CHIMERIC CHICKENS PRODUCED FROM INDUCED PLURIPOTENT STEM CELLS UTILIZING A NON-VIRAL APPROACH

By

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(Under the Direction of Franklin D. West)

ABSTRACT

Induced pluripotent stem cells (iPSCs) provide a powerful and novel approach to generating transgenic animals by enabling complex genetic manipulations. iPSCs from various species have been derived by introducing different combinations of reprogramming factors. However, chicken iPSCs have not been yet reported. In this study, we describe the generation of chicken induced pluripotent stem cells (ciPSCs) by transfection of a nonviral minicircle DNA construct into chicken embryonic fibroblast cells (CEFs). Following transfection, ciPSC colonies showed stem cell morphology. They expressed pluripotent markers such as POU5F1, SOX2, NANOG, SSEA-1. ciPSCs were capable of differentiate into 3 germ layers in vitro and in vivo. ciPSCs were capable of incorporating into tissues from all three germ layers of the chimeric chickens. This research demonstrates for the first time that ciPSCs may be a robust tool for the generation of transgenic animals with unique and specialized traits.

INDEX WORDS: Transfection, iPSCs, Minicircle DNA, Chicken, 2i/ LIF system, Chimera

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DEDICATION

I would like to dedicate this work to my parents, my mentor, my boyfriend, my brother, my aunt, and my grandma. Thank you all for indulging me in my desire to pursue research.

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CHAPTER 1

INTRODUCTION

Induced pluripotent stem cells (iPSCs) were obtained for the first time by transduction of mouse fibroblast cells with four reprogramming factors *Pou5f1, Sox2, c-Myc* and *Klf4* in 2006 [1]. Creation of iPSCs has paved the way to reprogram a cell in a restricted somatic cell state back to a pluripotent embryonic state. Since then, somatic cells have been reprogrammed by addition of selective transcription factors including *POU5F1, SOX2, KLF4, C-MYC, NANOG* and *LIN28* [2]. iPSCs have shown developmental potential equivalent to embryonic stem cells (ESCs) without the controversial use of embryos and have been heralded as a powerful tool to study regenerative medicine and modeling genetic diseases [3]. Stem cell therapies are attractive as a clinical treatment option for a variety of diseases and disorders. However, there are technical and ethical limitations for using adult or embryonic stem cells [4]. Lack of immunological compatibility has also hindered the use of embryonic stem cell for therapy as derived cells would be rejected by the body. Therefore, transplant of stem cells or their derivatives necessitate continual use of immunosuppressive drugs. However, iPSCs potentially overcome this challenge. Somatic cells isolated from a patient can be reprogrammed to "patient-specific iPSCs" that can be used to replace diseased cells in the same individual [5]. As the reprogrammed cells have been isolated from the patient's own body, theoretically these cells and cells derived from them would be recognized as self, thus eliminating potential immunological rejection. iPSCs can also

be utilized to generate different transgenic animals which have broad implications for agricultural and biomedical applications [6-7].

To create better chickens through transgenic means, chicken primordial germ cells (cPGCs) and embryonic stem cells (cESCs) have been generated using similar methods as previously done in the mouse [8-12]. However, these cells are often challenging to maintain in long term culture, have limited plasticity and produce highly variable and often low numbers of transgenic animals [9, 13], and their ability of forming transgenic animals will decrease or lose in a limited passage numbers. In addition, these systems often require mouse or rat feeder cells, adding a potential contaminating cell source, and complex media formulations and conditioning to maintain cESCs or cPGCs in a pluripotent state [13-15]. iPSCs could potentially overcome these challenges as they have proven to be relatively easy to culture long term in other species and maintain their potential to form chimeric animals [6-7, 16].

iPSCs are generated by the overexpression of a combination of exogenous pluripotent transcription factors in an adult somatic cell leading to the reversion of this mature cell to an embryonic or inner cell mass-like state [1]. The immortal and highly proliferative characteristics of iPSCs potentially make them uniquely suited for the development of transgenic animals. These characteristics allow for gene targeting and for complex genetic manipulation of these cells, which is not possible using current systems in avian species. iPSCs have been produced in other agriculturally important species including the pig [17-18], cow [19-20], sheep [21], horse [22] and of most relevance the quail [7], however, chicken iPSCs have never been reported. And in the case of the pig and quail, iPSCs have shown significant potential in producing transgenic animals [6-7]. This suggests that iPSCs may be a new and more robust cell type to create transgenic chickens with agriculturally and biomedically important characteristics.

This study sought to derive chicken induced pluripotent stem cells (ciPSCs) from fibroblast cells utilizing a minicircle DNA based reprogramming approach, maintaining ciPSCs in a pluripotent state in culture and study ciPSCs differentiation in vitro and in vivo with hopes of ultimately producing chimeric chickens that are capable of passing on transgenes to the next generation. This study were designed to test the following aims: 1) optimize transfection efficiency by using different transfection reagents and reprogram chickens embryonic fibroblasts using minicircle DNA, 2) re-transfect ciPSCs to improve their pluripotency and compare different established stem cell culture systems in maintaining ciPSC pluripotency, 3) determine proliferation and telomerase activity of ciPSCs and try to differentiate ciPSCs in the form of embryoid bodies, 4) inject ciPSCs into stage-X embryos and check their ability to incorporate into different tissues.

CHAPTER 2

LITERATURE REVIEW

GENERATION INDUCED PLURIPOTENT STEM CELLS FROM SOMATIC CELLS: PLURIPOTENCY NETWORK, DIFFERENT METHODS AND RECENT PROGRESS

Generation of Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) provide an exciting new tool to create transgenic animals, genetic disease models, to study differentiation in vitro and have broad implications for agricultural and biomedical applications. iPSCs are generated by the overexpression of a combination of exogenous pluripotent transcription factors in somatic cells leading to the reversion of this mature cell to an embryonic or inner cell mass-like state [1]. iPSCs are shown to be very similar to embryonic stem cells (ESCs). These cells are highly proliferative, express genes and surface proteins similar to ESCs and are capable of in vitro and in vivo differentiation into cells representing all three germ layers. Moreover, iPSCs from quail, mouse, rat and pig have been shown capable of incorporating into developing embryos and producing chimeric animals [7, 16, 18, 23-24]. The generation of chimeric animals that are then capable of passing on transgenes to the next generation is a critical step in the production of a stable line of transgenic animals. The immortal and highly proliferative characteristics of iPSCs make them uniquely suited for the development of transgenic animals. These characteristics allow for gene targeting and for complex genetic manipulation of these cells, which is not possible using current systems. The ability to delete, add or introduce targeted mutations in specific genes in a

permanent or conditional manner and then to generate animals from manipulated cells has revolutionized the way in which the mouse has been used as a biomedical and basic science tool [25-26]. This paradigm with iPSCs could be easily applied to other species, such as the chicken for basic science and agricultural purposes.

A variety of reprogramming methods have been developed to derive iPSCs with most approaches falling into one of four main categories: integrating, excisable, non-integrating and DNA free (Table 2.1) [27]. Originally, retroviruses were used to introduce transcription factors *Pou5f1*, *Sox2*, *Klf4* and *c-Myc* into the nucleus [1]. However, more recent attention has shifted to other techniques to perform reprogramming. These techniques include the use of lentivirus, adenovirus, plasmid, minicircle DNA, and the piggyback transportation system and even non-DNA methods such as the use of mRNA, protein and micro-RNA in reprogramming.

Table 2.1: Methods for reprogramming somatic cells to iPSCs [27].

Vector type	Cell type	Advantage	Disadvantage	References
Integrating	Retroviral	Reasonably efficient	Genomic integration, incomplete proviral silencing and slow kinetics	[28-31]
	Lentiviral	Reasonably efficient and transduces dividing and non-dividing cells	Genomic integration and incomplete proviral silencing	[32-35]
Excisable	Transposon	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines	[36]
	LoxP-flanked lentiviral	Reasonably efficient and no genomic integration	Labor-intensive screening of excised lines, and loxP sites retained in the genome	[37]
Non-integrating	Adenoviral	No genomic integration	Low efficiency	[38]
	Plasmid	Only occasional genomic integration	Low efficiency and occasional vector genomic integration	[39-40]
DNA free	Protein	No genomic integration, direct delivery of transcription factors and no DNA-related complications	Low efficiency, short half-life, and requirement for large quantities of pure proteins	[41-42]
	mRNA	No genomic integration, bypasses innate antiviral response, faster reprogramming kinetics, controllable and high efficiency	Requirement for multiple rounds of transfection	[43]
	MicroRNA	Efficient, faster reprogramming kinetics than commonly used lentiviral vectors, no exogenous transcription factors and no risk of integration	Lower efficiency than other commonly used methods	[44]

Integrating Methods

Retrovirus Mediated Ectopic Expression of Transcription Factors

Reprogramming Mouse Somatic Cells

Numerous studies have used transcription factors delivered through retrovirus to induce fibroblasts reversion into pluripotent stem cells [1, 45-46]. The initial contributors to this field were Yamanaka and Takahashi who successfully reprogrammed mouse embryonic and adult fibroblasts to a pluripotent stem cells like state after introducing *Pou5f1, Sox2, Klf4* and *c-Myc* through retrovirus mediated transduction [1]. These iPSCs exhibited the morphology and growth properties of ESCs and expressed ESC marker genes. Pluripotency was also confirmed by the ability of these cells to form embryoid bodies and teratomas that showed differentiation into ectoderm, endoderm and mesoderm cells. Even through these cells were similar to ESCs, they failed to generate chimeras following injection into blastocysts.

Germline-competent iPSCs where soon generated in 2007[16] by choosing Nanog as the selection marker to obtaining fully reprogrammed iPSCs. The Nanog selection system was achieved by inserting a green fluorescent protein (GFP) - internal ribosome entry site (IRES) – puromycin resistance gene ($Puro^r$) cassette into the 5' untranslated region of the Nanog gene. ESCs were then generated that carried the Nanog-GFP-IRES- $Puro^r$ construct that were GFP+, but became negative when differentiated. These modified ESCs were used to generate transgenic mice from which mouse embryonic fibroblast cells (MEFs) were isolated. The MEFs were then transduced with *Pou5f1, Sox2, Klf4* and *c-Myc* and GFP positive colonies were selected. Nanog GFP+ selected iPSCs were comparable to ESCs in morphology, proliferation, teratoma formation, gene expression and propensity to form chimeras. However, approximately 20% of chimeric offspring developed tumors attributed to reactivation of the *c-Myc* transgene. The reactivation of the c-Myc transgene potentially leading to tumor formation has lead to a concerted to replace retrovirus-mediated system with reprogramming systems that do not result

in the integration of reprogramming factors. This would eliminate the potential of reactivation of these genes in transplanted cells.

Mouse neural stem cells have been reprogrammed into iPSC exploiting endogenous gene expression of SOX2 and overexpressing other key reprogramming genes [47]. Mouse neural stem cells were reprogrammed in the presence of the transcription factors *Pou5f1, Klf4* and *c-Myc* and the endogenous *Sox*2. The reprogrammed cells differentiated into cells of the three germ layers in vitro and in vivo and contributed to mouse development in vivo. These results suggest that cell types that already express one of the four reprogramming factors at appropriate levels does not require its ectopic expression.

Mouse neural stem cells have also been reprogrammed into pluripotent stem cells using only two transcription factors [48].Mouse neural stem cells express higher endogenous levels of Sox2 and c-Myc than embryonic stem cells, which suggested that a two factor combination may be enough to reprogram these cells into iPSCs. Pou5f1 and Klf4 or Pou5f1 and c-Myc were used to transduce mouse neural stem cells and were shown to successfully generate iPSCs. These two-factor iPSCs were similar to embryonic stem cells at the molecular level, and contributed to the development of germline chimaeras. A similar study reported that exogenous expression of Pou5f1 alone was sufficient to generate pluripotent stem cells from adult mouse neural stem cells [49]. These one-factor induced pluripotent stem cells are similar to ESCs in vitro and in vivo. They can efficiently differentiate into cardiomyocytes and germ cells in vitro and are also capable of teratoma formation and germline transmission. Their result demonstrated that Pou5f1 is sufficient for reprogramming mouse neural stem cells to pluripotency. In this study, iPSCs could be generated without c-Myc, which is promising as reactivation of c-Myc has been closely linked to tumor formation [16]. It is also possible to generate iPSCs from mouse embryonic

fibroblasts without *c-Myc* transduction. Nakagawa et al demonstrated that Pou5f1, Sox2, and Klf4 could also generate iPSCs from MEFs [50]. The exclusion of *c-Myc* yielded less than 10% of the number of iPSCs when compared to cocktails including *c-Myc* and took twice as long. It may be that different cell types require different levels of expression for efficient reprogramming. Thus, cell type may dictate appropriate reprogramming factors.

Reprogramming Human Somatic Cells

Human iPSCs have been generated from adult human dermal fibroblasts through retroviral transduction of *POU5F1, SOX2, KLF4 and C-MYC*. These human iPSCs were similar to human ESCs in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. Furthermore, these cells could differentiate into cell types of the three germ layers in vitro and in teratomas [45]. Advances have now lead to the reprogramming of human fibroblasts into iPSCs with only *POU5F1* and *SOX2* and the addition of the histone deacetylase inhibitor valproic acid (VPA) [51]. VPA is a US Food and Drug Administration-approved treatment for epilepsy and therefore the use of VPA in the reprogramming phase may not limit the ability of these cells to be transitioned a clinical setting [52]. These results demonstrate that small-molecules can replace at least some of the transcription factors used to reprogram cells and moving the field towards a safer reprogramming system [51].

Other human cell types have been utilized in the reprogramming process and have demonstrated that cell source can have a significant effect on reprogramming efficiency. Human keratinocytes have also been reprogrammed to generate iPSCs by retroviral transduction with POU5F1, SOX2, KLF4 and c-MYC [53]. The result showed that the reprogramming of human

keratinocytes was at least 100-fold more efficient and two fold faster compared with reprogramming of human fibroblasts. These reprogrammed cells completely lost the keratinocyte specific marker expression and expressed the pluripotency markers REX1, CRIPTO, SSEA3, SSEA4, TRA1-60, TRA1-81 and showed stem cell developmental potential in vitro and in vivo. Reprogramming of keratinocytes to pluripotency provides a valuable experimental model to investigate the role of cell source on reprogramming and at a practical level to hasten the speed at which iPSCs can be generated for therapy. The majority of studies have used retrovirus to transduce cells for reprogramming. This approach has several disadvantages including the requirement of proliferating cells for infectivity and the occurrence of random insertional mutagenesis. As a result, other techniques are being explored to perform iPSC reprogramming.

Lentivirus Mediated Ectopic Expression of Transcription Factors

Lentivirus has been considered as a gene transfer vector since it can infect both proliferating and non-proliferating cells. The disadvantages of using a lentivirus vector include its limited insertion size and again the incorporation of the reprogramming factors DNA.

However this system is highly efficient relative to other approaches. Yu et al showed successful generation of human iPSCs from human fibroblasts by lentiviral transduction with *POU5F1, SOX2, NANOG* and *LIN28* [54]. These human iPSCs had normal karyotypes, expressed telomerase activity, expressed cell surface markers and genes identical to human ESCs. Similar to their mouse counterparts, these cells maintained their developmental potential to differentiate into advanced derivatives of all three primary germ layers. This method avoided the use of the c-MYC oncogene.

A drug inducible transgenic system has been used for direct reprogramming of multiple somatic cell types [55]. Primary fibroblasts were infected with dox-inducible lentiviruses encoding the four reprogramming factors *Pou5f1, Sox2, Klf4* and *c-Myc*. After blastocyst injection, chimeric mice were generated using puromycin selection. Cells derived from these chimeras were genetically homogenous secondary somatic cells that carry reprogramming factors as defined by doxyclycine (dox) inducible transgenes. These cells can be reprogrammed upon dox exposure and have reprogramming efficiencies 25 to 50 fold greater than observed by primary transduction. Thus reprogramming occurs without the need for viral infection. This technique facilitates the generation of large numbers of genetically identical donor cells and should be advantageous for genetic or chemical screening to improve reprogramming.

Most prior studies have required multiple viral vectors for reprogramming, resulting in high numbers of genomic integration sites in iPSCs. Sommer et al reported the use of a single lentiviral vector expressing a "stem cell cassette" composed of the four transcription factors and a combination of 2A peptide and internal ribosome entry site technology[56]. The derived iPSCs were revealed to have only a single integrated viral copy, in contrast to previous reports using multiple vectors, which required more than 15 viral integrations [1, 57]. This significantly reduces the risks of insertional mutagenesis and viral reactivation. Furthermore, a single vector encoding a stem-cell cassette represents an important step toward the removal of the viral genome and could potentially be achieve using loxP/Cre technology.

