migration could increase the efficacy of migration of exogenous PGCs when injected into the chick embryo. Creating a more comprehensive understanding of PGC communication and migration can only enhance the potential of using PGCs to successfully derive transgenic birds.

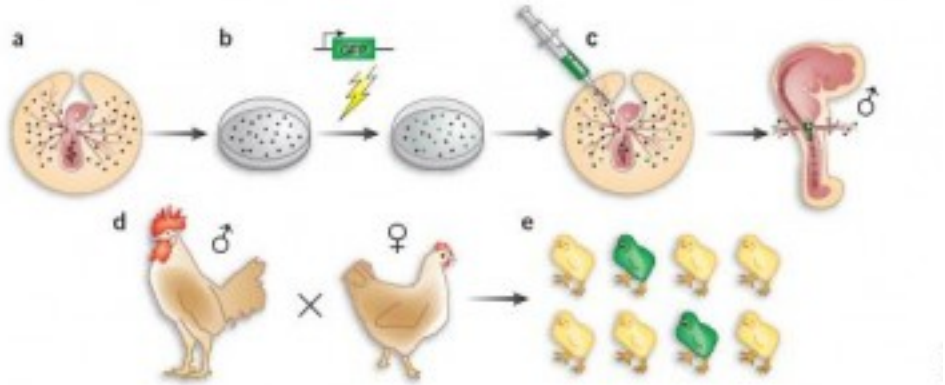

Figure 2.1 Using PGCs to Derive Transgenic Chickens [80]
First isolate PGCs from vasculature (a), transduce with gene of interest (b), inject into embryo (c), let transgenic embryo develop to hatching then sexual maturity and mate with wildtype hen (d), will produce transgenic progeny (e)

Non-viral Transfection

There are a few established non-viral transfection methods that create a safer system of to produce transgenic birds. Electoporation can be used to induce transient expression in chick embryos. In 2001, it was established as a highly efficient method to transfect the chick embryo [81] and has been used to selectively transfect particular cells and tissues during chick embryo development [82]. The system relies on a series of electric pulses to disrupt the plasma membrane of the

cell and introduce DNA into the cell [83]. Electroporation allows for a wide variety of expression vectors to be used, including plasmid and linear DNA vectors capable of carrying constitutive, cell-type specific, or inducible promoter/enhancer elements with no apparent constraint in vector size [51]. Unfortunately, this method can only induce transient expression with peak expression occurring 24 hours post-transfection and gradually declining, probably due to the dilution of the introduced DNA as replication occurred [84].

Another non-viral transfection method involves injecting DNA directly into a single-cell-stage oocyte. This method was originally established by the Sang lab in 1994 when they injected linear plasmid DNA coding for lacZ into chick zygotes [85]. Integration efficiency was disappointingly low with only about half of the injected embryos containing the transgene and with only one of the sexually mature chickens capable of transmitting the transgene to the next generation. Furthermore, the process of obtaining a single-cell-stage oocyte lacks finesse, as a hen must be killed immediately after ovulation, which yields only one oocyte. Also, hatchability is low as the oocytes never form albumen or a protective eggshell.

Inducible Expression of Biologically Active Proteins

The inducible expression of a transgene is especially desirable in the cases where the ubiquitous expression of a protein could be potentially toxic for chick embryo development. *Koo et al.* created a transfection system allowing for the expression of the human erythropoietin gene (hEPO) under the control of a tetracycline-inducible promoter [61]. The delivery of hEPO relied on a Moloney

murine leukemia virus retroviral vector system and the expression of hEPO was dependent upon a doxycycline feed additive. The inducible expression of hEPO was observed in the G1 progeny population, suggesting successful germline transmission and, upon the removal of doxycycline, expression levels of hEPO reverted back to a control state. Unfortunately, protein expression level was not as high as expression levels observed using the constitutive promoter cytomegalovirus but this system does provide an alternative to temporally uncontrollable, ubiquitous transfection.

Generation of Induced Pluripotent Stem Cells

Induced pluripotent stem cell technology is perhaps one of the most dynamic areas of transgenic research. First derived from mouse fibroblasts in 2006 [86]. Only a year later two groups, the Yamanaka group and the Thomson group, reported creating human iPSCs using two different combinations of reprogramming factors [87, 88]. The derivation of avian iPSCs was first reported in 2012 when *Lu et al.* reported the successful derivation of quail iPS colonies [95]. This has led to a shift of interest in the field of avian transgenics as culture of avian pluripotent stem cells has historically been difficult and the production of avian iPSCs could potentially offer an alternative in the generation of transgenic birds.

iPSCs are considered to have the same developmental potential as ESCs as both cell types are held in a pluripotent state. Pluripotency is loosely defined as the potential of a cell to differentiate into cells of the three germ layers: endoderm, mesoderm, and ectoderm [89]. However, a more stringent definition would limit the

term “pluripotent” to only cells capable of giving rise to an entire organism [90]. iPSCs are also highly proliferative and express genes and surface markers equivalent to ESCs. Furthermore, iPSCs have been used to produce chimeric offspring in a number of different species, including mouse, rat, pig, and quail [91-94]. The production of chimeric offspring proves that iPSCs are capable of integrating into a developing embryo and contribute to development alongside host cells. The ability to produce chimeric animals is an important facet in the potential uses for iPSCs as it provides a stable method of passing down essential transgenes from one generation to the next. This technology is essential in the ability to perform genetic manipulation, such as gene knockouts or knock ins, and has proved to be essential in continuing complex biomedical research using the mouse and human model [95]. With the generation of iPSCs from different species now occurring, it is becoming apparent that iPSC technology could be used for agricultural purposes as well as using large animals as better models for human disease research.

The key to generating induced pluripotent stem cells from somatic cells is to create exogenous expression of an appropriate set of transcription factors, usually a combination of *POU5F1 (Oct4), SOX2, KLF4, c-MYC, Lin28*, and *Nanog* [96]. Moreover, there are numerous methods in reprogramming somatic cells all of which fall under four different categories: integrating, excisable, non-integrating, and DNA free (Table 2.1) [90]. Many methods have been discovered to successfully generate iPSCs since the original derivation using retrovirus in 2006 [86], all providing new

advantages or disadvantages that have made it difficult for one method to emerge as superior in all aspects.

Table 2.1 – Methods for reprogramming somatic cells to iPS cells [90]

Vector Type		Factors	Efficiency (%)	Advantages	Disadvantages	
Integrating	Retroviral	OKSM, OSK, OSK+VPA or OS+VPA	~0.001-1	Reasonably efficient	Genomic integration, incomplete proviral silencing and slow kinetics	[86, 87, 91, 97-99]
	Lentiviral	OSK or *miR302/367* cluster+VPA	~0.1-1.1	Reasonably efficient and transduces dividing and non-dividing cells	Genomic integration and incomplete proviral silencing	[88, 100-102]
	Inducible Lentiviral	OSKM or OSKMN	~0.1-2	Reasonably efficient and allows controlled expression of factors	Genomic integration and requirement for transactivator expression	[103-105]
Excisable	Transposon	OSKM	~0.1	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines	[106, 107]
	loxP-flanked lentiviral	OSK	~0.1-1	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines and loxP sites retained in the genome	[108]
Non-Integrating	Adenoviral	OSKM	~0.001	No genomic integration	Low efficiency	[109, 110]
	Plasmid	OSNL	~0.001	Only occasional genomic integration	Low efficiency and occasional vector genomic integration	[111, 112]
DNA free	Sendai Virus	OSKM	~1	No integration	Sequence-sensitive RNA replicase, and difficulty purging cells of replicating virus	[113]
	Protein	OS	~0.001	No integration, direct delivery of transcription factors and no DNA-relation complications	Low efficiency, short half-life, requires large quantities of pure proteins and multiple applications	[114, 115]
	Modified mRNA	OSKM or OSKML+VPA	~1-4.4	No integration, bypasses innate antiviral response, faster reprogramming, high efficiency	Requirement for multiple rounds of transfection	[116]
	MicroRNA	miR-200c, miR-302s, or miR-369s	~0.1	Faster reprogramming than viral vectors, no exogenous transcription factors, no integration	Lower efficiency than other commonly used methods	[117]

Chapter 3:

The Carbohydrate Cell Surface, Its Role in Migratory Behavior, and How to Characterize It