Excisable Methods

Transposon-based reprogramming can generate iPSCs without transgene integration by removing transgenes through a transposase expression system, which is an important advance

towards clinically acceptable methods of deriving reprogrammed cells [58-59]. The Woltjen group demonstrated successful and efficient reprogramming of murine and human embryonic fibroblasts using a piggyBac (PB) transposon/ transposase system induced by doxycycline with individual PB insertions being successfully removed from established iPSC lines. Specifically, the genes (*c-Myc, Klf4, Pou5f1* and *Sox2*) were transferred into the PB-TET transposon plasmid under the transcriptional control of the tetO2 tetracycline/ doxycycline inducible promoter. The MEFs were transfected with the plasmid DNA in conjunction with a PB transposase expression plasmid in doxycycline-containing ESC culture condition. Colonies showed ESC like morphology and they were positive for the alkaline phosphatase, SSEA-1 and NANOG stem cell markers. RT-PCR revealed the expression of ESC pluripotency markers. Most importantly, the transgenes were able to be removed from the host genome. In many cases, researchers use transfection to deliver transposon/ transposase into cells, so this method eliminates the need for specialized biohazard containment facilities or the production of high-titer, limited-lifetime viral stocks [27]. This method enables accurate transgene removal through transposase expression system [59]. However, individual PB insertions cannot be removed entirely when transposase is added, so it needs further validation and labor-intensive screening of excised lines.

Another excisable version of reprogramming is the use of a single loxP-flanked lentiviral vector that upon removal generates iPSCs free of integrated transgene [60-61]. This approach successfully removes transgene sequences, but leaves behind residual vector sequences, which can still create insertional mutations. Somers et al reported the use of a single vector composed of a “stem cell cassette” (STEMCCA) encoding all four reprogramming factors, *POU5F1, SOX2, KLF4* and *C-MYC* which was flanked by loxP sites to achieve highly efficient reprogramming of postnatal human skin fibroblasts [60]. By using the single lentiviral vector,

iPSC clones containing a single integration were achieved. Transgene free subclones were achieved by transient transfection of iPSCs with a plasmid expressing Cre and a puromycin resistence (resistant??) gene in puromycin containing medium. Although exogenous transgenes were removed from the surviving subclones, about 200 bp of an inactive viral LTR remains in the host genome after excision, thus issue of insertional mutagenesis is not completely eliminated [60].

Non-integrating Methods

Adenoviral Mediated Method

The virus based reprogramming benefits from higher reprogramming efficiency compared to other methods. However, a major limitation is the use that these viral methods result in genome integration. Adenoviral reprogramming may provide an improved method for generating iPSCs as it does not cause integration of exogenous genes into the host genome. Integration-free mouse iPSCs have been derived from liver cells with adenoviral vectors [62], but the low frequencies obtained make it unclear how practical these approaches will be for human cells [54]. Matthias et al generated iPSCs from mouse liver cells using adenoviral vectors that allow for transient, high-levels of expression of exogenous genes without integrating into the host genome [62]. Mouse fetal liver cells were infected with the adenovirus containing *Pou5f1, Sox2, c-Myc* and *Klf4* under the control of the CMV promoter. iPSC colonies expressed pluripotency markers Oct4, Sox2, Klf4 and SSEA-1 and produced teratomas that can differentiate into three germ layers after injection into the nude mice. The efficiency of deriving iPSCs from fetal liver cells using adenovirus is 0.0001% to 0.001%, which is significantly lower than that obtained with integrating virus (0.01% to 0.1%). Lower reprogramming efficiency is

believed to occur because cells do not maintain viral expression long enough to trigger entry into a state sustained by endogenous pluripotency factors [63-64]. Genomic DNA PCR showed no exogenous genes integrated and southern blot analysis yielded no evidence for the continuous presence of the adenoviral sequences in iPSCs. However, the possibility that a small pieces of adenoviral DNA could have inserted into the genome of adeno-iPSCs could not be ruled out because of the detection limits of southern blot analysis.

Plasmid Mediated Method

Integration-free mouse iPSCs have also been derived from embryonic fibroblasts with repeated plasmid transfections [65]. Repeated transfection of the plasmids containing *Pou5f1*, *Sox2*, *Klf4* and *c-Myc* induced iPSCs from mouse embryonic fibroblasts. Mouse iPSC colonies were obtained without evidence of integration in 6 out of 10 experiments. Although iPSC colonies with genomic integration were frequently observed, its estimated rate was significantly lower than that of viral induction and genomic PCR screening can easily distinguish iPSCs that have integrated reprogramming factors. Therefore lines with integrated reprogramming factors could be potentially eliminated. Human iPSCs free of vector and transgene sequences have been derived from fibroblasts by a single transfection with oriP/EBNA1 (Epstein-Barr nuclear antigen-1) based episomal vectors [66]. Similar to mouse studies, the reprogramming efficiency of human fibroblasts with oriP/EBNA1 vectors is low (~3 to 6 colonies/ 10^6 input cells).

Minicircle Mediated Method

Minicircle DNA, a unique plasmid, has been used in producing non-integrating iPSCs in human cells. Plasmids currently used for gene transfer have the disadvantage of carrying a

bacterial origin of replication and an antibiotic resistance gene. Therefore there is a risk of uncontrolled dissemination of the therapeutic gene and the antibiotic resistance gene [67]. Minicircles are supercoiled DNA molecules for nonviral gene transfer which are free of th bacterial plasmid backbone elements and therefore do not activate silencing mechanisms to the same extent as plasmids [68]. They are thus smaller and potentially safer than the standard plasmids currently used for overexpression and benefit from longer ectopic expression due to its lower activation of silencing mechanisms [69]. In fact, minicircles exhibit two- to 10-fold higher reporter gene activity than standard plasmids resulting in improved gene delivery [67].

Wu reported the generation of transgene-free iPSCs from human adipose stromal cells (hASCs) using nonviral minicircle DNA [70]. The minicircles used to reprogram hASCs contain a single cassette of four reprogramming factors (*POU5F1, SOX2, NANOG* and *LIN28*) plus a green fluorescent protein (GFP) reporter gene, each separated by self cleavage peptide 2A sequence. Minicircle DNA was nucleofected into hASCs and resulted in iPSCs that displayed standard pluripotency character and functionality. The reprogramming efficiency was substantially lower (~0.005%) compared to integrating viral-based methods, which have typically been reported to be ~0.01% [16, 57, 71], however efficiency is still higher than previous plasmid-based transfection reprogramming methods [66, 72]. Thus, minicircle reprogramming provides a simple method for generating transgene-free iPSCs that requires only a single vector without the need for subsequent drug selection or vector-excision. Minicircle DNA is already FDA approved, giving its potential value in clinical and agricultural applications.

DNA Free Methods

mRNA Mediated Method

Although DNA transfection-based methodologies are relatively safe, they nonetheless entail some risk of genomic recombination or insertional mutagenesis and even the PiggyBac transposon system can potentially leave a foot print. An mRNA-based approach could offer several advantages in this case. First, mRNAs are directly translated into functional proteins in the cytoplasm with proper post-translational modification, which does not lead to any genetic modification of the host genome [73]. Second, mRNAs can be introduced into cells much easier than DNAs because of the smaller size, resulting in higher efficiency and much lower cytotoxicity. The disadvantage of mRNA is that they are degraded by the cells in 2-3 days so expression duration is very short, thus it requires multiple rounds of transfection leading to higher levels of toxicity and fewer reprogrammed cells. Plews et al reported transfection of human fibroblasts with a mixture of mRNAs encoding *POU5F1, SOX2, C-MYC, KLF4* resulted in significantly increased expression of endogenous pluripotent markers such as *POU5F1, NANOG, DNMT3β, REX1 and SALL4* [73]. Warren et al described a modified mRNA based method to reprogram human cells to pluripotent state with a surprisingly high reprogramming efficiency (1.4%) and rapid reprogramming kinetics [74].

Protein Mediated Method

Another non-DNA reprogramming method involves the delivery of combined reprogramming proteins directly into the target cells [75]. A major hurdle for this method is the difficulty to purify large amount of proteins and the limited ability for macromolecules such as proteins to cross the cellular membrane. Kim and his colleagues reported generation of stable

iPSCs from human fibroblasts by directly delivering four transcription factors (*POU5F1, SOX2, NANOG* and *LIN28*) fused with a cell penetrating peptide (CPP) [75]. These protein-induced human iPSCs exhibited similar characteristics to human embryonic stem cells in morphology, and express similar pluripotent markers. The DNA-free protein transduction system eliminates the potential risks of chromosome integrations and mutations caused by viral or any other DNA-based reprogramming methods. However, the generation of iPSCs is very slow and inefficient. In particular, the whole protein extracts limit the concentrations of reprogramming factors delivered in the target cells, thus purified proteins are required to deliver into cells in the future.

MicroRNAs Mediated Method

Recently, a new method using microRNAs to reprogram cells into iPSCs has been developed [76]. MicroRNA-iPSCs were generated by transfecting mature miRNAs (mir-200c, mir-302s and mir-369s) into human adipose stromal cells (ASCs) and human dermal fibroblast (HDFs). Although miRNAs-based methods have faster reprogramming kinetics than commonly used lentiviral or retroviral vectors and achieved non exogenous transcription factor integration, its efficiency is extremely low compared to other commonly used methods [27]. In all, DNA-based methods requires only minimal molecular biology background compared to mRNA and protein-based methods, so it may become a more attractive option for a wider population of researchers interested in reprogramming.

Pluripotency Network of the Induced Pluripotent Stem Cells

Somatic cells have been reprogrammed by the addition of selected transcription factors including *POU5F1, SOX2, KLF4, C-MYC, NANOG* and *LIN28* [2]. These factors alone or in

combination can be directly introduced into somatic cells through viral vectors or non-viral system and reprogram somatic cells into a pluripotent state. The mechanism of reprogramming is a complex process and it is unclear what role each transcription factor plays towards driving a somatic cell to pluripotency. Though, recent studied have shed light on the role played by transcription factors to promote pluripotency.

POU5F1is a member of the octamer-binding family of transcription factors and is a key regulator of stem cell pluripotency [77]. It has been shown that POU5F1 expression is limited to the inner cell mass (ICM) in the developing embryo and is maintained in adult germ cells but down regulated in differentiated somatic cells [77]. It has been well established that POU5F1 plays an essential role as a major regulator of pluripotency. POU5F1 expression levels are an important factor in determining the differentiation of somatic cells. If it is over expressed then ESCs will differentiate into primitive endoderm and mesoderm, while loss of expression results in spontaneous differentiation into ectoderm, endoderm and mesoderm. Thus a balance of POU5F1 is required in maintaining the pluripotency of ESCs [78]. Although different cocktails of transcription factors are reported in reprogramming, one of the characteristic features common to various studies is the invariable requirement of POU5F1 as a reprogramming factor. Recent genome-wide studies have identified the downstream targets of POU5F1 include genes encoding for self-renewal factors, lineage-specific factors, signaling molecules and DNA damage response sensors [79-80]. Thus POU5F1 is implicated in a broad spectrum of cellular processes that collectively specify the self-renewal state of the ESCs.

Another key reprogramming factor is SOX2. A transcription factor that belongs to a superfamily of proteins that all possess a high mobility group (HMG) box DNA-binding domain. It plays an important role in maintaining pluripotency in the early embryo. SOX2 is expressed in

the ICM, germ cells, epiblast of the early embryo [80-81]. SOX2 deficient mouse embryos fail to maintain a pluripotent epiblast, and die at the implantation stage [81]. Also, a decreased level of SOX2 in ESCs caused a loss of pluripotency and differentiation [82]. The role of SOX2 as a co-factor of POU5F1 in regulating downstream target genes in ESCs is well recognized. Genome-wide ChIP analysis revealed that POU5F1 and SOX2 bind DNA co-operatively by forming a heterodimers to control the expression of ES cell-specific genes [83]. By introducing combinations of three factors *POU5F1, KLF4* and *C-MYC*, iPSCs without *SOX2* failed to become pluripotent, highlighting the pivotal role of SOX2 [1].

NANOG is a homeobox transcription factor that works synergistically with POU5F1 and SOX2 in the activation of pluripotency networks. Nanog was discovered based on its ability to maintain ESC self-renewal in the absence of leukemia inhibitor factor, a critical signaling factor commonly used to maintain cultured mESC in an undifferentiated state [84]. NANOG was originally proposed as a transcription repressor that inhibits the expression of the genes important for cell differentiation [85]. However, Pan et al revealed that it actually contains two unusually strong transactivators at the C-terminus, suggesting that it primarily acts to activate those genes directly involved in maintaining stem cell pluripotency [86]. CHIP analysis has shown that the NANOG promoter is a direct target of the POU5F1/SOX2 proteins [80, 87]. NANOG has been shown to increase reprogramming efficiency and pluripotency with mouse iPSCs selected for NANOG expression resulting in germline-competent iPSCs, increased ESC gene expression and ESC DNA methylation patterning [16].

Two groups have mapped the transcriptional regulatory networks in embryonic stem cells [79-80]. Both studies have found POU5F1 and NANOG to co-occupy and share a substantial portion of their target genes [79-80]. POU5F1 and SOX2 maintain pluripotency by promoting

the expression of downstream self-renewal genes while simultaneously repressing the activity of differentiation-promoting genes. To map the interactome that defines pluripotency, Wang et al interrogated the protein interactions of key pluripotency genes. Genome-wide ChIP analyses showed that POU5F1, SOX2 and NANOG also bound the know pluripotency factors *Esrrb, Rif1* and *Sall4*[79-80]. In an independent study, Wu showed that SALL4 physically interacts with NANOG. ChIP analysis has further revealed considerable overlap in the binding sites of NANOG and SALL4, suggesting co-regulation of downstream target gene that include *Nanog, Sall4, Pou5f1* and *Sox2*. These findings indicate that auto- and co-regulatory feedback loops are common phenomena in the ESC transcriptional circuitry.

C-MYC, KLF4 and LIN28 have more recently been added to the list of critical pluripotency genes with the development of iPSCs. C-MYC is an oncogenic protein known to elicit oncogenic transformation, while KLF4 can function both as a tumor suppressor and an oncogene [88-89]. KLF4 promotes cell proliferation by suppressing p53 or suppress proliferation by activation p21 [89]. C-MYC acts as an opposing force by inducing p53-dependent apoptosis in primary fibroblasts [88]. Based on these observations, it has been postulated that the balance between KLF4 and C-MYC may be important in regulating growth and self-renewal of iPSCs [1]. Recent work indicates that NANOG can perform the roles of KLF4 and C-MYC in human somatic cell reprogramming, indicating that these genes are not necessary in the reprogramming process [54]. However, the inclusion of these genes increases efficiency of iPSC derivation. KLF4 promotes cell proliferation via the mediation of p53 suppression [89]. Since p53 is a negative regulator of NANOG [90], KLF4 may have a functional role in the activation of NANOG during reprogramming. Therefore, overexpression of NANOG could negate the need for activation through the Klf4/p53 pathway. Interesting, *c-Myc* is a target of NANOG in mouse

ESCs [79]. Taken together, NANOG may regulate and recruit the downstream endogenous expression of KLF4 and C-MYC during reprogramming. LIN28 may activate endogenous Myc proteins by enhancing translational of insulin-like growth factor-2 [91] .

In summary, the recent success in reprogramming somatic cells into a pluripotent state by introduction a few defined factors has initiated a new understanding of pluripotency. Basically, the expression of exogenous transcription factors will trigger the somatic cells entry into a state sustained by endogenous pluripotency factors. However, the molecular mechanisms driving reprogramming await further investigation. The reprogramming of somatic cells into pluripotent cells was originally achieved by transduction of retroviral or lentiviral vectors containing four transcription factors. These methods caused insertional mutagenesis by integration both viral backbones and transgenes permanently into the host genome. Recent studies have shown significant technical progress in improving reprogramming. Non-viral methods, excisable methods and DNA free methods such as mRNA-based, protein-based and microRNA-based approaches have been used in generating iPSCs aimed at causing minimum interference to the host genome. However, simple and efficient reprogramming approaches are still strongly required.

CHAPTER 3

PLURIPOTENCY IN AVIAN SPECIES AND AN EXAMINATION of THE EPIGENETIC CHARACTERISTICS OF IPSCs

Pluripotency in Avian Species

The great possibilities and potential gains of genetically engineered chickens has long been recognized with characteristics of interest including increased meat and egg production, development of eggs for generating recombinant and therapeutic proteins, monoclonal antibodies and other economically valuable traits [92-97]. To achieve this, a number of groups have used cellular approaches to generate transgenic animals utilizing cESC and primordial germ cell (PGC) lines. cESCs were first established in vitro from blastodermal cells taken from stage X embryos and maintained on murine embryonic fibroblasts feeders [8]. PGCs are precursors of sperm and eggs [98]. In avian embryos, PGCs are first identified in an extra-embryonic region, the germinal crescent, after approximately 18h incubation. After 50-55 h of development, PGCs migrate to the gonad and produce functional sperm and oocytes [99]. Although cESC and cPGC lines have been established and been incorporated into chimeric animals [10-12, 100], the use of these cells has not become widespread.

cESCs and cPGCs have proven to be challenging to culture with low proliferation rates, high levels of cell death and typically lose their ability to form chimeric animals after only a limited number of passages [9, 13]. A number of previous reports showed that chicken PGCs [9, 100] and ESCs [101] contributed to chimeras when injected into embryos immediately after

collection from the donor embryo or after only a few passages, normally < 10 passages. In addition, cESCs and cPGCs often require co-culture with mouse or rat feeder cells, potentially adding a contaminating cell source, and complex media formulations to maintain them in a pluripotent state [13-15]. It is currently unclear what kind of factors these feeder cells secrete into the media to keep cESCs and cPGCs pluripotent. Additionally, it is technically difficult and laborious to isolate and purify large quantities of cESCs and cPGCs because of the short window in which these cells can be collected and the limited number of cells in an embryo.

Given some of the issues that plague the isolation and development of avian ESCs and PGC lines, iPSCs maybe a superior option to generate transgenic animals as they have proven to be relatively easy to culture long term in other species and maintain their potential to form chimeric animals [6-7, 16]. Quail iPSCs (qiPSCs) have been generated by transduction with six human factors *POU5F1, NANOG, SOX2, LIN28, KLF4,* and *C-MYC [7]*. qiPSCs were strongly AP and PAS positive, proliferated at a high rate and possessed telomerase activity levels comparable to ESCs. They expressed ESC makers, form embryoid bodies and differentiated into cells of all three germ layers. Under directed neural differentiation conditions, qiPSCs differentiated into neurons, astrocytes, and oligodendrocytes. Most importantly, qiPSCs showed incorporation into chimeric embryo and generation of live chimeric offspring. These qiPSCs are relatively easy to genetically modify and clonally isolate compared to ESCs or PGCs. After serial subculture in vitro, qiPSCs still efficiently incorporated into tissues from all three germ layers in chimeric embryos at passage 26 and 45 [7]. With the successful generation of qiPSCs and incorporation into chimeras, the generation of chicken iPSCs should not be far away.

Assessment of Pluripotency

A variety of assessment assays have been developed and used for evaluating similarities between iPSCs and ESCs. Assessing reprogramming begins with stem cell like morphology. Pluripotent stem cells are expected to form compact colonies that have distinct borders with well-defined edges and are comprised of cells with large nucleoli and a high nucleus to cytoplasm ratio. Positive staining for alkaline phosphatase activity has been widely accepted as a marker of pluripotency. Fully reprogramming cells express a network of pluripotent markers including POU5F1, SOX2, NANOG, TRA-1-60, TRA-1-81,DNMT3β, and REX1[27]. Genome-wide epigenetic reprogramming is crucial for deriving fully reprogrammed cells which is often evaluated by assessing the methylation status of pluripotency gene promoters, as well as the genes driving differentiation. Pluripotent stem cells are expected to show low levels of methylation of pluripotency genes and high levels of methylation of genes associated with differentiation. Another pivotal event during epigenetic reprogramming is the reactivation of the silent X chromosome. It happens late in reprogramming and represents a hallmark of naïve state pluripotency, a state where cells have the highest level of plasticity [64, 102]. If iPSCs acquire all of these molecular features, they are expected to behave like ESCs and to demonstrate reprogramming factor independence, which is marked by silencing of the viral transgenes.

In addition to showing these molecular features, iPSCs also need to be assessed in various functional assays to be confirmed as truly pluripotent. Characterization of the functional abilities of iPSCs begins with in vitro differentiation, which normally include three dimensional embryonic body (compact balls of organized cells that are said to resemble the early developing embryo) differentiation in culture. This assay induces pluripotent stem cells to spontaneously differentiate into cells of all three germ layers. Then markers of the three gem layers are checked

by RT-PCR, immunocytochemistry or similar techniques. Teratoma formation is a more stringent in vivo differentiation assay when pluripotent stem cells are again stimulated to differentiate into cells of all three germ layers. In this assay, iPSCs are injected subcutaneously into immunodeficient mice leading to tumor or teratoma formation. Teratomas are dissected and then examined for ectoderm, endoderm and mesoderm cell types to determine iPSC differentiation potential.

The gold standard assay for assessing iPSC pluripotency is assessment of the ability of these cells to integrate into the developing embryo and form chimeric animals. iPSCs are introduced into blastocyst stage embryos and are then examined for normal contribution in embryos. In addition to assaying contribution to ecto-, endo-and mesoderm, emphasis is also placed on incorporation into the germline. Cells that have higher levels of plasticity are capable of contributing to the germline, while more restricted cell are not. Germline transmission is assessed by the ability of chimaeras to produce offspring that have the genome of the introduced iPSC line. The most stringent assay for iPSCs is tetraploid offspring generation. This assay measures the ability of the iPSCs to develop into the entire organism after injection of iPSCs into tetraploid blastocysts. This assay has only been successful with mouse iPSCs [103-104].

A Look at the Epigenetic Changes During Somatic Cell Reprogramming

Overcoming epigenetic barriers

Successful reprogramming requires the inactivation of the somatic cell program and reactivate of the ESC specific transcription programs of self-renewal and pluripotency. The mechanisms by which reprogramming occurs and the chromatin organization that underlies the reprogramming process are largely unknown. Generally, at the molecular level, completely

reprogrammed iPSCs show transcriptional patterns that are highly similar to those in ESCs, as well as DNA demethylation of the promoter regions of pluripotent genes and in female cells, the reactivation of the somatically silent X chromosome [71, 105-106]. In addition, iPSCs exhibit global patterns of histone methylation, including histones H3K4 and H3K27 trimethylation that are virtually indistinguishable from those in ESCs [16, 57].

DNA Demethylation

Reprogramming of somatic cells into iPSCs is accompanied by DNA demethylation of pluripotency genes at their promoter regions [71]. DNA methylation is one of the epigenetic processes by which gene expression is suppressed. This is achieved by recruiting methyl groups onto cytosine-C5 by DNA methyltransferases (Dnmts). The binding of the methyl group to cytosine residues cause suppression of specific gene expression. Demethylation of promoter in pluripotency related genes was required for fully reprogramming of iPSCs [107]. Partially reprogrammed cell lines often show DNA hypermethylation at pluripotency-related genes, it is hypothesized that loss of DNA methylation is a critical step in the transition from a partially reprogrammed state to pluripotency. The treatment of cells undergoing reprogramming with DNA methylation inhibitors enhanced the efficiency of reprogramming significantly and facilitated the conversion of partially reprogrammed cells into a fully pluripotent state [51, 106, 108]. Bisulfite genomic sequencing analysis of the Pou5f1 promoter showed that it was highly unmethylated in pig iPSCs established by transduction with six reprogramming factors, whereas CpG dinucleotides in these regions were highly methylated in parental pig fibroblast cells [109]. Deng and colleagues generated buffalo iPSCs (biPSCs) from buffalo fetal fibroblasts (BFFs) with four buffalo defined factors [110]. Methylation assay revealed that the promoters of Pou5f1

and Nanog were hypomethylated in biPSCs, whereas hypermethylated in BFFs and pre-biPSCs. Thus, demethylation of the promoter of endogenous pluripotent genes is likely necessary for full reprogramming of iPSCs.

Inactive X Chromosome (Xi) Reactivation in Female Cell Reprogramming

Another criterion in female cell reprogramming is the reactivation of the inactive X chromosome (Xi). Female mammals silence one of their two X chromosomes in a process called X chromosome inactivation (XCI) during early embryonic development as a mechanism to equalize X-linked gene dose between the two sexes [111]. XCI is regulated by an X-inactivation center, a locus that contains the Xist, Tsix and Xite genes, which produce non-coding regulatory DNAs. Undifferentiated female ESCs carry two active X chromosomes (Xa) and express Tsix from both X chromosomes to repress Xist expression. Upon differentiation, Xist becomes strongly upregulated to induce silencing, whereas Tsix disappears and is absent in somatic cells [112]. Since XCI is one of the most dramatic forms of heterochromatin formation associated with differentiation of pluripotent cells, an interesting question has been whether the inactive X chromosome (Xi) reactivates during reprogramming to the iPSC state. Using mouse fibroblasts, it was found that reactivation of the Xi occurs in a late step in reprogramming that roughly coincided with the reactivation of the endogenous Nanog and Pou5f1 loci [64, 71]. The recent study linked acquisition of the pluripotent state with X chromosome inactivation (XCI), which was mediated by pluripotency factors acting specifically on noncoding genes of the X-inactivation center to initiate XCI [113]. However, during the induction of pluripotency by defined factors, X chromosome reactivation is a late event in the reprogramming process [64]. These studies underscore the tight linkage between X chromosome state and pluripotent state.

Global Reprogramming to Histone Methylation Pattern in iPSCs

In addition to DNA demethylation of the pluripotent gene promoters and the reactivation of the Xi in female cells, histone patterning must also change to favor a pluripotent fate. Histone methylation plays a crucial role in epigenetic regulation of gene expression during mammalian development. In general, transcribed genes are associated with H3K4 trimethylation [114-115], whereas many silenced genes are associated with H3K27 trimethylation [116]. Genome-wide location analysis for H3K4 and H3K27 trimethylation in the Nanog-selected iPSCs, MEFs, and ESCs using ChIP [71] revealed 94.4% of the signature genes in iPSCs carried a methylation pattern virtually identical to ESCs, whereas only 0.7% of histones were methylated in a more MEF-like pattern. Pearson correlation revealed that iPSCs and ESCs were similar in their H3K27 methylation patterns, whereas MEFs clearly differed to the same extent from both iPSCs and ESCs. The treatment of cells undergoing reprogramming with histone methylation inhibitors has enhanced the efficiency of reprogramming significantly and facilitates the complete conversion of partially reprogrammed cells that would otherwise fail to reprogram [108].

In summary, ciPSCs may provide a novel and efficient approach to generate transgenic chickens compared to cESCs or cPGCs. cESCs and cPGCs are not easy to culture in vitro. First, they have high death rate and low proliferation rate. Second, their will typically lose their ability to form chimeric animals after only a limited number of passages. Third, these cells often require to be grown on a feeder layers, adding a potential contaminating cell source. In contract, iPSCs may overcome these challenges because iPSCs from other species have been reported to be relatively easy to culture long term in vitro and maintain their potential to form chimeric animals. Generally, at the molecular level, completely reprogrammed iPSCs show epigenetic patterns similar to ESCs. Reprogramming of somatic cells into iPSCs is accompanied by DNA

demethylation of pluripotency genes at their promoter regions. Another criterion in female cell reprogramming is the reactivation of the inactive X chromosome (Xi). Besides, fully reprogrammed iPSCs show histone methylation pattern similar to ESCs. The use of a cocktail of small molecules that are linked with epigenetic modifiers, such as inhibitors of histone demethylases and inhibitors of DNA methylation were shown to significantly improve reprogramming efficiency.

CHAPTER 4

NA ÏVE AND PRIMED STEM CELLS AND SIGNALING PATHWAYS INVOLVED IN PLURIPOTENCY AND SELF-RENWAL IN STEM CELLS

Somatic cells from mouse, human and other livestock have been reprogrammed using different factors combinations, including *POU5F1, SOX2, KLF4, C-MYC, LIN28* and *NANOG*. And iPSCs of different species show various levels of pluripotency reflected by fulfilling different pluripotency criterion (Table 4.1).

Table 4.1: Species-specific iPSCs show various levels of pluripotency.

Species	Express ES Cell Genes	Form EBs	Teratomas	Chimeras	Germline Chimeras	Tetraploid Offspring	References
Mouse	Yes	Yes	Yes	Yes	Yes	Yes	[1], [16]
Human	Yes	Yes	Yes	No	No	No	[45], [54], [117]
Pig	Yes	Yes	Yes	Yes	Yes	No	[6], [118]
Sheep	Yes	Yes	Yes	No	No	No	[119], [21]
Horse	Yes	Yes	Yes	No	No	No	[120], [121]
Bovine	Yes	Yes	Yes	No	No	No	[19], [20], [122]
Quail	Yes	Yes	Yes	Yes	No	No	[7]
Rabbit	Yes	Yes	Yes	No	No	No	[123]

Primed Versus Naïve Pluripotency in Reprogramming

Mouse epiblast stem cells (EpiSCs) are derived from the post-implantation epiblast of day 5.5 embryos and they depend on the FGF4 signaling pathway. In contrast, mouse ESCs are obtained from epiblast progenitors of the earlier blastocyst (day 3.5) which require LIF signaling to maintain pluripotency [124]. EpiSCs are able to differentiate in vitro into the three germ layers similar to ESCs and therefore are considered pluripotent, but opposed to ESCs, EpiSCs are almost unable to contribute to chimera [124].Therefore, EpiSCs are commonly referred to as "primed" pluripotent cells as opposed to the "naïve" pluripotency of mouse ESCs [125-126]. Mouse EpiSCs express many of the same genes as mouse ESCs including *Pou5f1, Sox2* and *Nanog* and can be induced to revert back to a naïve state when culture conditions are changed, and/ or transcriptional factors such as *KLF4, C-MYC*, or *NANOG* are overexpressed [127-128]. However, reprogramming is still not very efficient when changing EpiSCs to ESCs and the limitations are not well understood [128].

The original key to successful derivation of ESCs was co-culture with mouse embryonic fibroblasts, now known to produce the cytokine leukemia inhibitory factor (LIF). LIF activates STAT3 and either serum or bone morphogenetic protein (BMP) to induce inhibition-of-differentiation proteins [129]. Although ESCs and EpiSCs are both pluripotent as they are capable of generating derivatives of the three germ layers upon differentiation, important molecular and functional differences exist between these two pluripotent states. At the molecular level, the ESC pluripotent state is maintained by a combination of LIF/JAK/STAT3 and BMP4 signaling, while EpiSCs require a combination of bFGF and TGFβ/ Activin signaling for their continued self-renewal. The different culture conditions that maintain ESC and EpiSCs are reflected in the morphological, molecular and functional properties of these cells.

LIF and BMP Signaling

Initially, mouse ESCs were derived and cultured on a layer of mouse embryonic fibroblasts in serum-containing medium [130]. The fibroblasts were thought to provide trophic factors that support self-renewal and were hence described as feeders. Subsequent fractionation of this conditioned medium identified leukemia inhibitory factor (LIF) as the active component in the maintenance of pluripotency [131]. In 2003, it was demonstrated that serum could be replaced by the addition of BMP4 and for the first time ESCs could be grown in fully defined conditions [132].

LIF is a member of the IL6 family of cytokines that signal through the transmembrane receptor gp130 [133]. The gp130 homodimers or the heterodimers consisting gp130 and other receptors including the LIF receptor can mediate signaling that directs self-renewal. Following ligand-induced receptor dimerization, there are two main signaling pathways that become activated (Figure 4.1). The intracellular domain of gp130 recruits the tyrosine kinases Janus Kinases (JAKs) [134]. JAKs phosphorylate tyrosine residues on the intracellular domain of gp130, creating binding sites for STAT3. Receptor-bound STAT3 is phosphorylated on Tyr705 by JAKs and becomes dimerized and translocates to the nucleus where it acts to modulate transcription of target genes.

STAT3 homodimers function as transcription factors and it is critical in directing self-renewal. One group has identified c-Myc as a target of STAT3 in murine ESCs, and c-Myc played a central role in maintenance of ESCs self-renewal and in the generation of iPSCs [135]. They proposed that c-Myc functioned by blocking differentiation, but it is not clear how this is achieved nor is it clear if the effect remains dependent on the presence of serum in the culture medium. The iPSC reprogramming gene Klf4 has also been shown to be a downstream target of

LIF. Klf4 overexpression in ESCs leads to decreased differentiation in EBs and the high capacity to generate secondary EBs suggesting that it promotes self-renewal [136]. The recent identification of cMyc and Klf4 as two of the four factors in reprogramming somatic cells to iPSCs further confirmed their role in the self-renewal of ESCs, however, their mechanisms of reprogramming need to be further explored [1].

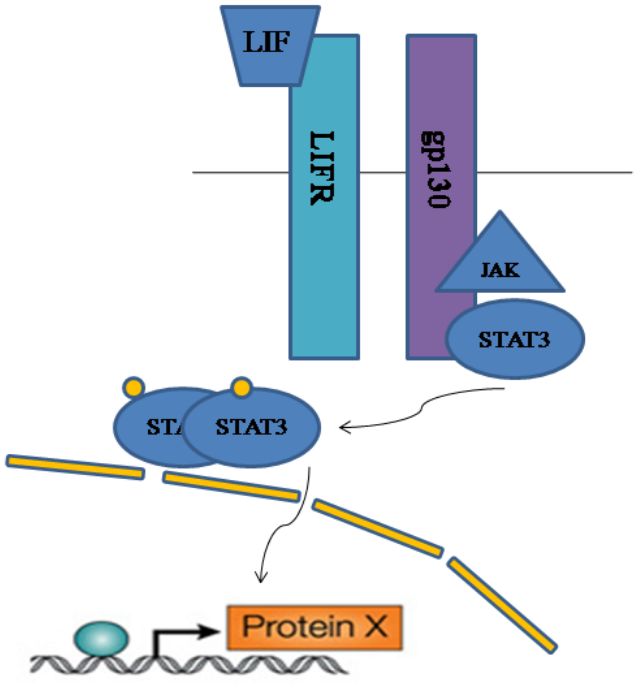

Figure 4.1: **LIF signaling.**
LIF binds to its receptor and induces dimerization with gp130. Janus Kinases (JAKs) constitutively associated with gp130 become activated and phosphorylate Tyr residues on the receptor creating docking sites for SH2 domain-containing proteins including STAT3 and Shp2. Receptor-bound STAT3 is phosphorylated by JAKs, dimerizes, translocates to the nucleus where it activates transcription of target genes presumed to promote self-renewal (adapted from [137]).