Glycoconjugates and Cell Signaling

Glycoproteins play a role in the migration of primordial germ cells and gonad assembly [128]. The exact mechanisms of PGC migration still remain elusive but two theories have been established. The first theory suggests that a chemotactic substance originating from the gonadal ridge guides movement. The second suggests that PGCs are constantly guided by cells from the epithelial and mesenchymal layers and are also guided by signals deriving from the basement membrane and extracellular matrix of the mesentery tissue [129]. The glycoproteins of the extracellular matrix have long been considered to play a role in PGC migration across many species. It is thought that oligosaccharide chains derived from glycoproteins and glycolipids on the cell surface are involved in cell linkage, adhesion, and growth control as PGCs find their way to the genital ridge [130]. Fibronectin, laminin, and collagen type IV have been found dispersed throughout tissues along the migratory route of chick PGCs suggesting participation in guiding migration [131]. The interactions between the PGCs themselves have also been discovered to play an important role in migration in the mouse model. Using confocal microscopy and the carbohydrate antigen, Stage-Specific Embryonic Antigen-1 (SSEA-1), the Heasman lab was able to demonstrate that the PGC cell

surface evolves in morphology as the mouse embryo develops [132]. As mouse PGCs grow closer to the genital ridge, they begin to form long processes and use the processes to reach out and selectively adhere to another PGC. This allows for the formation of PGC aggregates, informing migratory PGCs that their destination has been reached. This suggests that PGC migration is not an independent phenomenon but relies on extensive networking of neighboring cells.

Additionally, it appears that the glycosylation of proteins is important for the migration of cells. The role of the SDF-1/CXCR4 pathway in the migration of PGCs has been described in an earlier chapter but it appears that interactions between SDF-1 and glycosaminoglycans actually serve to enhance migration of cells[133]. In a study analyzing the migratory behavior of hematopoietic progenitor cells, researchers analyzed the ability of the cells to migrate in transwell filters precoated with various glycosaminoglycans versus untreated filters. The results found that the addition of glycosaminoglycans did enhance migration in hematopoietic progenitor cells [134]. So far, the effects of the glycosylation of SDF-1 have not been analyzed in primordial germ cells but the SDF-1/CXCR4 complex seems to be conserved in many different migratory cell types, including leukoctyes, hematopoietic progenitors, pre-B cells, and cancer stem cells [135-137],

However, to better understand the factors that drive PGC migration, the identification of cell surface molecules responsible for cell adhesion is necessary. Many of the suggested molecules are proteins, including N-cadherin, a transmembrane protein that has been found to be present on the surface of chick PGCs [138]. However, a multitude of glycans have been found on the cell surface of

germ cells and other pluripotent cells from a few different species. For example, in *Xenopus,* neutral glycolipids were discovered on the cell surface of germ cells at the time period in which they migrate from the gut [139]. Other examples include the SSEA family originally found in mouse [140] and the keratin sulfate antigens, TRA-1-60 and Tra-1-81 conserved across many species [141]. Elucidating further glycan biomarkers could be useful in further understanding the molecular signals that drive PGC formation and migration, potentially leading to a better grasp on causes of infertility.

Lectins

First discovered in plants over one hundred years ago, lectins have since been discovered to be ubiquitous throughout nature [142]. Ricin is considered to be the first lectin discovered. Then referred to as hemagglutinins (due to their ability to agglutinate erythrocytes), ricin was first described in 1888 by a Peter Hermann Stillmark in his PhD thesis [143]. Their ability to distinguish between erythrocytes of human blood types became crucial in the investigation of the specificity of antigens within the ABO blood group system and greatly increased their exposure for other uses.[144]. The use of lectins to probe the carbohydrate cell surface has long been established as an effective tool to study the changes that occur with many cell processes, including differentiation and mitogenesis [145]. Capable of recognizing many diverse sugar structures, lectins are carbohydrate binding proteins of non-immune origins [146]. These “selectins” recognize and agglutinate specific mono- and oligosaccharides making them a promising tool in the fields of

biotechnology and medicine [147]. Profiling the specificity of lectin binding to various mono- and oligo-saccharides is also considered key in the area of developmental biology as glycoproteins and carbohydrates are known to have altered distributions based on developmental stage, age, and sexual maturity [148].

The avian embryo and developing reproductive system has been described in many papers throughout the years, answering many developmental questions along the way. For example, lectin histochemistry has been used to define not only the phases of maturity for oogonia during chick embryonic ovarian development but also the stages of maturity of spermatogonia during testis development [149, 150]. Many different labs have also explored the carbohydrate cell surface of chick and quail germ cells. *Armengol et al.* used lectin binding profiles to establish dramatic differences in sugar residue distribution of PGCs when compared to differentiated gonads from both sexes [130]. The lectins LEA, WGA, RCA-I, PNA, and WFA were all found to be present at some point in germ cell development (see Table 2). According to this table and data found in the Armengol paper, lectins specific for N-acetylglucosamine (LEA, WGA, and RCA-I) reacted positively in all stages of PGC development, regardless of sex. This suggests that these carbohydrate residues play an important role in PGC migration and in the colonization of the gonad. PNA, the lectin specific for β-Gal(1-3)-GalNAc reacted only in the differentiated stage of female embryos, suggesting that structural changes in regards to this sugar residue are important for the development of oogonia in quail.

Table 3.1 Summary of the lectin binding pattern of PGCs during quail gonad differentiation [130]

	PGC Migration			Differentiated Gonads					
	20HH	24HH	28HH	30HH		35HH		38HH	
PGCs				Male	Female	Male	Female	Male	Female
LEA	+	+	+	+	+	+	+	+	+
WGA	+	+	+	+	±	+	+	±	+
RCA-I	+	+	+	+	±	+	+	±	+
PNA	-	-	-	-	-	-	+	-	+
WFA	+	+	+	-	-	±	±	-	+

(-) No labeling, (±) Weak labeling, (+) Intensive labeling

Another study established the lectins STA and DBA as potential markers for chicken PGCs. *Jung et al.* collected PGCs from a 5.5 day old chick embryo, cultured *in vitro* for three passages, then characterized the cells. PGCs were stained for pluripotency markers, SSEA-1, SSEA-3, SSEA-4, and EMA-1 as well as the lectins WGA, ConA, STA, and DBA. The PGCs reacted strongly to after FITC-staining with STA and DBA whereas, only weak fluorescence intensity or nonspecific binding occurred DBA, ConA, and WGA. Double staining with STA and SSEA antibodies was also achieved. This study suggests that lectin binding profiles can be used in identifying germ cell populations. However, to date, there have not been any studies on PGCs that have been cultured for prolonged periods of time or investigation as to how these lectins might influence migratory behavior.

The lectin binding profiles of PGCs have also been analyzed in other food production animals, with a focus on analyzing the carbohydrate cell surface of the pig. The lectin DBA has been shown to have a specific affinity for porcine PGCs in the genital ridge and is considered to be a specific marker of PGCs in the pig [151]. Additionally, the specificity of DBA for PGCs was also confirmed in the cow [152]. *Tagaki et al.* compared the lectin binding profiles of PGCs in the pig embryo to the mouse embryo at comparable developmental time periods [153]. Table 3.2 contrasts the lectin binding profiles between the mouse and the pig. With the exception of DBA and UEA-1, the lectin binding profiles between the two species were almost identical. However, mouse PGCs did not react with DBA, suggesting that DBA is not an appropriate universal marker for migratory PGCs.

Since PGCs express many of the same protein markers of ESCs, it is important to consider the lectin binding specificities of this cell type in comparison to PGCs [154]. Chicken ESCs are difficult to isolate and maintain *in vitro* so most lectin binding studies have been analyzed using human and mouse ESCs. One interesting application emerging from using lectins to analyze the cell surface of pluripotent stem cells is the ability to use a lectin to isolate a pure population of a certain cell type. *Wang et al* used lectin microarrays to compare lectin specificity in 12 different ESC lines, 14 separate iPSC lines, and 13 differentiated cell types [155]. Of the 13 lectins used, UEA-1 was determined to bind the most selectively to pluripotent cell types. This led to the isolation of pluripotent stem cells from a mixed population of cells based solely on UEA-1 expression. *Dodla et al.* used a similar approach to isolate neural progenitors from a population of cells containing ESCs, neural

progenitors (NPs), and mesenchymal progenitors (MPs) [146]. The ability to isolate cells based on lectin binding specificity could have many advantages. First, lectins can be easily removed from the cell surface and have little to no toxicity, making cell sorting significantly less stressful on the cells. Additionally, lectins are considerably less expensive than protein antibodies. The affordability of lectins could make large-scale screening of cells an option in areas where cost would be a factor [155].

In this study, we plan to further investigate the roles of the carbohydrate cell surface by using immunocytochemistry and flow cytometry to provide quantitative and qualitative data as to which lectin binding profiles are expressed in PGC cultures that have been propagated *in vitro* for a prolonged period of time. It is our aim to establish a correlation between a specific lectin binding profile and migratory potential, perhaps providing a novel marker to characterize cells with a greater migratory potential. This could greatly increase the efficiency of deriving transgenic birds.

Table 3.2 Lectin Reactivity with Pig and Mouse Embryos [153]

Pig 26-day embryo				Mouse 12.5-day embryo		
	Genital Ridge			Genital Ridge		
Lectin	**PGC**	**Whole Area**	**Mesonephros**	**PGC**	**Whole Area**	**Mesonepros**
LTA	+/-	-	+	-	-	+
DBA	+	-	+/-	-	-	-
UEA-1	+	+	+	-	-	-
SBA	-	-	++	-	-	+/-
PNA	++	++	+	-	-	+
VVL	+	+	+	+	+	+
ConA	++	++	++	++	++	++
RCA	++	++	++	++	++	++
WGA	++	++	++	++	++	++
LEL	++	++	++	++	++	++
PSA	++	++	++	++	++	++
SJA	++	++	++	++	++	++

CHAPTER 4

LECTIN BINDING PROFILES OF CHICKEN PRIMORDIAL GERM CELLS

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To be submitted to *Stem Cells and Development*

Abstract

The ability to stably and efficiently derive transgenic birds is a technology that has great potential in many areas of research. One method often used is the isolation and manipulation of avian primordial germ cells. Unfortunately, this method suffers from a lack of efficiency that is partially caused by not understanding the molecular mechanisms that drive the successful migration of PGCs to the gonads. In this study, we characterized PGCs cultured long term *in vitro* that retain the ability to migrate to the gonad. To characterize, several lectins including STL, DBA, ConA, WGA, MAA, SBA, PNA, and RCA were used. This panel of lectins was chosen based on previous studies analyzing primordial germ cells *in vivo* as well as used to analyze the cell surface of embryonic stem cells. Two lines of cPGCs were analyzed, one line expressing a mixed population of the chemokine receptor CXCR4 and another negative for CXCR4. Nonmigratory CEFs were used as a negative control. The cells were stained to determine if a lectin binding profile could predict migratory potential. Both lines of PGCs were positive for STL, ConA, WGA, MAA, and RCA while negative for DBA, SBA, and PNA. CEFs were positive for STL, ConA, WGA, and MAA and negative for DBA, SBA, PNA, and RCA. The differences in specificity for RCA between the CEFs and PGCs indicate that RCA could potentially be a marker for migration in avian PGCs.

Introduction

It has long been established that the chick embryo is one of the foremost models to study developmental biology. Aristotle first used the chick embryo in 350 BC to study development by opening chick eggs at different stages and observing the results of each opening [156]. From that first series of experiments, the chick embryo has been used to understand cell patterning during development, the fate of neural crest cells, and as a disease model to understand retinal degeneration [157-159]. The use of the chick embryo also has applications beyond the developmental biology world, with many researchers striving to create an efficient avian transgenic system. Genetic manipulation of chickens could lead to more efficient and higher quality chicken production, which is critical as chickens are considered to be an important source of protein for people across the globe. Furthermore, the production of transgenic chickens is considered to be a promising and efficient method of producing animal bioreactors [160]. The creation of a robust system to generate transgenic birds would be beneficial for researchers attempting to create chickens with enhanced biomedical and agricultural characteristics.

For the past two decades, attention and effort has been directed at the genetic manipulation and transfer of primordial germ cells (PGCs) into host embryos to produce germline chimeras [161-163]. PGCs are early germ cells that eventually develop into sperm or eggs in the adult bird. These cells can be detected as early as the blastodermal stage (Stage X) [12] and can be isolated from the vasculature as PGCs begin to migrate to the genital ridge (Stages 15-16) [164].

While we know the physical mechanisms as to how PGCs migrate from the germinal crescent to the vasculature then the genital ridge, the signaling mechanisms that direct this migration remain elusive. Additional characterization of chicken PGCs is essential for better understanding of the mechanisms that influence germ cell migration. Elucidating these factors will be essential in further developing an efficient avian transgenic system. In this study we aim to identify new markers to characterize migratory avian PGCs through the use of lectin binding profiles.

It is well established that the carbohydrate cell surface evolves throughout development and cell differentiation[165, 166]. Furthermore, the glycans of the extracellular matrix are considered essential for guiding germ cells during their journey to the genital ridge [131]. Previous studies have characterized the carbohydrate surface of avian PGCs *in vivo* and in *in vitro* cultures. However, these analyses determined the lectin binding profiles of endogenous PGCs in the tissues of chick embryo or germ cells displaying a morphology more similar to an embryonic germ cells [167-169]. To improve the efficacy of manipulating PGCs to derive transgenic birds, it is essential to probe the carbohydrate cell surface of PGCs that can proliferate in an *in vitro* culture while retaining a PGC morphology.

To characterize the carbohydrate cell surface of our PGCs we analyzed the lectin binding profiles of two lines of PGCs compared to the lectin binding profiles of non-migratory CEFs. Lectins are ubiquitous proteins that bind specific carbohydrate structures and are considered a useful tool to characterize glycosylation patterns on the cell surface [170]. We chose a panel of eight lectins based on previous studies analyzing lectin binding patterns of PGCs and other pluripotent cell types [130, 146,

153, 169, 170]. Each lectin had been determined to bind to pluripotent cells similar to germ cells, germ cells, or other migratory cell types. In this study, we aimed to determine if our *in vitro* cultured non-adherent cells retained a similar carbohydrate profile as the cells used in previous reports.

Additionally, in this study we analyzed two lines of PGCs for the expression of the chemokine receptor CXCR4. Both CXCR4 and its ligand SDF-1 have been documented as essential for proper PGC migration in many species, including xenopus, mouse, and chick [29, 171, 172]. SDF-1 serves as a homing protein that signals CXCR4 expressing cells toward the genital ridge and knockouts of either protein create severe directional aberrations in the migration patterns of PGCs[31]. We aimed to determine if there was a correlation between the expression of CXCR4 and lectin binding profiles that could enhance migration in PGCs.

In this study, we determined that cells negative for the expression of CXCR4 still retained the ability to migrate to the embryonic gonad in the chick. This finding suggests that there might be a better marker for migratory potential. We used lectin binding profiles to potentially identify this improved migratory marker. From our findings, we determined that there is little difference in expression of carbohydrates between PGCs and CEFs with the exception of the lectin RCA. The positive expression of RCA in both lines of migratory PGCs indicates the up-regulation of β-galactose in PGCs but not CEFs. This finding suggests that RCA could potentially function as a marker to identify highly migratory PGCs. Enriching a PGC population with cells having a greater potential to migrate could greatly increase the efficacy of creating transgenic birds using PGCs.

Materials and Methods

Culture of PGCs and CEFs

Chicken PGCs were isolated from a male White Leghorn chicken and maintained in suspension on a mouse embryonic fibroblast feeder layer and cultured in cKO germ cell media. The cKO media contains Knockout Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies), 10% feeder conditioned KO-DMEM, 7.5% defined fetal bovine serum (HyClone), 2.5% chicken serum (Sigma), 1X Pen/Strep (Gibco), 1X GlutaMAX (Gibco), 1X GS nucleoside supplement (Millipore), 4 ng/mL bFGF, and 0.1 mM β-mercaptoethanol. PGCs were maintained at 5% CO_2 at 37° C and passaged every 3-5 days at a 1:3 concentration using manual passaging.

CEFs were isolated from day 11 Barred Rock embryos and cultured in fibroblast media containing DMEM High Glucose (HyClone), 10% FBS (HyClone), 4mM L-Glutamine (Gibco) and 1x Pen/Strep. CEFs were passaged every 3 days at a 1:3 concentration using trypsin enzymatic passage.

Embryo Injection

Before injection, cells were labeled with GFP (System BioSciences) using a piggyBac transposon transfected according to the manufacturer's instruction. H&H Stage 15 White Leghorn embryos were used as the host for the injection. A window with 1-cm diameter is made on the blunt end above the air cell to expose the embryos. A total of $1x10^4$ cells loaded in the micro glass needle were injected into the vasculature system of each embryo and then sealed the window by applying 2 layers of parafilm. The injected embryos were incubated for 6 days and then

euthanized to isolate the gonads under stereomicroscope. Image of gonads were captured under an inverted microscope.