LIF is not strictly sufficient for self-renewal since the addition of serum to the medium is still required to support self-renewal. Upon removal of serum from the culture, mESCs undergo

neural differentiation, even in the presence of LIF. Ying et al proposed that inhibition of neural differentiation might support serum-free self-renewal [129]. Bone Morphogenetic Proteins (BMPs) were known to inhibit neural differentiation. They found that addition of BMP to serum-free medium containing LIF could support self-renewal of the mESCs. The BMP pathway signals through SMAD 1,5 and 8 to induce expression of ID proteins [138]. Constitutive expression of ID 1, 2 or 3 is sufficient to replace the requirement for BMP or serum. BMP ligand binds to a type II TGFβ receptor, which allows the receptor to bind to a type I TGFβ receptor and activated type I receptor by phosphorylation. The activated type I receptor can now phosphorylate the Smad proteins. Smads 1,5 and 8 are activated by the BMP family of TGFβ factors. There phosphorylated Smads bind to Smad4 and form the transcriptional factor complex that will enter the nucleus and suppress the genes committed for neural differentiation [129].

ERK1/2 Signaling

Extracellular signal-regulated protein kinase-1 and -2 (ERK1/2) are two MAPKs involved in the regulation of ESC self-renewal and in early embryonic cell fate choice. ERK1/2 function downstream of MEK1/2 and are activated in response to many extracellular molecules including FGFs. FGF ligands bind with FGF receptors resulting in activation of the receptor tyrosine kinase (RTK) activity. The phosphorylated tyrosine on the receptor is then recognized by an adaptor protein and in turn it recruits SOS which activates Ras and initiates the MAPK cascade to activate ERK1/2. Activated ERK1/2 is translocated into the nucleus and phosphorylates certain transcription factors (Figure 4.2) [139].

It has been shown that a MEK-ERK inhibitor PD inhibits differentiation of mouse ESCs because of the persistence of POU5F1 expression during EB differentiation [140]. Stimulation of

the ERK signaling pathway triggers the transition of the pluripotent ESCs from self-renewal to lineage commitment [141]. In addition, MEK-ERK pathway inhibition has been shown to be important in various reprogramming steps. Sheng Ding et al demonstrated that the reprogramming of human fibroblast cells was significantly improved by adding PD 0325901 into the medium, which inhibits MEK-ERK pathway [142]. Therefore, by blocking lineage commitment induced by MEK-ERK signaling pathway, the ground state of ESC self-renewal could be maintained [143].

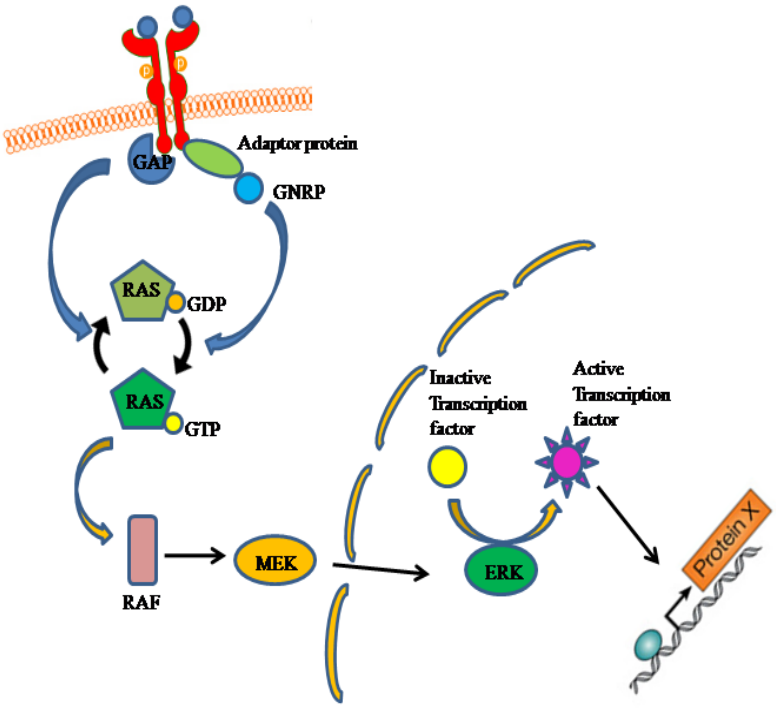

Figure 4.2: The RTK pathway.
Ligands such as FGF, EGF, and stem cell factor bind to receptor tyrosine kinase (RTK), RTK undergoes dimerization and autophosphorylation. The phosphorylated tyrosine on the receptor is then recognized by an adaptor protein. The adaptor protein activates a G protein, called RAS. The active RAS in turn activates a kinase RAF. Then the RAF protein activates the MEK protein by phosphorylating it. MEK is itself a kinase, which activates ERK. And ERK can enter the nucleus and phosphorylate certain transcription factors (adapted from [137]).

The Wnt pathway

The Wnt pathway is another key pathway in maintaining ESC and iPSCs in a pluripotent state. Members of the Wnt family of paracrine factors interact with transmembrane receptors of the Frizzled family (Figure 4.3). In most instances the binding of Wnt by the Frizzled protein causes the Frizzled protein to activate the Disheveled protein. Once the Disheveled protein is activated, it inhibits the activity of the glycogen synthase kinase-3 (GSK3) enzyme, preventing the degradation of β-catenin protein from the APC protein. Then β-catenin can enter the nucleus and activates the Wnt-responsive genes promoting the self-renewal of both mouse and human ESCs through the inhibition of GSK3 and the subsequent nuclear accumulation of β-catenin [144-145].

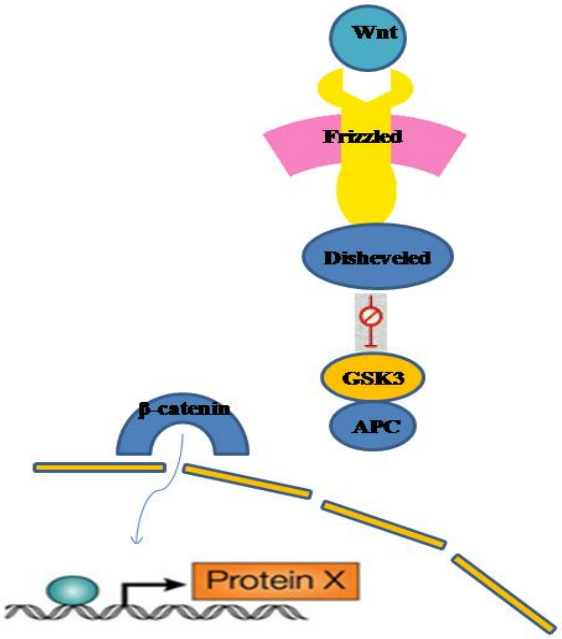

Figure 4.3: The model of Wnt pathway
The binding of Wnt by the Frizzled protein causes the Frizzled protein to activate the Disheveled protein. Once the Disheveled protein is activated, it inhibits the activity of the glycogen synthase kinase-3 (GSK3) enzyme, preventing the degradation of β-catenin protein from the APC protein. Then β-catenin can enter the nucleus and activates the Wnt-responsive genes, such as c-Myc (adapted from [137]).

Once the β-catenin is inside the nucleus, it can form a heterodimer with an LEF or TCF DNA-binding protein, becoming a transcriptional factor, binding to and activating the Wnt-responsive genes [146]. CHIR99021, a GSK3 inhibitor, had been used in ESC derivation and iPSC reprogramming. The inhibition of the EMF and GSK3 pathways enabled the derivation and maintenance of genuine rat ESCs that were capable of contributing to germline competent chimeras [147]. Smith and colleagues reported treatment of 2i combination (MEK inhibition Pd0325901 and GSK3 inhibitor CHIR99021) and LIF modulated the transition of mouse pre-iPSCs to fully competent iPSCs that satisfied stringent criteria of pluripotency [143].

A prominent downstream regulator of the Wnt pathway is c-Myc [148]. It is reported that dox-inducible OSK and OSK-mediated reprogramming of MEFs cultured using Wnt3a conditioned medium resulted in subtle increases in colony formation upon dox induction [149]. Therefore, it is possible that the effect of Wnt3a on reprogramming was at least partially mediated by c-Myc.

TGFβ/ Activin/ Nodal Signaling

While mouse ESCs are maintained by the LIF/STAT3 and BMP signaling pathways, human ESCs and mouse EpiSCs depend on fibroblast growth factor (FGF) and TGFβ/ Activin/ Nodal signaling [150]. TGFβ/ Activin/ Nodal signal through the TGFβ super-family of the receptors to activate downstream signaling including phosphorylation of Smad2 and Smad3 (Figure 4.4) [151].

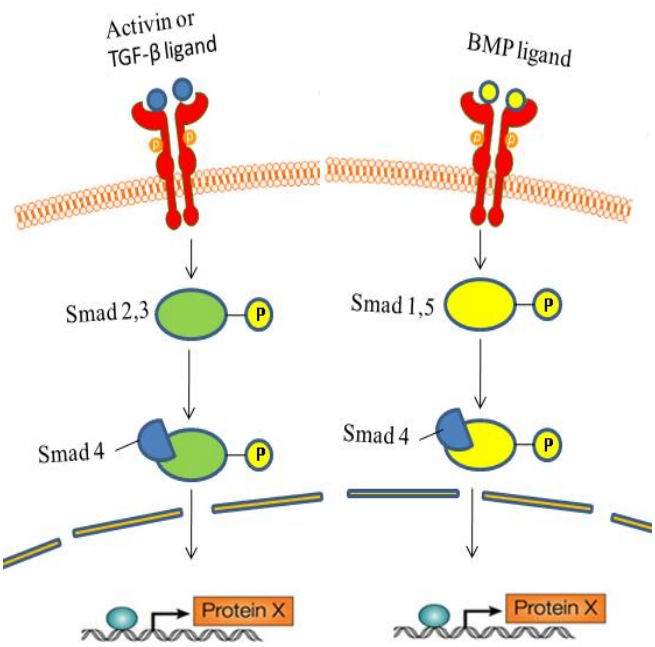

Figure 4.4: The Smad pathway
The TGF-β ligand binds to a type II TGF-β receptor, which allows that receptor to bind to a type I TGF-β receptor. Once the two receptors are in close contact, the type II receptor phosphorylates a serine or threonine on the type I receptor, thereby activating it. The activated type I receptor can now phosphorylate the Smad proteins. Smads 1 and 5 are activated by the BMP family of TGF-β factors, while the receptors binding activin and the TGF-β family phosphorylate Smads 2 and 3. These phosphorylated Smads bind to Smad 4 and form the transcription factor complex that will enter the nucleus. In vertebrates, the TGF-β superfamily ligand Nodal appears to activate the Smads pathway in those cells responsible for the formation of the mesoderm and for specifying the left-right axis in vertebrates (adapted from [137]).

Several groups have reported a positive role for TGFβ mediated signaling in the maintenance of pluripotency in hESCs [152-154]. Amit et al demonstrated cells kept long-term self-renewal in a feeder and serum-free system. Cells were cultured on a fibronectin substrate in media containing serum-replacement, TGFβ, LIF and FGF2, and this condition supported long-term self-renewal on hES cells [152]. Another recent paper reports that Activin A/ Nodal signaling maintains pluripotency by controlling Nanog expression in human ESCs [155]. Additional they found that the function to promote Nanog expression was mediated by Smad2/3 proteins, which bond to Nanog promoter in hESCs. This is also supported by the report that

pharmacological inhibition of Smad2/3 phosphorylation results in differentiation of hESCs cultured on Matrigel in MEF conditioned medium [153].

2i/ LIF Feeder Free Pluripotent Stem Cell System

Later research found that in combination with LIF, selective small molecule inhibitors could replace the requirement for serum/BMP and supported robust long-term ES-cell propagation. Recently, it has been found that suppression of GSK-3 (glycogen synthase kinase-3) and MEK (mitogen activated protein kinase) / ERK (extracellular signal regulated kinase) pathways with selective small molecule inhibitors (a culture system known as the 2i/LIF system) is sufficient to stabilize and sustain mouse ESCs with full pluripotency (Silva and Smith, 2008; Ying et al., 2008). Specifically, 2i stands for two small molecules, PD0325901 and CHIR99021. CHIR99021 is a GSK3 inhibitor by which the β-catenin accumulates in the nucleus and activates the Wnt-responsive genes promoting the self-renewal of both mouse and human ESCs and enhances cell growth capacity and viability at low cell density. PD0325901 functions in selecting or stabilizing of true iPSCs by inhibiting MEK/ ERK kinases which are known to activate the genes responsible for lineage commitment [156-157].

Several groups have reported 2i/LIF culture system promotes pluripotency in mouse, bovine and other species [156, 158]. Austin Smith et al found that a culture system containing PD0325901, CHIR99021 and LIF can sustain efficient mouse ESCs self-renewal and pluripotent state. These mouse ESCs expressed *Pou5f1*, *Nanog* and *Rex1* with minimal levels of lineage commitment markers. Mouse ESC injection into blastocysts yielded high-grade chimaeras and germline transmission [143]. When mouse brain-derived neural stem (NC) cells were cultured in the 2i/LIF system they acquired an undifferentiated morphology, expressed a number of key

pluripotency markers and showed reactivation of the X chromosome after transduction with only *POU5F1* and *KLF4* [156]. These cells proved to be chimeric competent and capable of significant contribution to all 3 germ layers. In addition, 2i/LIF culture system was reported to increase the efficiency of iPSC generation in bovine and pig species [20, 159]. Huang et al. generated the first bovine iPSC-like cells by using transfection and 2i/LIF system. Under this culture condition, bovine iPSC-like cells were capable to differentiate into all three germ layers in vitro and in teratomas [20]. Naive porcine iPSCs which resemble naïve mouse ESCs were successfully generated using 2i/LIF medium [159]. These porcine iPSCs exhibited high telomerase activity, a short cell cycle interval, and a normal karyotype, and are able to generate teratomas.

In this study, we transfected CEFs with minicircle DNA comprised of human POU5F1, SOX2, NANOG and LIN28 toultimately generating ciPSCs without transgenes integration. In order to develop naïve ciPSCs and maintain these cells in an undifferentiated state in a long term culture, different kinds of established stem cell systems, including 2i/LIF medium, were tested in cultured ciPSCs.

CHAPTER 5

CHIMERIC CHICKENS PRODUCED FROM INDUCED PLURIPOTENT STEM CELLS UTILIZING A NON-VIRAL APPROACH[1]

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Introduction

Over 50 billion chickens for meat and eggs are reared annually and serve as a major food source and a critical component of many country economies worldwide. Due to the importance of the chicken as a food source and to the economy, a number of efforts have been made to generate transgenic animals that produce higher quality and quantity of product or animals with unique characteristics such as reduced waste [160-162]. Chickens are also biomedically important as they have been used in basic developmental biology studies of the nervous system, to understand cell patterning during embryo development and to study diseases such as retinal degeneration, Hashimoto's thyroiditis and sex-linked dwarfism [163-168]. They are also used for the production specific pathogen-free (SPF) eggs to make vaccines, which is a critical component of disease prevention worldwide [169]. To create better chickens through transgenic means, chicken primordial germ cells (cPGCs) and embryonic stem cells (cESCs) have been generated using similar methods as previously done in the mouse [8-12]. However, these cells are often challenging to maintain in long term culture, have limited plasticity and produce highly variable and often low numbers of transgenic animals [9, 13]. Induced pluripotent stem cells (iPSCs) are a new class of pluripotent cell that have been recently developed in the mouse and human [1, 54]. iPSCs are generated by the overexpression of a combination of exogenous pluripotent transcription factors in an adult somatic cell leading to the reversion of this mature cell to an embryonic or inner cell mass-like state [1]. iPSCs have been produced in other agriculturally important species including the pig [17-18], cow [19-20], sheep [21], horse [22] and of most relevance the quail [7]. And in the case of the pig and quail, iPSCs have shown significant potential in producing transgenic animals [6-7]. This suggests that iPSCs may be a

new and more robust cell type to create transgenic chickens with agriculturally and biomedically important characteristics.

iPSC technology was first established when *Pou5f1, Sox2, c-Myc and Klf4* pluripotency reprogramming genes were introduced into mouse embryonic fibroblasts (MEFs) leading to the reversion of these somatic cells to pluripotent stem cells [1]. These mouse iPSC lines were highly proliferative, expressed a number of important pluripotency markers and were capable of significant in-vitro differentiation into cells representing all three germ layers. Ultimately these mouse iPSCs were proven capable of contributing to chimeric animals, demonstrating an extremely high level of plasticity [1, 16]. iPSCs have now been derived from quail, mouse, rat, sheep, pig, non-human primate and human somatic cells utilizing different factor combinations of POU5F1, SOX2, NANOG, KLF4, C-MYC and LIN28 [1, 7, 54, 118-119, 170-171]. iPSC technology has proven to be highly robust, enabling the generation of pluripotent stem cell lines from non-permissive mouse strains, rats and pig, species which have all proven to be extremely difficult to isolate ESCs using traditional inner cell mass isolation approaches [18, 24, 172-173]. iPSCs from many of these species have shown various levels of plasticity, but only quail, mouse, rat and pig have been shown capable of incorporating into a developing embryo and producing chimeric animals [7, 16, 18, 23-24]. The generation of chimeric animals that are then capable of passing on transgenes to the next generation is a critical step in the production of a stable line of transgenic animals. In addition to being capable of contributing to chimeric animals, the immortal and highly proliferative characteristics of iPSCs potentially make them uniquely suited for the development of transgenic animals. These characteristics allow for gene targeting and for complex genetic manipulation of these cells, which is not possible using current systems in avian species. The ability to delete, add or introduce targeted mutations in specific genes in a

permanent or conditional manner and then to generate animals from manipulated cells has revolutionized the way in which the mouse has been used as biomedical and basic science tool [25-26]. This paradigm with iPSCs could be applied to other species, such as the chicken for basic science and agricultural purposes.

The great possibilities and potential gains of genetically engineered chickens have long been recognized with interest including increased efficiency of meat and egg production, development of eggs for generating recombinant and therapeutic proteins, monoclonal antibodies and other economically valuable traits [92-97]. To achieve this, a number of groups have used cellular approaches to generate transgenic animals utilizing cESCs and primordial germ cell (PGC) lines. These cells have shown similar characteristics to their rodent counterparts expressing high levels of pluripotency markers and the capacity to contribute to chimeric animals [10-12, 100]. However, the use of these cell lines have not become widespread as they have proven to be challenging to culture with low proliferation rates, high levels of cell death and typically losing their ability to form chimeric animals after only a limited number of passages [9, 13]. In addition, these systems often require mouse or rat feeder cells, adding a potential contaminating cell source, and complex media formulations and conditioning to maintain cESCs and cPGCs in a pluripotent state [13-15]. iPSCs could potentially overcome these challenges as they have proven to be relatively easy to culture long term in other species and maintain their potential to form chimeric animals [6-7, 16].

In this study, we derived chicken induced pluripotent stem cells (ciPSCs) for the first time from fibroblast cells utilizing a minicircle DNA based reprogramming approach. These highly proliferative cells showed classical stem cell character including morphology,

immunoreactivity and in vitro differentiation potential. Ultimately these cells proved to be capable of successfully incorporating into chick embryos and producing live chimeric offspring.

Materials and Methods

Cell Culture and Transfection

Chicken embryonic fibroblast (CEF) cells from Black Australorp chickens were isolated from day-11 embryos and cultured in fibroblast medium [Dulbecco's modified Eagle's medium (DMEM) high glucose (Hyclone) with 10% fetal bovine serum (Hyclone), 4mM Lglutamine (Gibco), and 50U/mL penicillin and 50 mg/mL streptomycin (Gibco)] in 5% CO2 at 37 ℃. Cells were typsinized and passaged using 0.05% trypsin (Gibco) upon reaching confluence. For transfection, a total of $1x10^6$ CEF cells were plated in 35mm dishs. After 24 hrs, CEF cells were transfected with Minicircle DNA (System Biosciences) containing the four reprogramming factors *POU5F1*, *SOX2, LIN28, NANOG* and the green fluorescent protein (GFP) reporter gene all driven by the constitutively active cytomegalovirus (CMV) promoter. Cells were transfected using one of four types of transfection reagents: Lipofectamine (Invitrogen), Xfect (Clontech), Purefection (System Biosciences) and Genejammer (Agilent Technologies). 5 µg of minicircle DNA was diluted in 250ul DMEM/F12 and mixed with transfection reagent per transfection reagent manufacturer instructions. The mixture was incubated at room temperature for 20 min. The mixture was added to the CEF dish drop wise. After 24 hrs, the transfection reagent mixture was removed and replaced with fresh medium. The CEF cells were transfected a total of 3 times in this manner every other day over a 5 day period in. At day7, the CEFs were trypsinized and plated onto inactivated MEF feeder cells in 20% KSR stem cell medium [DMEM/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2mM L-glutamine (Gibco),

0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM b mercaptoethanol (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich and R&D Systems)]. To assess transfection efficiency, a subset of cells before plating in stem cell conditions from each transfection reagent treatment at day 6 were analyzed by flow cytometry for GFP expression. ciPSCs were maintained on feeders and were mechanically dissociated using a glass Pasteur pipette or passaged using 0.05% trypsin every 4–5 days. After 10 passages on feeders, colonies were picked up and dissociated in 0.05% trypsin into single cells, and ciPSCs were directly passaged into feeder free conditions on Matrigel (BD Biosciences; diluted 1:100 in DMEM/F12) coated dishes in 20% KSR plus 10ng/ml bFGF.

Alkaline Phosphatase and Periodic Acid Schiff's Staining

Alkaline phosphatase (AP) staining was carried out with the VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Laboratories) according to the manufacturer's instructions. Periodic acid Schiff (PAS) staining was performed by first fixing cells with 4% PFA for 5min. The PAS solution (Sigma-Aldrich) was added to the plate and incubated at room temperature for 5 min, followed by 3 PBS -/- washes. Schiff's reagent (Sigma-Aldrich) was added and incubated at room temperature for 15 min followed by 3 washes with PBS -/-. Plates were then observation.

RNA Isolation and PCR

RNA was isolated using the RNeasy QIAprep Spin miniprep Kit (Qiagen) according to manufacturer's instructions. Genomic DNA was removed using gDNA eliminator columns (Qiagen). The RNA quality and quantity was determined using the NanoDrop 8000 (Thermo Scientific). Total mRNA (500ng) extractions were reverse transcribed into cDNA using iScript

cDNA Synthesis Kit (Bio-Rad Laboratories). PCR amplification was performed using GoTaq Green master mix (Promega). Primers used in RT-PCR are listed in Table 4.1. PCR reactions were performed by initially denaturing cDNA at 95°C for 3 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 30 sec, polymerization at 72°C for 30 sec and a final 10-min extension at 72°C. PCR products were loaded into 2% agarose gels containing 0.6 μg/mL ethidium bromide and run in Tris-acetate-ethylenediaminetetraacetic acid buffer for 45 min. The Alpha Innotech gel documentation station was used to observe PCR products.

Immunocytochemistry

Cells were passaged onto Matrigel coated chamber slides. Cells were washed with PBS+/+ and fixed with 4% PFA at room temperature for 15 min. For intracellular staining, cells were permeabilized with 0.1% Triton X-100, 1% PVP in a PBS blocking solution containing 4% normal fetal bovine serum. For extracellular staining, cells were blocked in a PBS containing 4% normal fetal bovine serum. Fixed cells were blocked for 45 min and were incubated with primary antibodies for 1 hour. Primary antibodies used were POU5F1 (1:500; Santa Cruz), SOX2 (1:200; R & D Systems), NANOG (1:200; Millipore), β III-Tubulin (1:200; Neuromics), Brachyury (1:200; Santa Cruz), Vimentin (1:100; BD Pharmingen), SSEA-1 (1:200; Developmental Studies Hybridoma Bank), SSEA-4 (1:200; Developmental Studies Hybridoma Bank). Primary antibodies were detected using a fluorescently conjugated secondary antibody, Alexa Fluor 488 (Molecular Probes, 1:500) and 594 (Molecular Probes, 1:500). Cells were observed and images were captured on the Ix81 microscope with Disc-Spinning Unit (Olympus) using Slide Book Software (Intelligent Imaging Innovations).

Flow Cytometry

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. Cells were washed 3 times in phosphate-buffered saline (PBS; Hyclone) without calcium and magnesium (-/-) and were blocked in 6% fetal bovine serum (FBS) 94% PBS blocking solution for 45min. SSEA-1 (1:200; Developmental Studies Hybridoma Bank) primary antibody was added and cells were incubated for 1 hour. Cells were washed 3 times with blocking solution. Primary antibody was detected using fluorescently conjugated secondary antibody Alexa Flour 488 (1:1000; Invitrogen). Cells were analyzed using a Dakocytomation Cyan (DakoCytomation) and FlowJo Cytometry analysis software (Tree Star).

Proliferation and Telomerase Activity

ciPS cells were plated on 6-well plates at day 1 with $1x10^5$ cells per well. Proliferation assay was performed by manual counts (n=3) at 12, 24, 36 and 48 hours after plating. Population doubling time was determined using an exponential regression curve fitting (www.doubling-time.com/compute.php).

Telomerase activity of CEFs, ciPSCs, WA09 human embryonic stem cells (hESCs) and Hela cells (positive control) were determined using TRAPeze XL Telomerase Detection Kit (Millipore) following the manufacturer's instructions.

Embryoid Body Formation and Differentiation

Embryoid bodies (EBs) were formed by plating 3.6 $x10^6$ ciPSCs in 20% KSR medium and 0.1 mM Y-27623 ROCK inhibitor (Calbiochem) in an AggreWell plate (Stemcell Technologies). After 24 hours, cell aggregates were harvested and cultured in differentiation

medium [DMEM/F12 (Gibco), supplemented with 20% fetal bovine serum (FBS; Hyclone), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM b mercaptoethanol (Sigma-Aldrich)] for 10 days. Differentiation was assessed by RT-PCR using the primers in Table 1 to assess the differentiation by immunostaining, EBs were replated in 4 well chamber slides and differentiate further for 4 days in differentiation medium. They were then stained for germ layer markers as previously described.

Re-Transfection and Medium Comparison

To compare several established stem cell culture system in maintaining ciPSCs pluripotency, $1x10^{6}$ cells were plated on Matrigel coated 35mm dish and re-transfected with Minicircle DNA as before and were passaged directly into 1 of 5 feeder free culture systems. The medium components of the five culture system were as follows:

Control Group: 20% KSR medium: DMEM/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM b mercaptoethanol (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich and R&D Systems);

Group 1: TGFβ1/LIF medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM b mercaptoethanol, 0.12ng/ml TGFβ1 (Pepro Tech), 1000 unites/ml LIF(Millipore);

Group 2: LIF/ Wnt3a medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM b mercaptoethanol, 100ng/ml Wnt3a (R&D Systems), 1000 unites/ml LIF.

Group 3: 2i/LIF medium: DMEM/F12 supplemented with N2 (Gibco) and mix 1:1 with Neurobasal medium (Gibco) supplemented with B27 (Gibco), 1mM L-glutamine, 0.8μM PD0325901(Sigma), 3μM CHIR99021 (Selleckchem), 20ng/ml LIF:

Group 4 TGFβ1/activin A/nodal medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM b mercaptoethanol, 0.12ng/ml TGFβ1 (Pepro Tech), 10ng/ml Activin A (R& D Systems), 50ng/ml mouse recombinant nodal (R & D systems).
Matrigel was used as the substrate for all systems.

Chimera Production

Stage-X White Leghorn chicken embryos were used to produce chimeras. Small injection windows were drilled into injection egg shells using a Dremel rotary tool. ciPSCs from Black Australorp chickens were transduced with Turbo-GFP Lentiviral Vector (Thermo Scientific Open Biosystems) before injection according to the manufacturer's instructions. ciPSCs were injected into the subgerminal cavity using a glass micropipette with pressure controlled microinjector (Parker Automation). Each embryo was injected with 10,000 cells. The window was sealed by using a hot glue gun after injection and eggs were incubated at 37.8 ℃.

Eggs were opened and dissected at day 5 to determine if GFP positive ciPSCs successfully incorporated into chick embryos or eggs were allowed to hatch at day 22. Hatched chicks were checked for feather chimerism.

DNA Isolation, PCR and Sequencing Analysis

Chicks were sacrificed and brain, liver, muscle, heart and gonad tissues were collected for PCR analysis for the integrated GFP gene (see primers in Table 1). DNA was isolated from organs using a DNeasy kit (Qiagen) following the manufacturer's instructions. PCR reactions were performed by initially denaturing DNA at 95°C for 3 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 58°C for 30 sec, polymerization at 72°C for 30 sec and a final 10-min extension at 72°C.

Sequencing verification of GFP gene was performed by extracting DNA from agarose gels after electrophoresis. DNA was extracted from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen) per manufacturer's instructions. Purified DNA was sequenced at the Georgia Genomic Facility and resulting sequence was compared by Blast in the NCBI database.

Statistical Analysis

Flow cytometry, proliferation and telomerase activity data were analyzed using a one-way ANOVA and Tukey pair-wise comparisons between each population. Statistically significant differences are defined at the 95% confidence index ($p<0.05$). Data shown are means ± standard error of the mean.

Results

ciPSCs Generated by Minicircle Transfection Express Pluripotent Markers

A recent report successfully demonstrated the generation of iPSCs from human adipose stromal cells (hASCs) using a non-viral minicircle DNA reprogramming approach [70]. The use of non-viral approaches provides a significant advantage as viral DNA can trigger an immune response that raises biosafety concerns for agricultural applications where the products (e.g. meat and eggs) of iPSC-derived farm animals or their offspring ultimately enter the human food chain. Minicircle DNA has already been approved by the Food and Drug Administration (FDA) for similar commercial applications, paving the way for rapid utilization [70, 174]. Additionally, Minicircle DNA results in longer ectopic expression of transgenes due to its lower activation of exogenous silencing mechanisms relative to standard plasmid systems [69]. In this study, we transfected chicken embryonic fibroblasts (CEF; Figure 5.1A) isolated from day-11 Black Australorp chicken embryos with minicircle vectors containing the *POU5F1*, *SOX2*, *NANOG* and *LIN28* reprogramming genes and the GFP reporter using four different transfection reagents: Lipofectamine, Xfect, Purefection and Genejammer. CEFs were transfected a total of 3 times every other day over a 5 day period. The GFP expression reached a peak at 72 hours post first transfection (Figure 5.1B). The lipofectamine transfected group showed the highest transfection efficiency with 16.4% population being GFP+, while transfection with GeneJammer, Xfect and Purefection resulted in 2.3%, 2.1%, and 15.1% GFP+ cells respectively (Figure 5.1C, D).

At day 7, the cells were trypsinized and plated onto inactivated feeder cells in 20% KSR stem cell medium. Compact colonies began to emerge around two weeks after replating with colonies showing well defined borders (Figure 5.1E). To determine the effect of transfection reagents on colony formation, the number of colonies was manually counted. Cells transduced

with lipofectamine resulted in significantly (p-value < 0.05) higher levels of colony formation (21.7 ± 1.0) relative to cells transduce with GeneJammer (11 ± 2.1) and Xfect (10.7 ± 1.8; Figure 1G). Transfection with purefection resulted in similar levels of colony formation (17.3 ± 1.4) as lipofectamine. The compact colonies were mechanically isolated and replated on feeder plates in 20% KSR medium. After passaging, ciPSC displayed morphological characteristics consistent with iPSCs including a colonial growth pattern with colonies forming highly refractive colonies with well defined boarders (Figure 5.1F). At the single cell level, ciPSCs had a high nucleus to cytoplasm ratio and possessed large nucleoli indicative of a stem cell fate.

ciSPCs were tested for stem cell markers and were found to be strongly positive for alkaline phosphatase (AP) and periodic acid Schiff staining (PAS, Figure 5.2A, B respectively), while CEF control cells were negative for both AP and PAS (Figure 5.2C, D respectively). To determine if pluripotent genes were activated in ciPSCs, RT-PCR was performed. RT-PCR analysis revealed that the ciPSCs expressed ES cell markers *Pou5f1, Sox2, NanoG, Rex-1, Slc2a3, Dnmt3b and Terf1* as did the chicken primordial germ cell (PGCs) positive controls, while CEF cells were negative (Figure 5.2E).Immunostaining showed that POU5F1 (Figure 5.3A, B), SOX2 (Figure 5.3D, E) and NANOG (Figure 5.3G, H) proteins were highly expressed in ciPSCs, but were absent in CEFs (Figure 5.3C, F, I). However, immunostaining of ciPSCs showed cells were negative for the pluripotent markers SSEA-1 (data not shown).