Immunocytochemistry for CXCR4

PGCs were fixed in 4% paraformaldehyde in 1X $PBS^{-/-}$ for 15 minutes. Cells were then washed twice with $PBS^{-/-}$ then blocked with 6% goat serum in $PBS^{-/-}$ solution for 45 minutes. Primary and secondary antibodies were diluted in the same block solution. After blocking, cells were stained with CXCR4 (1:300, ECM Bioscience) for 1 hour at room temperature. After 1 hour, the cells were washed twice in $PBS^{-/-}$ and incubated in secondary antibody AlexaFluor 488 (1:1000, Molecular Probes) for 1 hour at room temperature. Cells were again washed twice in $PBS^{-/-}$ and post-stained with 4'-6-Diamidino-2-phenylindole (DAPI, Invitrogen) to detect cell nuclei. All slides were mounted and visualized using a Zeiss LSM 710 confocal microscope and images were processed using Zen software.

Flow cytometry analysis to determine percentage cell-lectin binding

Table 4.1 describes the lectins used in this study, their saccharide specificities, and the inhibitory sugars used as negative controls to determine specific binding.

PGCs and CEFs were harvested, collected in 15 mL conical tubes, and fixed in 4% paraformaldehyde in 1X $PBS^{-/-}$. After fixing, cells were washed twice in $PBS^{-/-}$ and placed in block solution. Then each cell type was stained with one of the 8 lectins described in Table 1 at 10 μg/mL for one hour at room temperature. Cells were washed twice in $PBS^{-/-}$ then stained with secondary antibody streptavidin 594 (1:1000, BD Biosciences). Cells stained with inhibitory sugars and secondary only

were used as negative controls. Flow cytometry was performed using a CyAn cytometer (Beckman Coulter) using two lasers turned to 405 nm and 594 nm. Data analysis was performed using FlowJo (Tree Star, Inc) software. Percentage of cells expressing fluorescence intensity greater than control cells were calculated using FlowJo.

Significance was determined using a One-way ANOVA (Microsoft Excel) and Tukey's Pair-Wise analysis (StatPlus). Results where a P-value<0.05 were considered to be significantly different.

Immunocytochemistry to characterize cell-lectin binding

For PGCs, the cells were harvested and immunostained following our flow cytometry protocol as described above. After the secondary antibody incubated for 1 hour, the cells were washed twice in PBS $^{-/-}$, resuspended in DAPI, and transferred to a slide. Slides were mounted then imaged using a Zeiss 710 LM confocal microscope.

CEFs were plated into a 4-well chamber slide then fixed, washed, and blocked as described previously. The CEFs were then stained for an individual biotinylated lectin (10 μg/mL) for 1 hour at room temperature, washed twice, then incubated with secondary antibody streptavidin conjugated Alexafluor 594 (1:1000). Cells were then washed twice with PBS $^{-/-}$ and post-stained with DAPI before mounting and imaging. Each lectin's competitive sugar was used to verify the specificity of each lectin for its carbohydrate.

Results

Characterization of PGCs for CXCR4

Previous publications indicated that expression of the chemokine receptor CXCR4 was necessary for the successful migration of chick PGCs to the gonads [173]. To identify a CXCR4 negative strain we tested our line of PGC (L1) and a sub-line (SL1) derived from the L1 population for CXCR4 expression. Both lines of cells were maintained as nonadherent cultures (Fig 4.1A) on feeder layers. The PGCs were immunostained for CXCR4 then analyzed by flow cytometry. L1 contained a mixed population of cells displaying positive expression of CXCR4 (65.3%) (Fig 4.1C) whereas the SL1 line of cells was negative for expression of CXCR4 (2.82%) (Fig 4.1D). Fluorescent imaging confirmed the results determined by the flow cytometry analysis (Fig 4.1E,F).

PGCs migrate to the embryonic gonad *in vivo*

To determine if both the L1 and SL1 line were capable of migrating through the vasculature system to the gonads cells were labeled with GFP using a piggyBac transposon vector then injected into the vasculature of stage 15 chicken embryos. The embryos injected with both lines of PGCs showed expression of GFP-positive cells in the gonad or mesentery tissue surrounding the gonad (Figure 4.2). This suggests that cell populations negative for expression of CXCR4 can still migrate to the embryonic gonads.

Analysis of carbohydrate expression by flow cytometry

The presence and percent binding of the panel of lectins were analyzed on both lines of PGCs as well as a line of chicken embryonic fibroblasts (CEFs) (Table

4.1). Both the L1 and SL1 line of PGCs reacted very strongly with the lectins STL and MAA with binding percentages ranging from 98-100%. The PGCs also reacted to the lectins ConA, WGA, and RCA. The lectins DBA, PNA, and SBA did not bind to the PGCs with histograms from the flow cytometry analysis showing no peak shifts when compared to the secondary only control (Fig 4.3). The lectin binding profiles of the CEFs were largely similar to what was observed in the PGCs with the exception of RCA expression, suggesting that CEFs do not contain galactose residues that specifically bind to RCA on the cell surface (Fig 4.4).

Analysis of carbohydrate expression using immunocytochemistry

The PGCs and CEFs were further analyzed using immunocytochemistry to determine if any localization or staining patterns of the carbohydrates on the cell surface occurred. The immunostaining results of both the PGC lines and CEFs supported the flow cytometry analysis (Fig 4.4). The lectins STL (Fig 4.4A, 4.4I), ConA (Fig 4.4C, 4.4J), WGA (Fig 4.4D, 4.4K), MAA (Fig 4.4E, 4.4L), and RCA (Fig 4.4H, 4.4P) appeared to bind uniformly to the PGC L1 and SL1 cell surface with the intensity of staining appearing to be highest for STL and MAA. The lectins DBA, SBA, and PNA (Fig 4.4B, 4.4J, 4.4F, 4.4N, 4.4G, and 4.4O, respectively) had little or no binding with the PGC cells corroborating the flow cytometry data.

For the CEFs, the lectins STL (Fig 4.4Q), ConA (Fig 4.4S), WGA (Fig 4.4T), and MAA (4.4U) appeared to bind uniformly to the cell surface with staining intensity the highest for MAA. Expression of the lectins DBA, SBA, PNA, and RCA was very low or nonexistent, corroborating again the flow cytometry data.

Analysis of carbohydrate expression and expression of CXCR4 using flow cytometry

Here we tested whether there is a correlation between CXCR4 expression and lectin binding expression in our L1 line of PGCs. The PGCs were stained for CXCR4 and one lectin then analyzed using flow cytometry (Fig 4.6). The flow cytometry analysis did not reveal a subpopulation of cells that were CXCR4+/lectin-. However, for the lectins STL, ConA, WGA, MAA, and RCA (Fig 4.6 A, C, D, E, H, respectively) it was revealed that there is a population of CXCR-/lectin+ cells. We did not find any interesting correlations between the expression of CXCR4 and the individual lectin but these results did confirm that a significantly higher population of cells expressed RCA over CXCR4 (Fig 4.6H).

Discussion

Our results report the first cell surface carbohydrate characterization of nonadherent migratory PGCs cultured *in vitro* for a prolonged period of time. In this study, we probed the carbohydrate cell surface of two lines of migratory PGCs, one positive for expression of CXCR4 and one negative, and one line of CEFs to determine if the carbohydrate cell surface might play a role in determining migratory potential. We used a panel of 8 lectins based on previous studies involving lectin binding profiles of PGCs and other pluripotent cell types [130, 146, 153, 169, 170]. This panel of lectins allowed us to determine differences in glycan expression between the non-migratory mesenchymal cells and our migratory PGC

lines. Furthermore, we also determined that expression of CXCR4 is not required for the migration of avian PGCs to the gonad.

Our characterization of the PGC lines SL1 and L1 for the expression of the chemokine receptor CXCR4 demonstrated two differing expression profiles. Line L1 contained a mixed population for CXCR+/CXCR4- cells while the SL1 line contained a completely negative population of cells expressing CXCR4. Then, since it is well known that glycoproteins influence the migration of PGCs in many species [34, 174, 175], we decided to probe the carbohydrate cell surface to determine if there are any key differences between the two lines of PGCs and non-migratory CEFs.

Our comparison of the lectin binding profiles of the PGC lines and CEFs revealed no significant differences between the PGC lines and only one difference in expression between the PGCs and the CEFs. Thus, both our migratory PGCs and the non-migratory CEFs express carbohydrate moieties represented by N-acetyl-D-lactosamine (STL), α-mannose (ConA), Neu5Ac (WGA, MAA), and N-acetyl-glucosamine (WGA). Both the PGCs and CEFs were found not to express complex N-glycan structures containing N-acetyl-galactosamine (DBA, SBA, PNA). The only difference in carbohydrate expression found between the PGC lines and the CEFs was the expression of terminal β-Galactose (RCA) (Fig 4.5). Expression of RCA has been reported in a number of *in vivo* avian primordial germ cell studies[149, 176]. This could make the lectin RCA a useful tool in tracking the migration of PGCs in the avian embryo.

Our results indicate a few differences previously reported in papers characterizing avian PGCs. This could be due to the differences in culture conditions.

For example, *Jung et al* reported the positive expression of DBA in their PGCs with minimal expression of both ConA and WGA [169]. However, as those cells were cultured as an adherent, colony-forming culture indicating a morphology more similar to an embryonic germ cell line.[177]. It is entirely feasible that the profile of the cell surface evolves as these cells lose their ability to be cultured as a nonadherent culture. *Didier et al.* reported the positive expression of the lectin PNA in migrating chick PGCs [167]. These results are rather controversial as many more labs have reported the absence of expression of PNA in PGCs [130, 149, 176]. However, this could also be due to the extensive culturing of our PGCs *in vitro* compared to the *in vivo* immunohistochemistry studies reported in the Didier paper.

For future studies, it will be important to characterize a nonmigratory germ cell line with a more extensive panel of lectins to identify additional glycan structures that might be upregulated in migratory PGCs. This could be helpful in determining if the changes in carbohydrate expression is a result of the differentiation of PGCs to a more developed fate or if these glycans do influence the PGCs to successfully migrate to the genital ridge. In this study we have identified glycan structures that are expressed in migratory PGCs even when CXCR4 expression is quenched. Further studies could help in identifying any additional markers of migratory PGCs or perhaps identify the individual roles these glycans play in cell migration.

Acknowledgments

We would like to thank the Bill and Melinda Gates Foundation for their financial support and the lab of James Petitte and North Carolina State University for providing the primordial germ cells for analysis. We also thank Julie Nelson and the Center for Tropical and Emerging Global Diseases Flow Cytometry Facility for her technical support.

Table 4.1 Features of reviewed plant lectins

Lectin Name	Abbreviation	Monosaccharide Specificity	Inhibitor
Solanum tuberosum Lectin	STL	GlcNAc	Chitin Hydrolysate
Dolichos biflorus agglutinin	DBA	GalNAc	200 mM GalNAc
Concanavalin A	ConA	Mannose	200 mM Lactose
Wheat germ agglutinin	WGA	GlcNAc	Chitin Hydrolysate
Maackia amurensis agglutinin	MAA	Galactose	200 mM Lactose
Soybean agglutinin	SBA	GalNAc	200 mM GalNAc
Peanut agglutinin	PNA	Galactose	200 mM Galactose
Ricinus comunis agglutinin	RCA	Galactose	200 mM Galactose

Figure 4.1 PGCs Display Mixed Levels of Expression of CXCR4

Brightfield images of L1 (**A**) and SL1 (**B**). Flow cytometry analysis showed that Line 1 contained a mixed population of CXCR+/CXCR4- cells (**C**) while Sub-Line 1 was negative for CXCR4 expression (**D**). Immunocytochemistry confirmed CXCR4 expression in Line 1 (**E**).

Both flow cytometry analysis and immunocytochemistry were performed three times. All images are representative images from the three replicates.

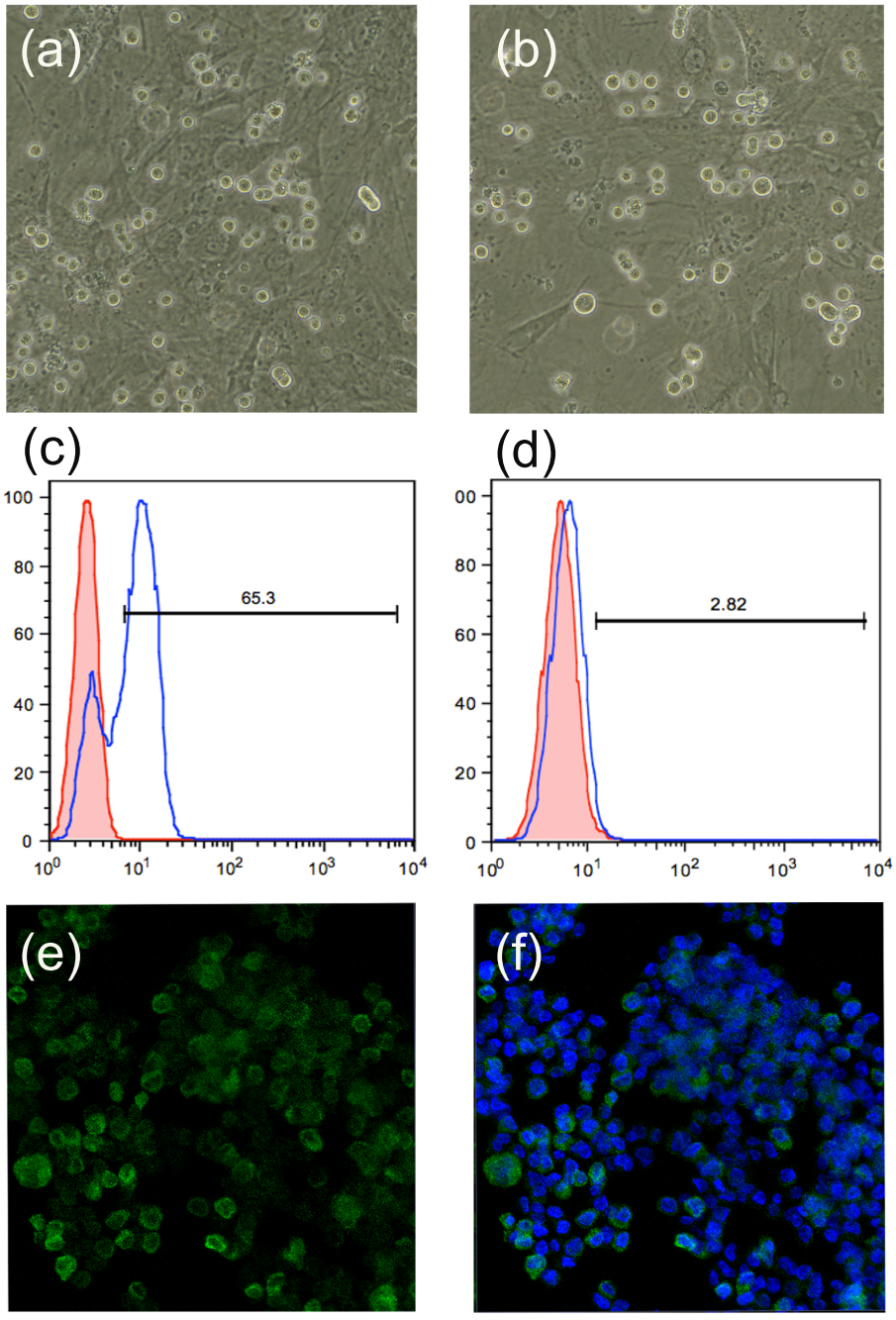

(a)
(b)
(c)
(d)
65.3
2.82
(e)
(f)

Figure 4.2 PGCs Migrate to the Embryonic Gonad

PGCs labeled with GFP were injected into the Stage 15 chicken embryo. Embryos were opened and the gonads isolated 6 days post-injection. GFP-positive cells from both the SL1 line (**A,B**) and L1 line (**C,D**) were observed in the gonads.