Re-Transfection Increases of Pluripotent Marker Expression in ciPSCs

ciPSCs generated from the first transfection were negative for the pluripotent stem cell marker SSEA-1. One potential reason for the lack of SSEA-1+ cells is that the media conditions were not optimum for ciPSCs. Therefore, we tested alternative stem cell culture systems. To

potentially increase the pluripotency of partially reprogrammed ciPSCs, cells were re-transfected as before with Minicircle DNA three times using the Lipofectamine transfection reagent. Cells were then directly expanded in five different established feeder free stem cell culture systems. The five different mediums were control group: 20% KSR with 10ng/ml bFGF, group 1: TGFβ1/LIF, group 2: LIF/ Wnt3a, group 3: 2i/LIF, group 4: TGFβ/activin A/nodal. Flow cytometry was then performed for SSEA-1 at passage 6 and 12. ciPSCs cultured in 2i/LIF medium showed typical stem cell like colonies (Figure 5.4A) and immunostainig result of ciPSCs showed expression of SSEA-1 at passage 6 (Figure 5.4B). Flow cytometry data showed 2i/LIF medium was marginally better than other medium in maintaining SSEA-1 expression. However, SSEA-1 positive percentage is not significantly different among these medium with the highest SSEA-1 percentage 4.8% (Figure 5.4C). All treatments with the exception of group 3, showed a significant decrease ($p< 0.05$) in the number of SSEA-1 positive cells at passage 12 relative to passage 6. This suggested that the media types were not optimum for maintaining these cells in a pluripotent state. Alternatively, it was potentially possible that these cells were reprogrammed at a low efficiency and that FACs sorting a pure population of these cells could lead to higher levels of SSEA1+ cells. To test this hypothesis, SSEA1+ cells were FACs sorted into each media type. However, cells failed to proliferate, differentiated and ultimately died (data not shown).This further supported that the media types currently used were not optimum for the chicken system.

ciPSCs are Highly Proliferative and Have High levels of Telomerase Activity

Rapid proliferation and high levels of telomerase activity are hallmarks of pluripotent stem cells. To determine the doubling time of ciPSCs, cells were plated and counted every 12

hours for 48 hours. The population doubling time of ciPSCs was 19.45 hrs, which was significantly faster than the CEF parent cell line (28.46 hrs, $P< 0.01$) and similar to the WA09 hESCs line (20.08 hrs; Figure 5.5A). Telomerase activity revealed a significant ($P<0.01$) increase of > 4-fold of total product generated (TPG) (TPG is directly proportional to telomerase activity) from 58.5 in CEFs to 233.9 TPG in ciPSCs (Figure 5.5B). This further supported the transformation of CEFs to ciPSCs.

In previous studies utilizing the minicircle system for reprogramming, it was found that minicircle DNA had spontaneously incorporated into the genome of successfully reprogrammed cells at a high rate [20, 65]. Therefore ciPSCs were tested for integration of the human reprogramming factors using genomic PCR. The results showed integration of human NANOG and LIN28 into the chicken genome (Figure 5.5C). This may in part explain the increased levels of SSEA1 pluripotent marker expression in cells after the second round of transfection.

ciPSCs Form All Three Germ Layers During Embryonic Body Differentiation

To assess whether ciPSCs were capable of differentiating into all 3 germ layers, ciPSCs were plated into Aggrewell plate and centrifuged to form embryoid bodies (EBs) (Figure 5.6A). After 24 hours, compact EBs were collected and were cultured in suspension for 10 days in differentiation medium (Figure 5.6B). EBs were collected for RT-PCR analysis (See Table 1 for primer sequences). EB demonstrated strong up regulation of ectoderm (*TH, GFAP, CNP*), mesoderm (*PPARγ, LPL, GLUT1*) and endoderm (*CYP7A1, HNF1α, HNF4α*) genes, whereas ciPSCs and CEFs were negative for these markers (Figure 5.6C).

EBs were then tested at the protein level for the expression of ectoderm, endoderm and mesoderm differentiation. EBs were replated and allowed to further differentiate for 4 days in

differentiation medium. Immunostaining showed differentiated EBs were positive for Tuj-1 (ectoderm, Figure 5.6D, E), brachyury (mesoderm, Figure 5.6F, G) and vimentin (endoderm, Figure 5.6H, I). These results indicated that ciPSCs could differentiate into various cell types from all three germ layers.

Incorporation of ciPSCs into Chimeric Embryos

To determine if ciPSCs could be incorporated into different regions of the developing embryos and generate chimeric chickens, 10,000 GFP+ ciPSCs at passage 47 were injected into the subgerminal cavity of stage X embryos. Embryos were incubated for 5 or 22 days and were then dissected to determine GFP+ ciPSCs incorporation into embryos. GFP+ ciPSCs were only lowly visible indicating either silencing of the GFP gene or limited incorporation. However, GFP expression was observed in the tail bud (Figure 5.7A, B) and liver (Figure 5.7C, D) of a day 5 embryo. Out of 15 embryos, 3 (20%) showed integration of GFP+ cells in developing embryos. Five out of 15 embryos developed to term and hatched after 22 days of incubation. However, no feather chimerism was observed from the hatched offspring. Brain, liver, heart, muscle and gonad were collected from the hatched chicks and PCR was performed for the turbo GFP gene that was transduced into the ciPSCs to further determine if ciPSC incorporated into the chimeric animals. The ciPSCs were present in the brain (ectoderm), heart (mesoderm) and the gonad (mesoderm/germline) (Figure 5.7E). PCR products from ciPSCs from the brain, heart and gonad were sequenced to validate that PCR primers were solely expanding the turbo GFP sequence. Comparison of the sequenced DNA amplified from these tissues and turbo GFP sequence (Figure 5.8) by two-sequence BLAST analysis reveals 99% to 100% (Figure 5.9) identity between the sequences. Upon inspection, no bird showed overt formation of tumors despite the

fact that ciPSCs demonstrated stable integration of reprogramming genes *NANOG* and *LIN28*. The combined results from pre- and post-hatch chicks indicate that ciPSCs incorporated and contributed to chicken embryonic tissues from all 3 germ layers and potentially the germline.

Discussion

Our results show the novel generation of chicken iPSCs that possessed morphological characteristics, immunoreactivity and developmental potential of a pluripotent stem cell fate. ciPSCs were highly positive for stem cell markers AP, PAS, POU5F1, SOX2 and NANOG, similar to mouse and human iPSCs and chicken ESCs and PGCs [1, 10, 14, 45]. Upon transplantation of ciPSCs into stage X embryos, ciPSCs were found to contribute to tissues from all three germ layers and potentially the germline in offspring. This demonstrated that these cells had achieved a high level of pluripotency. A number of previous reports showed that chicken PGCs [9, 100] and ESCs [101] contributed to chimeras when injected into embryos immediately after collection from the donor embryo or after only a few passages. ciPSCs were still capable of incorporating into chimeric embryos even at late passages. The highly proliferative (doubling time of 19hrs) and pluripotent nature of ciPSCs will further facilitate genetic manipulations enabling complex genetic modifications using approaches such as gene targeting. This will allow for the development of transgenic animals with multiple manipulations, which is a key aspect as many traits of interest such as reproduction are controlled by several genes [118, 175-176].

The production of chimeric offspring validates the potential of ciPSCs as a tool for the creation of complex transgenic chickens for agricultural and biomedical purposes. In this study, the highly proliferative ciPSCs with a doubling time similar to hESCs can be continually expanded for >40 passages without showing any indication of senesces. This is an important

attribute of ciPSCs which will enable complex genetic manipulation of these cells. In previous reports, chicken PGCs and ESCs showed limited proliferation and were unable to be maintained in extended culture [9, 14]. ciPSCs had a doubling time of 19 hrs, while cPGCs [14] and cESCs [13] showed poor proliferation rates in culture and frequently shown to senesce after 10 to 20 passages [9, 13]. Overall, PGCs and ESCs have shown high variability in proliferation rates and expandability making them difficult to maintain [10, 14]. In addition, ciPSCs demonstrated high levels of developmental plasticity. In vitro ciPSCs were capable of differentiating into all 3 germ layers. In vivo, these cells were able to contribute to all 3 germ layers and potentially the germline in chimeric chicks at an efficiency of 33.3% with late passage cells. Reports have regularly shown that cPGCs and cESCs have limited ability to form chimeric animals with the number of chimeric animals ranging from 3 to 20% with early passage cells and even fewer birds being generated with late passage cells [14-15, 177]. Techniques such as magnetic activated cell sorting (MACS) and florescence activated cells sorting (FACS) based on specific markers, bisulfite treatments and coring of the pellucid have increased chimera generation in chickens [100] [10, 178]. These same techniques maybe applied to the generation of chimeras with ciPSCs with potentially a greater effect as ciPSCs appear to be primed for chimerism.

ciPSCs provide new opportunities to study avian diseases, developmental biology and generation of transgenic animals both in vitro and in vivo. For example, ciPSCs were capable of differentiating into Tuj-1 positive neural cells which could be used to study avian bornavirus (ABV) which exhibit a high tropism in the central nervous system infecting neurons and astrocytes [179-180]. This model could easily be extrapolated to other diseases such as avian influenza or Newcastle Disease. Due to extensive characterization of chick embryo development and the easy accessibility of the avian embryo, important developmental questions could be

answered by the removal or introduction of genes in cells and then transferring these cells [181]. In this study, we showed incorporation of cells in multiple tissue types of all 3 germ layers and potentially the germline suggesting that these cells can successfully incorporate. ciPSCs could also be utilized in large scale screens such as ENU mutagenesis screens to further elucidate gene functions. The accessibility of the chicken embryo and its lack of immune response provide a popular model for cell transplantation of ciPSCs or ciPSC derivatives [182].

Chicken pluripotent stem cell culture conditions have remained elusive with culture systems only being able to maintain cells for short time periods before changes in plasticity, proliferation and general character occur cells [14-15, 177]. These culture systems are typically complex, even beyond what is used for standard mouse and human pluripotent cells, requiring feeder cells, specialized media and the use of animal products that have shown significant lot-to lot variability such as fetal bovine serum [183-186]. To develop a system with defined culture conditions, several established feeder free culture systems were tested for their ability to maintain pluripotency as indicated by SSEA1 expression in ciPSCs including the 2i/LIF system and the 20% KSR system supplemented with TGFβ, activin A, nodal, LIF, Wnt3a [143, 145, 152-153, 155]. The 2i/LIF culture system has been previously shown to maintain mESCs in a naïve state, as opposed to a primed state. Naïve ESCs are characterized as having higher levels of plasticity being capable of forming chimeras with high efficiency, domed colony morphology , LIF dependency and expression of high levels of REX1 and NANOG relative to primed ESCs [187]. ciPSCs cultured in 2i/LIF medium detached from the dish leaving a few colonies showing domed stem cell like morphology and immunostaining result of these colonies showed expression of SSEA-1. However, these colonies expand very slowly in 2i/LIF medium. Domed morphology was not observed in other conditions as cells were mostly flattened as expected of

primed stem cells. Flow cytometry data showed that the 2i/LIF medium was only marginally better than other medium in maintaining SSEA-1 expression. However, SSEA-1 positive percentage is not significantly different among these mediums with the highest SSEA-1 percentage being 4.8%. Huang et al reported generation bovine iPSCs under 2i/LIF medium [20]. The bovine iPSCs in 2i/LIF were neither proliferating nor apoptotic but quiescent. These results were similar to the ones found in this study with ciPSCs. Perhaps this indicates species specific differences between mouse and other species in media composition needs to maintain cell in a naïve state.

Minicircle-based iPSC technology provides a non-viral and potentially non-DNA integrating approach to making ciPSCs as an alternative to the commonly used retroviral and lentiviral strategies [1, 45-46, 54]. Although these viral-based methods have relatively high reprogramming efficiency, they usually generate iPSCs with both the vector backbone and transgenes permanently integrated into the genome [54]. The permanent integration of these genes often under the control of a powerful constitutively active promoter (e.g. CMV) can lead to gene reactivation and the formation of tumors, as these genes have been closely linked to tumorigenicity [16, 188-190]. Although the minicircle reprogramming process used to generate ciPSCs was non-viral, integration of 2 out of 4 reprogramming genes was observed. These results were similar to those previously found in the mouse [20, 65]. In a previous study transgenes were found to be integrated into iPSCs colonies in 4 out of 10 experiments using plasmid transfection [65]. The generation of bovine iPSCs using a similar DNA base approach also resulted in the stable integration of exogenous reprogramming genes as well [20]. These findings suggest that DNA based approaches may be useful for overcoming the need for viral based reprogramming, however have the significant downside of DNA integration.

Conclusion

Chickens are a major economic and food source throughout the world and have been utilized as a key part of developmental biology research [191] [163-168]. ciPSCs are a highly proliferative and stable cell populations that offer a better opportunity for genetically manipulating chickens to improve traits, to study gene function and gene interactions. In addition, these cells potentially have great utility for in vitro studies of bird development being capable of in vitro differentiation and to potentially study avian diseases using specialized cell types derived from ciPSCs. These cells have a tremendous potential to enhance improvements and discoveries in a broad range of fields from stem cell biology to animal production.

Acknowledgments

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Figure 5.1: Generation of ciPSCs from CEFs**.** (**A**) CEFs were transduced with the minicircle plasmid which contains the *POU5F1, SOX2, NANOG* and *LIN28* reprogramming genes and the GFP reporter gene utilizing Genejammer, Xfect, Purfection and Lipofectamine to determine the optimum transfection reagent. Transfected CEFS expressed GFP (**B**) and flow cytometry demonstrated that Lipofectamine resulted in the highest percentage of GFP+ cells 72 hours post-transfection (**C, D**). Putative ciPSCs grew as colonies showing well defined borders at day 20 post-transfection (**E**) with cell displaying typical iPSC morphology of large nuclolus and high nucleus to cytoplasmic ratio (**F**). Quantification of colony demonstrated that Lipofectamine resulted in the highest average number of ciPSC colonies formed relative to Genejammer, Xfect and Prufection (**G**; *= p-value <0.05).

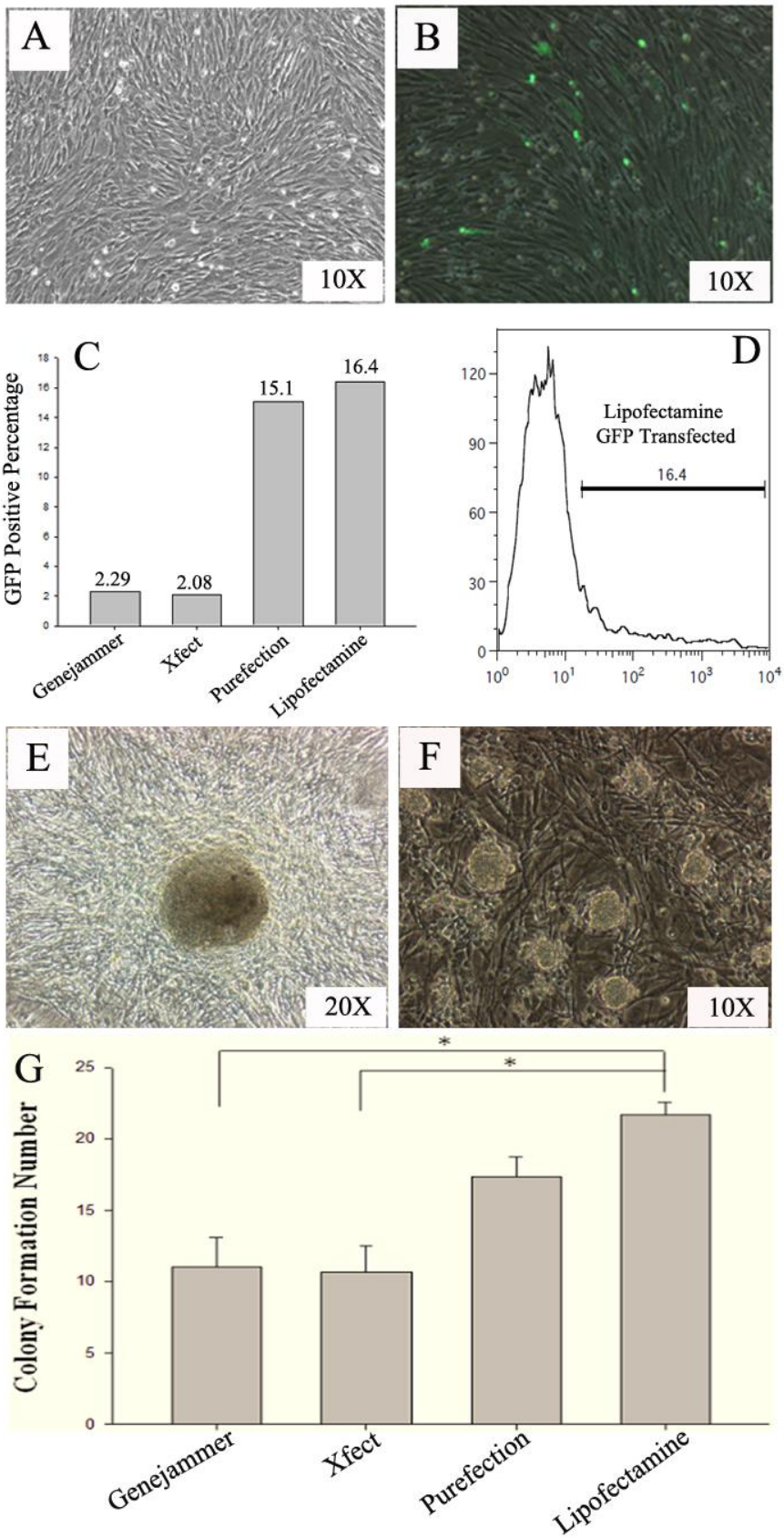
A
10X
B
10X
C
GFP Positive Percentage
2.29
2.08
15.1
16.4
Genejammer
Xfect
Purefection
Lipofectamine
D
Lipofectamine
GFP Transfected
16.4
E
20X
F
10X
G
Colony Formation Number
*
*
Genejammer
Xfect
Purefection
Lipofectamine

Figure 5.2: ciPSCs express pluripotency markers AP and PAS and show activation of pluripotency network**.** ciPSCs stained positive for AP **(A)** and PAS **(B)**, while CEFs were negative for AP **(C)** and PAS (**D**) staining. **(E)** RT-PCR analysis of ES cell genes showed significant up-regulation in ciPSCs similar to that found in cPGCs, while CEFs were negative for all markers tested.

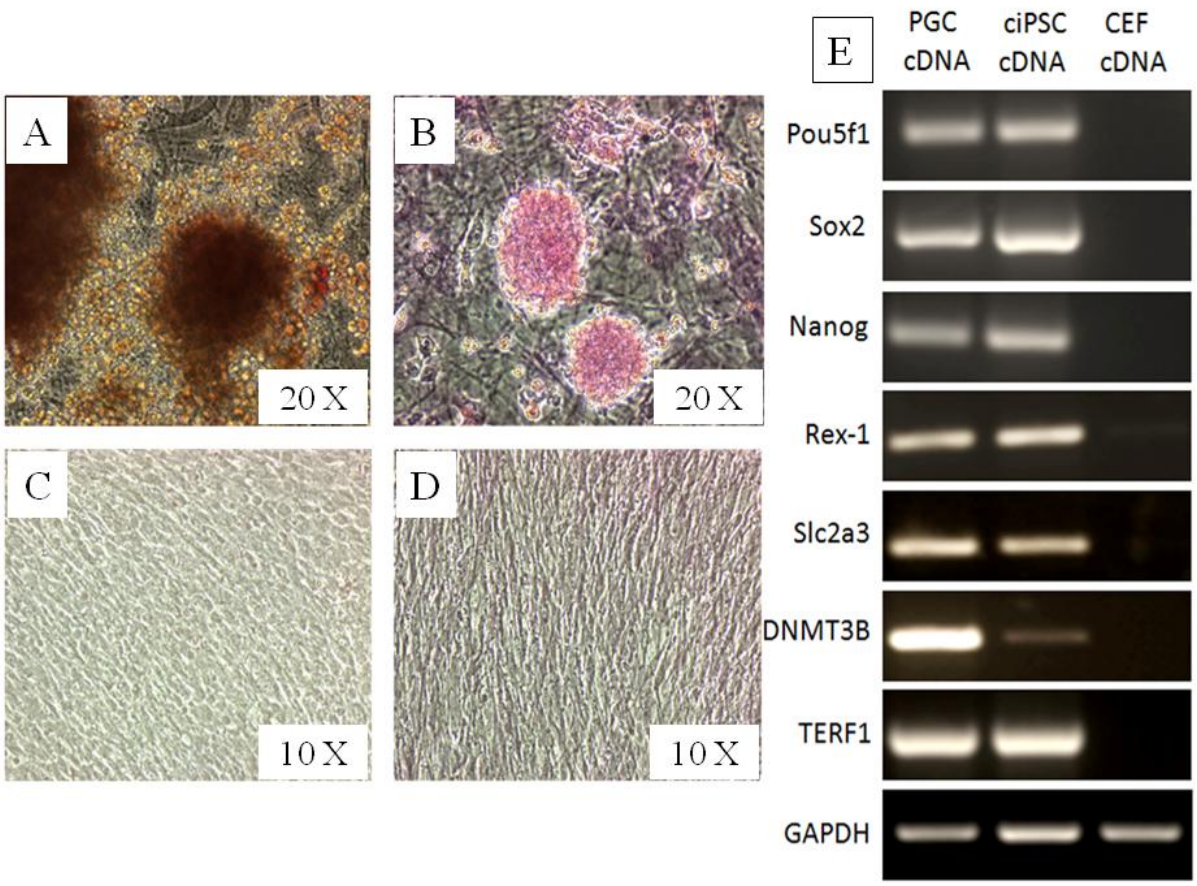
A
20 X
B
20 X
C
10 X
D
10 X
E
PGC cDNA
ciPSC cDNA
CEF cDNA
Pou5f1
Sox2
Nanog
Rex-1
Slc2a3
DNMT3B
TERF1
GAPDH

Figure 5.3: ciPSCs express pluripotency transcription factors POU5F1, SOX2 and NANOG. Immunostaining demonstrated that ciPSCs were POU5F1 (**A, B**- dapi merge), SOX2 (**D, E**- dapi merge) and NANOG (**G, H**- dapi merge) positive, while CEFs were POU5F1 (**C**- dapi merge), SOX2 (**F**- dapi merge) and NANOG (**I**- dapi merge) negative. Scale bars=50um.

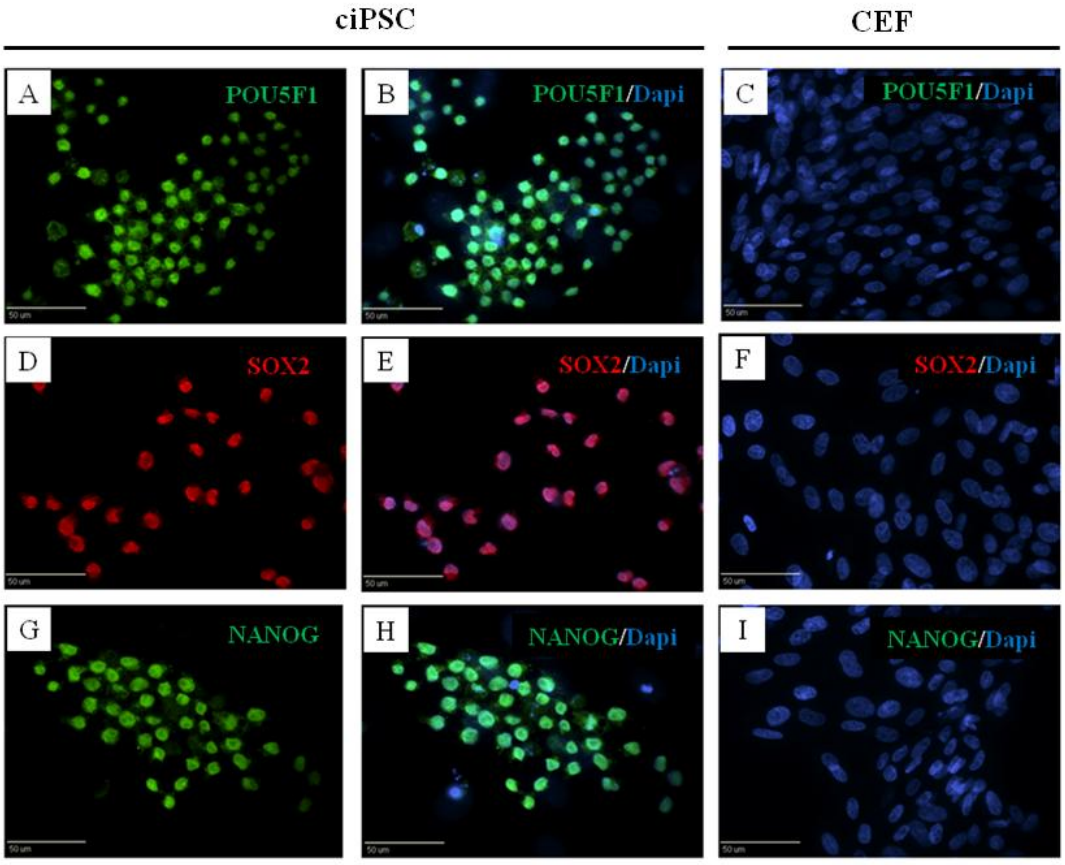
ciPSC
CEF
A
POU5F1
B
POU5F1/Dapi
C
POU5F1/Dapi
D
SOX2
E
SOX2/Dapi
F
SOX2/Dapi
G
NANOG
H
NANOG/Dapi
I
NANOG/Dapi

Figure 5.4: Culture of ciPSC in defined conditions**.** ciPSCs underwent a second round of reprogramming with minicircle DNA and were passaged on Matrigel in one of 5 media conditions: 20% KSR (control), TGFβ1/LIF (Group 1, G1), LIF/ Wnt3a (Group 2, G2), 2i/LIF (Group 3, G3) and TGFβ1/activin A/nodal (Group 4, G4). CiPSCs showed stem cell colony expansion and morphology (**A**) and SSEA1 expression (**B**). Flow cytometry analysis of cells at passage 6 and 12 showed that all conditions resulted in less than 5% SSEA1+ cells (**C**). However, only 2i/LIF (G3) media did not result in a significant (*= p-value < 0.05) reduction in SSEA1+ cells from passage 6 to 12.

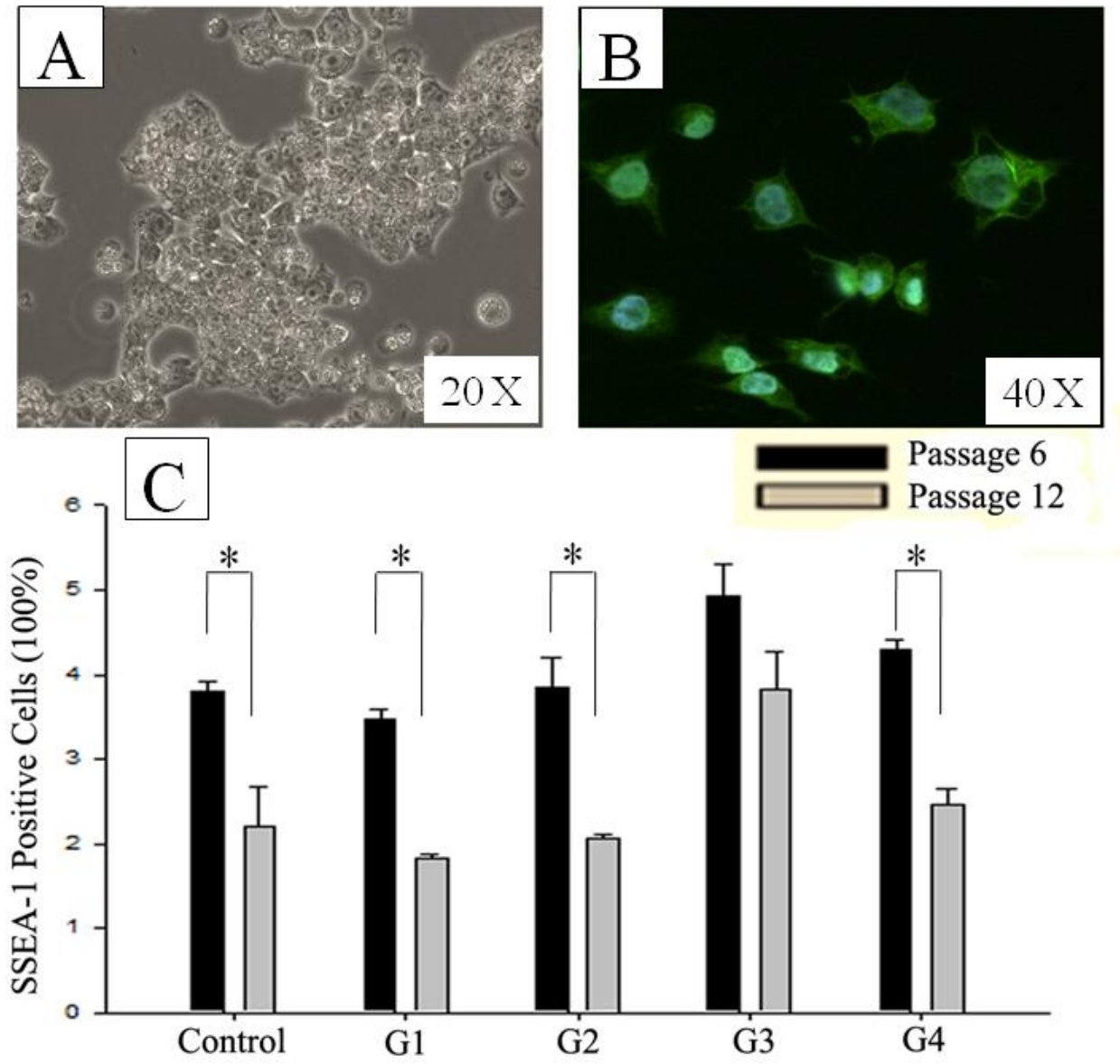
A
20 X
B
40 X
C
Passage 6
Passage 12
SSEA-1 Positive Cells (100%)
0
1
2
3
4
5
6
*
*
*
*
Control
G1
G2
G3
G4

Figure 5.5: ciPSCs demonstrate rapid proliferation and high levels of telomerase activity. ciPSC doubling time was 19.45 hrs (**A**), significantly (p-value < p<0.01) faster than the CEFs (28.46 hrs) and similar to the hESCs (20.08 hrs). Telomerase activity in ciPSCs (**B**) was significantly (p-value < p<0.01) higher than CEFs. However, the telomerase activity in hESCs was higher than that in ciPSCs, while comparable to that in Hela cells. (**C**) PCR of Genomic ciPSC genomic DNA showed integration of the human NANOG and LIN28 transcription factors. hESCs were used as positive control for human POU5F1, SOX2, NANOG and LIN28, and CEFs were used as negative control.

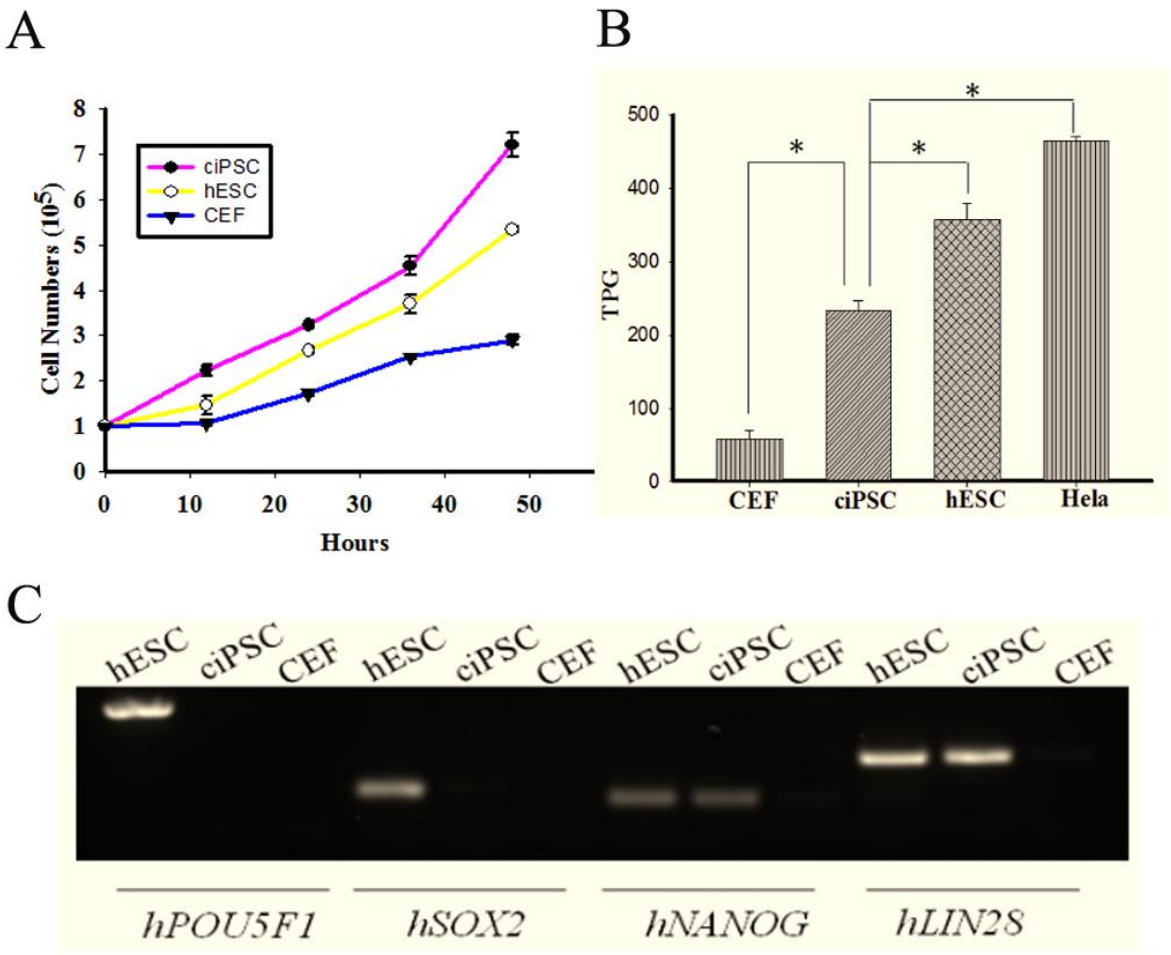
A
B
C
Cell Numbers (10^5)
Hours
ciPSC
hESC
CEF
TPG
CEF
ciPSC
hESC
Hela
hESC
ciPSC
CEF
hPOU5F1
hSOX2
hNANOG
hLIN28

Figure 5.6: Differentiation of ciPSCs into cells representing all 3 germ layers. ciPSCs were plated into an AggreWell plates (**A**) in differentiation media and were then expanded in suspension culture. After 5 days, ciPSCs formed compacted embryoid body (**B**) and after 15 days ectoderm, endoderm and mesoderm genes were expressed. Immunostaining of plated EBs showed that cells were positive for the ectoderm marker Tuj1 (**D, E**- Dapi merge), mesoderm marker brachyury (**F, G**- Dapi merge) and endoderm marker vimentin (**H, I**- dapi merge). Scale bars=50um.