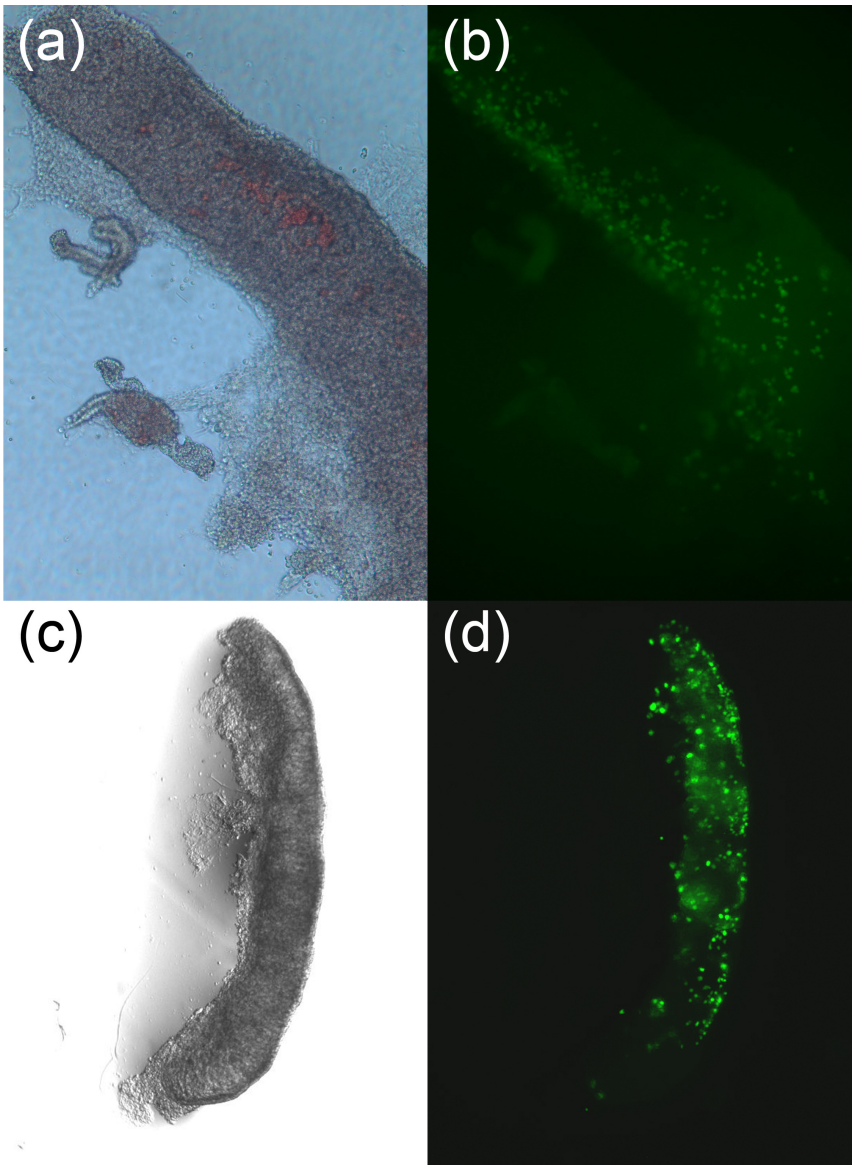

(a)
(b)
(c)
(d)

Figure 4.3 Flow Cytometry Histograms of Lectin Binding in PGCs and CEFs

Each histogram displays the percentage of each cell line binding to eight different lectins. The far left red filled peak represents cells stained with secondary antibody only. Peaks outlined in blue represent cells stained for a lectin. Gating for each histogram represents the percentage of cells positive for each lectin. Each histogram is a representative image for one of the three replicates performed. Panels in the left column are for L1, panels in middle column are for SL1, and panels in the left column are for CEFs.

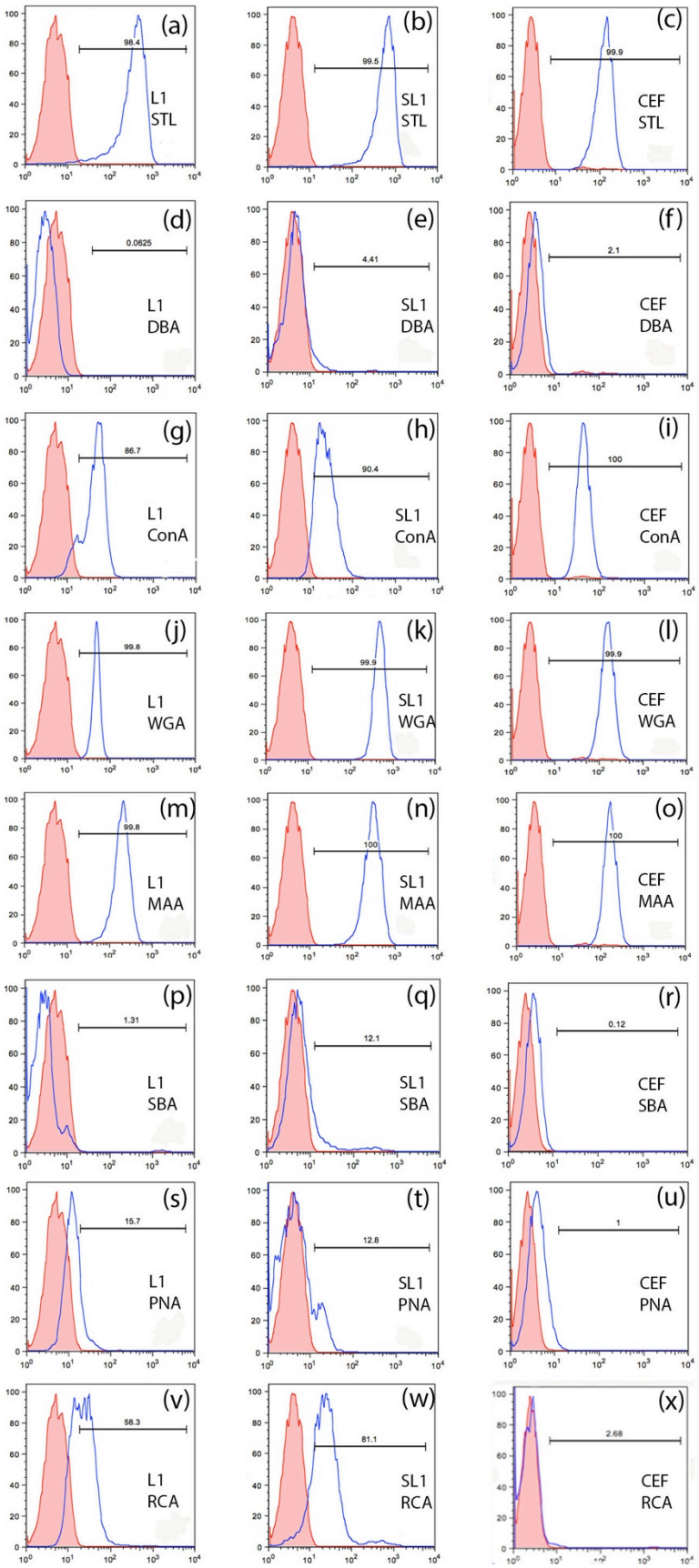

(a)
98.4
L1
STL
(b)
99.5
SL1
STL
(c)
99.9
CEF
STL
(d)
0.0625
L1
DBA
(e)
4.41
SL1
DBA
(f)
2.1
CEF
DBA
(g)
86.7
L1
ConA
(h)
90.4
SL1
ConA
(i)
100
CEF
ConA
(j)
99.8
L1
WGA
(k)
99.9
SL1
WGA
(l)
99.9
CEF
WGA
(m)
99.8
L1
MAA
(n)
100
SL1
MAA
(o)
100
CEF
MAA
(p)
1.31
L1
SBA
(q)
12.1
SL1
SBA
(r)
0.12
CEF
SBA
(s)
15.7
L1
PNA
(t)
12.8
SL1
PNA
(u)
1
CEF
PNA
(v)
58.3
L1
RCA
(w)
81.1
SL1
RCA
(x)
2.68
CEF
RCA

Figure 4.4 Quantification of lectin binding

The percent of cells with specific carbohydrate expression was determined using our panel of 8 different lectins and analyzed with flow cytometry. Each cell type was stained with an individual lectin. The data is represented as average +/- SD of 3 independent assays of L1, SL1, and CEF cells. Means with different letters are significantly different, * indicates $p<0.05$ compared to L1 and # indicates $p<0.05$ compared to SL1.

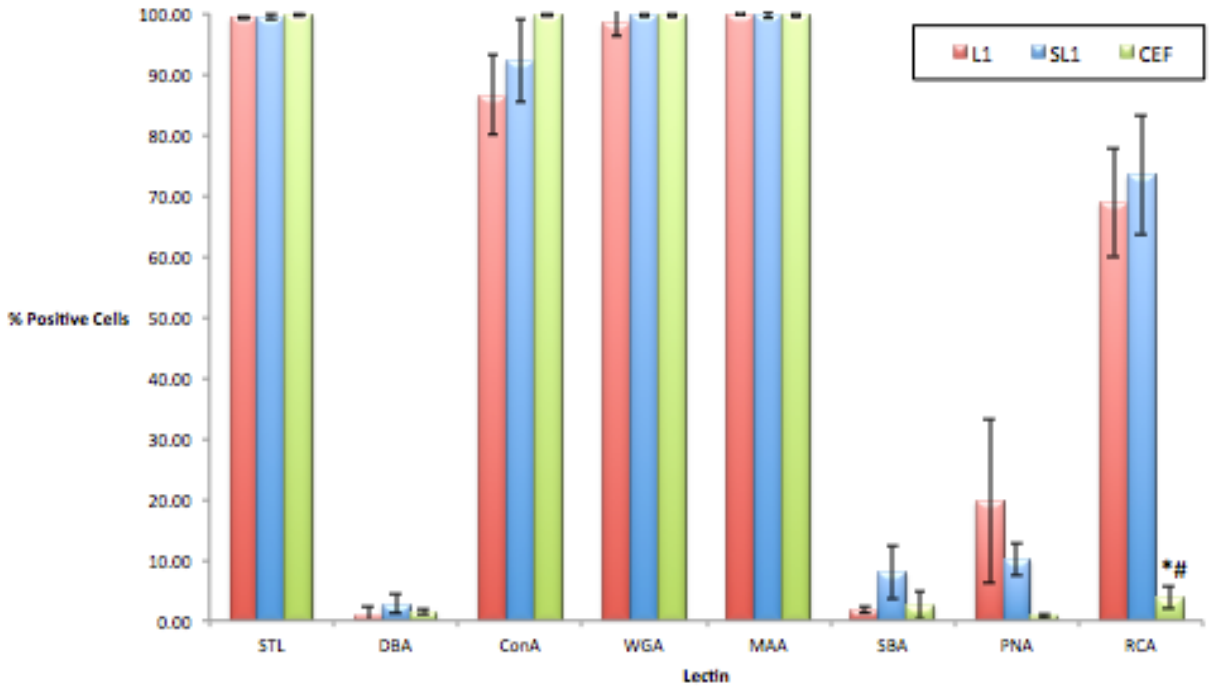

100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% Positive Cells
L1
SL1
CEF
*#
STL
DBA
ConA
WGA
MAA
SBA
PNA
RCA
Lectin

Figure 4.5 Immunocytochemistry of PGCs and CEFs for binding to lectins

The panels in the left column are lectin binding profiles of L1, the panels in the middle column are SL1, and the panels in the right column are of the CEFs. Immunostaining roughly correlates with flow cytometry analysis.

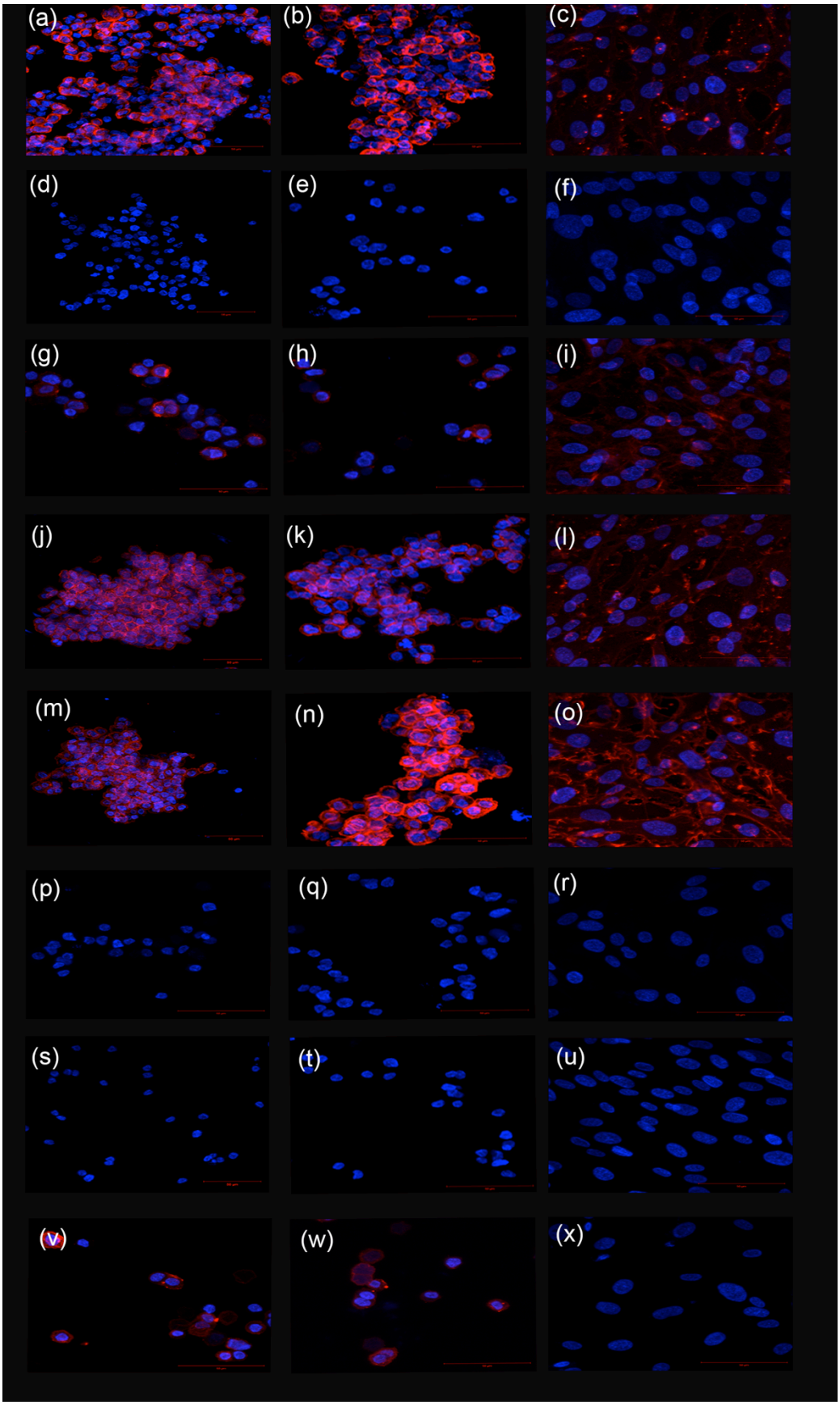

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)
(s)
(t)
(u)
(v)
(w)
(x)

Figure 4.6 Expression of RCA

The only lectin binding profile that differs between the L1 and SL1 lines of PGCs and the CEFs is the expression of lectin RCA. The immunostaining and flow cytometry analysis in the first row represents L1 (**A**,**B**) the second row represents SL1 (**C,D**), and the third row represents the CEFs (**E, F**)

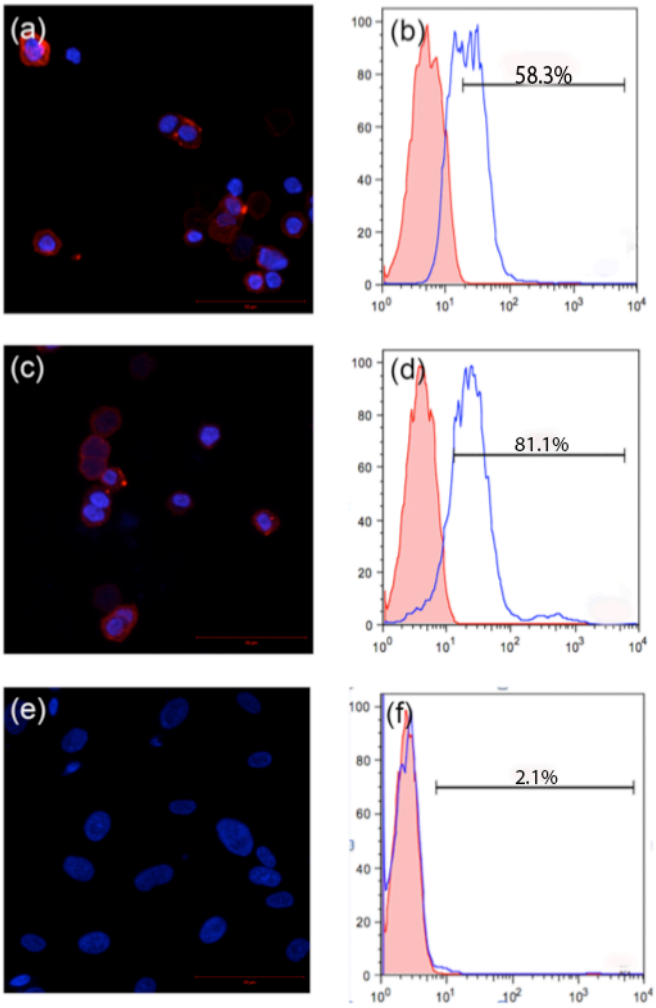
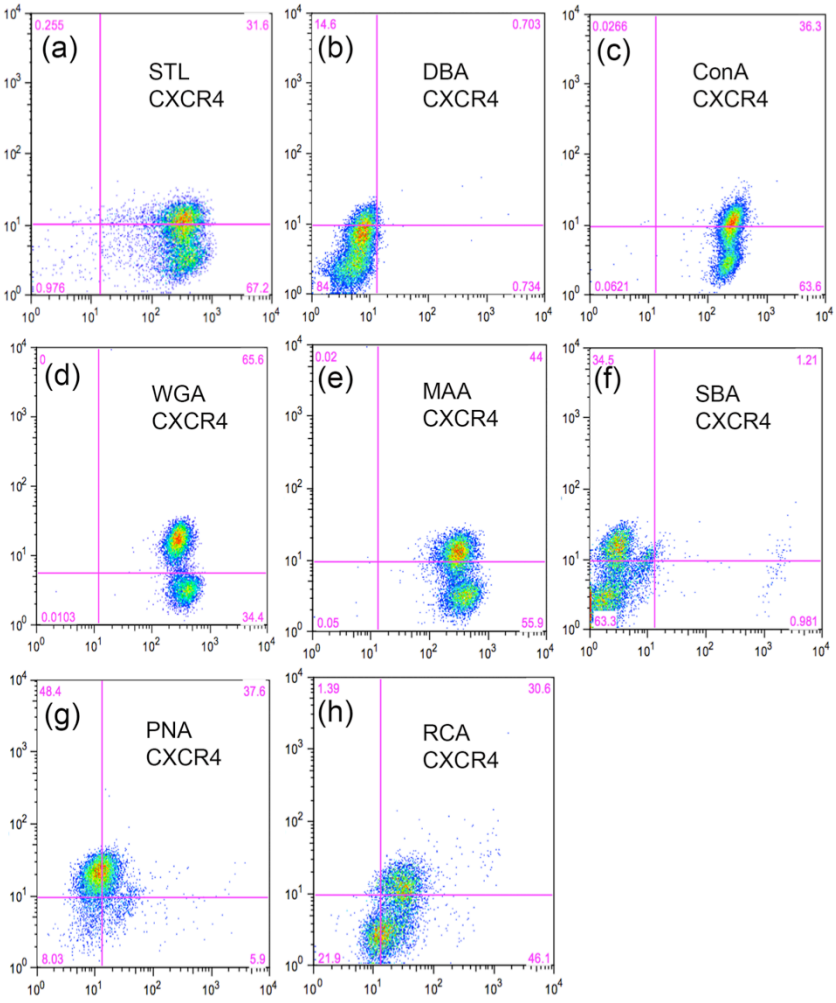

(a)
(b)
58.3%
(c)
(d)
81.1%
(e)
(f)
2.1%

Figure 4.7 Flow Cytometry Analysis of Co-localization for CXCR4 and Lectins

Flow cytometry analysis was performed on the L1 line of PGCs to determine if any lectin binding patterns emerged when co-localized with CXCR4. The y-axis represents CXCR4 expression whereas the x-axis represents lectin expression. It appears that there are no CXCR+/lectin- populations with most cells either appearing to be CXCR+/lectin+ or CXCR4-/lectin-.


(a)
STL
CXCR4
0.255
31.6
0.976
67.2
(b)
DBA
CXCR4
14.6
0.703
84
0.734
(c)
ConA
CXCR4
0.0266
36.3
0.0621
63.6
(d)
WGA
CXCR4
0
65.6
0.0103
34.4
(e)
MAA
CXCR4
0.02
44
0.05
55.9
(f)
SBA
CXCR4
34.5
1.21
63.3
0.981
(g)
PNA
CXCR4
48.4
37.6
8.03
5.9
(h)
RCA
CXCR4
1.39
30.6
21.9
46.1

Chapter 5

Conclusion

The ability to efficiently create transgenic chickens is an attractive possibility for researchers in many fields. For scientists in the agriculture sector the ability to generate birds capable of producing higher quality or more meat and eggs is exciting. For researchers trying to generate large amounts of recombinant proteins, creating transgenic birds to serve as animal bioreactors is a promising approach. For biomedical purposes, transgenic avian embryos can help researchers better understand development and disease. However, technical difficulties have prevented progress in achieving avian transgenesis as successfully as their mammalian counterparts. However, none of these ideas will be able to come to fruition without a stable, more efficient system in generating avian chimeras. For this to occur it is crucial that researchers continue to delve into better understanding the molecular pathways that drive and influence germ cell migration.

Our results exhibit for the first time the lectin binding profiles of nonadherent, *in vitro* cultured chick PGCs, providing insight as to the morphology of the carbohydrate cell surface. Furthermore, we prove that PGCs negative for the expression of CXCR4 can still successfully migrate to and integrate into the gonads of the avian embryo. This finding suggests that there are other chemotactic factors that influence the migratory potential of avian PGCs. Here, we suggest that the

communication between cell surface glycoproteins and glycoproteins found in tissues along the migratory route to the genital ridge is essential for migration.

We chose a panel of eight lectins to probe the carbohydrate cell surface of two lines of PGCs, one containing a mixed population of cells positive for expression of CXCR4 and one negative for expression of CXCR4. We also analyzed a non-migratory line of CEFs to determine if we could isolate a lectins as a possible biomarker for migratory potential. Of our eight lectins, only 5 reacted with the PGC lines, STL, ConA, WGA, MAA, and RCA. This staining demonstrates that migratory PGCs express LacNAc (STL), alpha-linked mannose (ConA), Neu5Ac residues (MAA), beta-4 linked N-acetyl-glucosamine (WGA) and beta-linked galactose (RCA) on their cell surface. Both lines of PGCs were negative for expression of the lectins DBA, SBA, and PNA suggesting that PGCs do not express alpha-linked N-acetylgalactosamine (DBA, SBA), and Gal-(β1-3)-GalNAc (PNA). The CEF line had almost identical lectin binding profiles as the PGCs with the exception of RCA expression. It appears that CEFs do not express beta-linked galactose, potentially making RCA a useful marker for migratory cells.

There are many options to further this work in the future. First, as no one lectin stood out as an ideal marker for migratory potential, a more extensive panel of lectins could be analyzed. At the very least, adding to our panel of original lectins will provide further insight as to what glycoproteins exist on the cell surface of migratory PGCs. Second, there have been many papers detailing the lectin binding profiles of PGCs either *in vivo* using immunohistochemistry or PGCs that form adherent, colony-forming cultures. As no one paper has reported as extensive a

panel of lectins used as our we could analyze the lectin binding profiles of lectins from our panel of PGCs either *in vivo* or in an adherent culture to provide a further comparison, especially in the case of expression of RCA. Our last option moving forward is to characterize the lectin binding profiles of cells recently derived in our lab that appear germ cell like in morphology and migratory behavior. These cells, coined iPGCs, are derived from CEFs using lentiviral vectors containing the human stem cell genes, *Pou5F1,Sox2, Klf4, c-Myc, Nanog*, and *Lin28*. These cells are competent to migrate to the gonad and integrate when injected into the Stage 15 chick embryo. Characterizing these cells for the expression of lectin binding profiles could provide some insight as to the morphology of migratory cells that were not originally committed to the germ cell fate.

By better understanding the function of the carbohydrate cell surface of primordial germ cells researchers will be able to obtain a greater understanding of cell communication. This better understanding will provide insight into the maintenance of migratory potential as well as possibly introducing new tools to increase efficiency in the production of transgenic birds. The ability to efficiently create transgenic birds on a large-scale basis holds tremendous potential for many applications. However, until we gain a comprehensive understanding of the molecules that influence germ cell proliferation and migration that potential will remain unrealized.

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