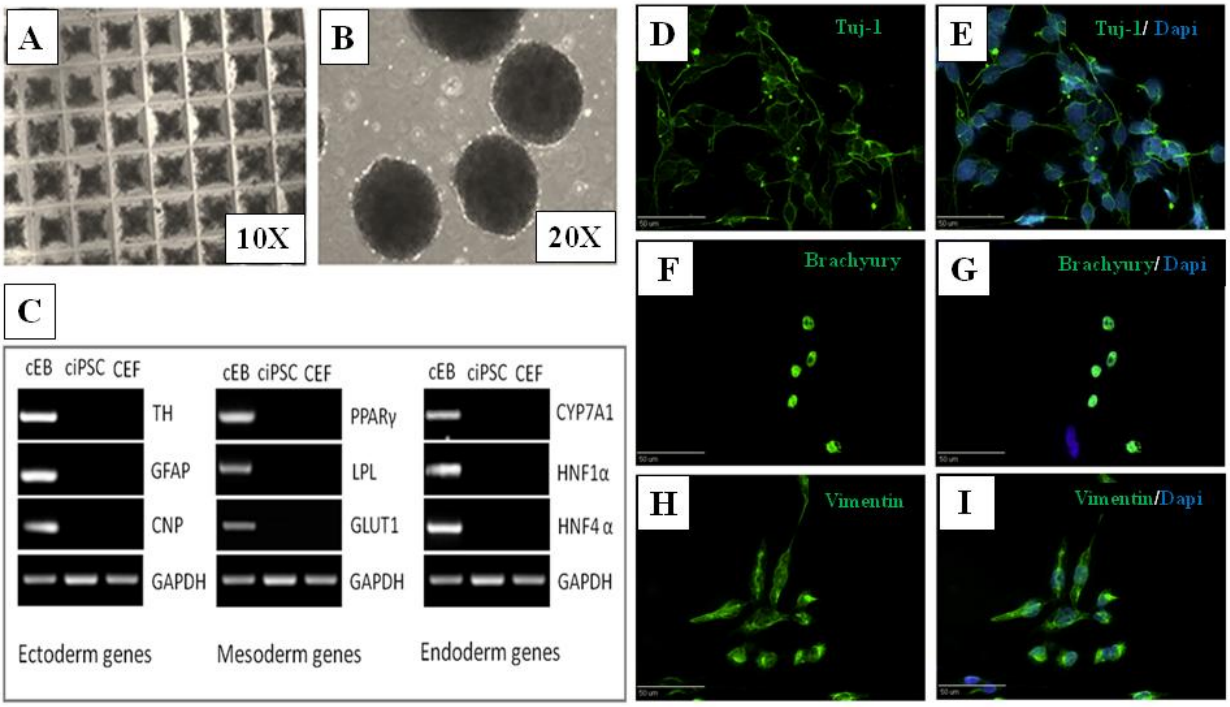
A
10X
B
20X
C
cEB ciPSC CEF
TH
GFAP
CNP
GAPDH
cEB ciPSC CEF
PPARγ
LPL
GLUT1
GAPDH
cEB ciPSC CEF
CYP7A1
HNF1α
HNF4 α
GAPDH
Ectoderm genes
Mesoderm genes
Endoderm genes
D
Tuj-1
E
Tuj-1/ Dapi
F
Brachyury
G
Brachyury/ Dapi
H
Vimentin
I
Vimentin/Dapi

Figure 5.7: Chimeric chicks derived from ciPSCs. 10,000 GFP+ ciPSCs were injected into stage x embryos and at day 5 were opened and inspected for GFP expression. GFP+ ciPSCs were found to be incorporated into the tail bud (**A, C**- phase) and liver (**B, D**- phase) of chimeric embryos. After 22 days, 5 chicks were hatched and brain (ectoderm), liver (endoderm), muscle, heart (mesoderm) and gonad (germline/mesoderm) tissues were collected from each (**E**). PCR was performed with GFP specific primers and brain, heart (mesoderm) and gonad tissues were positive for the GFP transgene similar to ciPSC positive control. CEF negative controls were negative for the GFP transgene.

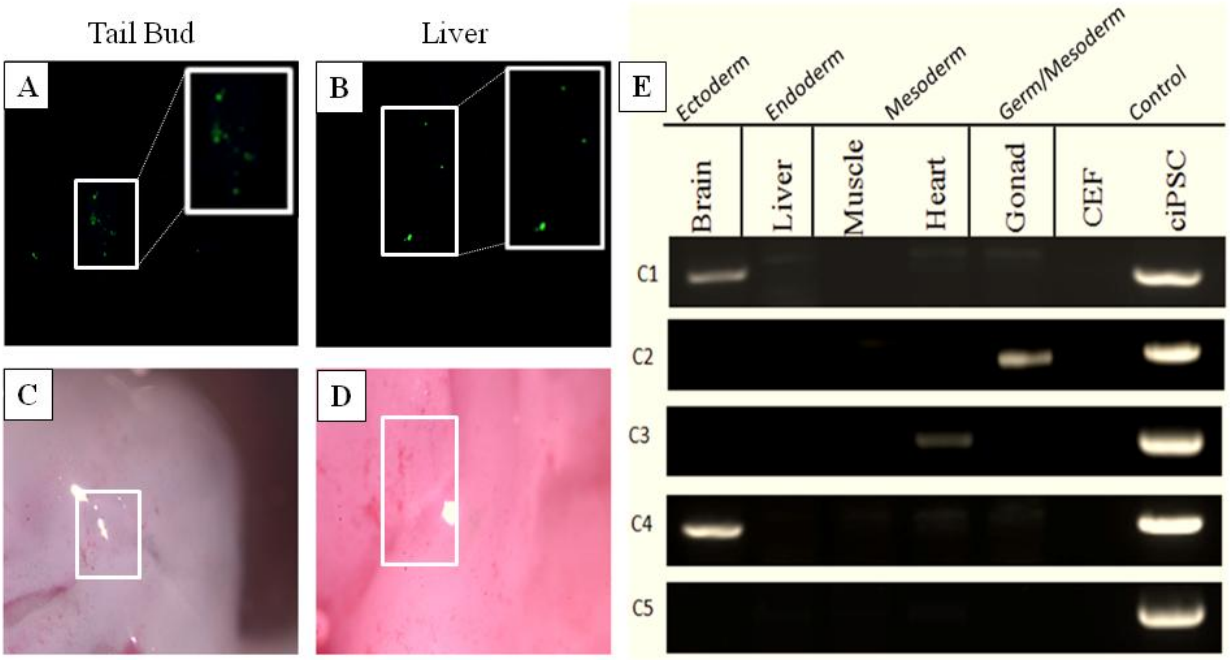
Tail Bud
Liver
A
B
C
D
E
Ectoderm
Endoderm
Mesoderm
Germ/Mesoderm
Control
Brain
Liver
Muscle
Heart
Gonad
CEF
ciPSC
C1
C2
C3
C4
C5

Figure 5.8: Two sequence BLAST analysis**.** Comparison of the sequenced DNA amplified from tissues of brain, heart and gonad from hatched offspring and turbo GFP sequence by two-sequence BLAST analysis reveals 99% to 100% identity between the sequences.

```
 Score =  555 bits (300),  Expect = 2e-162
 Identities = 300/300 (100%), Gaps = 0/300 (0%)
 Strand=Plus/Plus

Query  1    TACCGCTACGAGGCCGGCCGCGTGATCGGCGACTTCAAGGTGATGGGCACCGGCTTCCCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  TACCGCTACGAGGCCGGCCGCGTGATCGGCGACTTCAAGGTGATGGGCACCGGCTTCCCC  387

Query  61   GAGGACAGCGTGATCTTCACCGACAAGATCATCCGCAGCAACGCCACCGTGGAGCACCTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  GAGGACAGCGTGATCTTCACCGACAAGATCATCCGCAGCAACGCCACCGTGGAGCACCTG  447

Query  121  CACCCCATGGGCGATAACGATCTGGATGGCAGCTTCACCCGCACCTTCAGCCTGCGCGAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448  CACCCCATGGGCGATAACGATCTGGATGGCAGCTTCACCCGCACCTTCAGCCTGCGCGAC  507

Query  181  GGCGGCTACTACAGCTCCGTGGTGGACAGCCACATGCACTTCAAGAGCGCCATCCACCCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508  GGCGGCTACTACAGCTCCGTGGTGGACAGCCACATGCACTTCAAGAGCGCCATCCACCCC  567

Query  241  AGCATCCTGCAGAACGGGGGCCCCATGTTCGCCTTCCGCCGCGTGGAGGAGGATCACAGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568  AGCATCCTGCAGAACGGGGGCCCCATGTTCGCCTTCCGCCGCGTGGAGGAGGATCACAGC  627


 Score =  551 bits (298),  Expect = 2e-161
 Identities = 299/300 (99%), Gaps = 0/300 (0%)
 Strand=Plus/Minus

Query  1    AAGGNGAACATGGGGCCCCCGTTCTGCAGGATGCTGGGGTGGATGGCGCTCTTGAAGTGC  60
            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  AAGGCGAACATGGGGCCCCCGTTCTGCAGGATGCTGGGGTGGATGGCGCTCTTGAAGTGC  543

Query  61   ATGTGGCTGTCCACCACGGAGCTGTAGTAGCCGCCGTCGCGCAGGCTGAAGGTGCGGGTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  ATGTGGCTGTCCACCACGGAGCTGTAGTAGCCGCCGTCGCGCAGGCTGAAGGTGCGGGTG  483

Query  121  AAGCTGCCATCCAGATCGTTATCGCCCATGGGGTGCAGGTGCTCCACGGTGGCGTTGCTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  AAGCTGCCATCCAGATCGTTATCGCCCATGGGGTGCAGGTGCTCCACGGTGGCGTTGCTG  423

Query  181  CGGATGATCTTGTCGGTGAAGATCACGCTGTCCTCGGGGAAGCCGGTGCCCATCACCTTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  CGGATGATCTTGTCGGTGAAGATCACGCTGTCCTCGGGGAAGCCGGTGCCCATCACCTTG  363

Query  241  AAGTCGCCGATCACGCGGCCGGCCTCGTAGCGGTAGCTGAAGCTCACGTGCAGCACGCCG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  AAGTCGCCGATCACGCGGCCGGCCTCGTAGCGGTAGCTGAAGCTCACGTGCAGCACGCCG  303
```

Figure 5.9: Turbo GFP sequence. The sequence of Turbo GFP contained in lentivirus vector used in transduction of ciPSCs.

Turbo GFP sequence:

atggagagcgacgagagcggcctgcccgccatggagatcgagtgccgcatcaccggcacc
ctgaacggcgtggagttcgagctggtgggcggcggagagggcaccccgagcagggccgc
atgaccaacaagatgaagagcaccaaaggcgccctgaccttcagccctacctgctgagcca
cgtgatgggctacggcttctaccacttcggcacctaccccagcggctacgagaaccccttcctg
cacgccatcaacaacggcggctacaccaacacccgcatcgagaagtacgaggacggcggc
gtgctgcacgtgagcttcagctaccgctacgaggccggccgcgtgatcggcgacttcaaggtg
atgggcaccggcttccccgaggacagcgtgatcttcaccgacaagatcatccgcagcaacgc
caccgtggagcacctgcaccccatgggcgataacgatctggatggcagcttcacccgcacctt
cagcctgcgcgacggcggctactacagctccgtggtggacagccacatgcacttcaagagcg
ccatccaccccagcatcctgcagaacgggggccccatgttcgccttccgccgcgtggaggag
gatcacagcaacaccgagctgggcatcgtggagtaccagcacgccttcaagaccccggatgc
agatgccggtgaagaa

CHAPTER 5

CONCLUSION

Chickens are important farm animals for meat and eggs worldwide. Studies have been conducted to generate transgenic chickens that produce higher quality product. Chicken iPSCs provide an exciting new tool to create transgenic chickens and have broad implications for agricultural and even biomedical applications in the future. Furthermore, due to the relative size, the short generation interval and ease of access to the embryo for manipulations of avian species [192-193], chicken can be a biomedically important model for basic developmental biology studies and chicken iPSCs serve as an excellent system to study differentiation, cell patterning during development and avian diseases in vitro and in vivo.

Our results showed for the first time the novel generation of chicken iPSCs that possessed morphological characteristics, immunoreactivity and developmental potential of a pluripotent stem cell fate. ciPSCs were highly positive for stem cell markers AP, PAS, POU5F1, SOX2 and NANOG similar to mouse and human iPSCs and chicken ESCs and PGCs [1, 10, 14, 45]. Upon transplantation of ciPSCs into stage X embryos, ciPSCs were found to contribute to tissues from all three germ layers and potentially the germline in offspring. This demonstrated that these cells had achieved a high level of pluripotency. A number of previous reports showed that chicken PGCs [9, 100] and ESCs [101] contributed to chimeras when injected into embryos immediately after collection from the donor embryo or after only a few passages. ciPSCs were still capable of incorporating into chimeric embryos even at late passages. The highly proliferative (doubling

time of 19hrs) and pluripotent nature of ciPSCs will further facilitate genetic manipulations enabling complex genetic modifications using approaches such as gene targeting and the development of transgenic animals with multiple mutations [118, 175-176].

Initially, we investigated whether the presumed ciPSC shared characteristics of pluripotent stem cells from other species. Morphologically, ciPSCs formed rounded colonies with well defined edges similar to mouse ESCs [194]. Individually, ciPSCs showed large nucleus-to-cytoplasm ratio shared by both mouse and human ESCs [194-195]. As with human and mouse iPSCs, ciPSCs expressed pluripotent markers Pou5f1, Sox2 and Nanog. Similar to mouse iPSCs, they were also positive for the cell surface marker SSEA-1 and were negative for SSEA-3 and -4 [196]. The ciPSC also lack TRA-1-60 and TRA-1-81, which are characteristics of human cells [197]. The expression of SSEA-1 is consistent with reports showing the antigen to be expressed on chicken ESCs and PGCs which also lack SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 [13]. Together, these data are consistent with the pluripotent nature of ciPSC and their resemblance to ESC. Another feature common to ciPSC and human and murine ESC is their relatively rapid rate of proliferation. The calculated 19-h doubling time is slightly greater than that of mouse ESC (11.4-15.7h) [198] and that of human (15-16h) [199].

The ciPSCs expressed much the same spectrum of pluripotent genes as human and mouse iPSCs including Pou5f1, Sox2, Nanog, Rex-1, Slc2a3, DNMT3B and TERF1[28, 196]. Telomerase-associated factor TERF1 is controlled by Pou5f1 and Sox2 genes, and its expression was consistent with high telomerase activity measured with the TRAP assay [200-201]. Slc2a3, gene encodes for glucose transporter type 3, one of the protein that facilitates transport of glucose across the plasma membrane, is highly expressed in most kinds of cancer cells and stem cells [202-203]. Dnmt3b, one of the three CpG DNA methyltransferases, is strongly expressed in

ESCs, early embryos, and developing germ cells but are expressed at low levels in differentiated cells. Indeed, genetic studies have demonstrated that Dnmt3b is involved in maintaining global DNA methylation patterns in ESCs, and inactivation of Dnmt3b in ESCs resulted in progressive demethylation of repetitive elements, imprinted genes and nonimprinted genes [204-205]. All of these gene expressions suggest ciPSCs have achieved a gene expression profiles similar to other pluripotent cell types.

However, the low expression and gradual loss of the reprogramming factor independent pluripotent marker SSEA-1 raised significant concerns pertaining to the pluripotent nature of ciPSCs. One possibility is that culture conditions for growing ciPSCs were not optimized for maintaining the cells in an undifferentiated state, and the SSEA-1 expression will be improved as culture conditions improve. Following re-transfection, five different established culture mediums were used to expand ciPSCs. However, all culture conditions showed a significant decrease in the number of SSEA-1 positive cells during 6 passages. 2i/LIF medium achieved the best results when maintaining ciPSCs. 2i/LIF medium contains small molecules that selectively inhibit the MEK/ERK signaling cascade and glycogen synthase kinase 3 (GSK3), which provides, in combination with LIF, an optimal environment for derivation and propagation of ESCs from different rodent backgrounds in serum-free medium [140, 143]. 2i/LIF condition also promotes the generation of authentic iPSCs from partially reprogrammed cells [156]. After transfer into 2i/LIF, ciPSCs began to lift off of culture plates and underwent cell death with only a few colonies surviving. Species specific traits have a strong influence on the derivation and propagation of ESCs and on iPSC generation. Therefore it is probable that the pathways which chicken naïve pluripotent stem cells rely on for maintaining pluripotency are different than those of mouse cells and the 2i/ LIF needs to be modified to maintain chicken cells. Another feasible

possibility is that PD and CHIR concentration used in rodent iPSC generation is not appropriate for derivation of ciPSCs. Cell death was alleviated when PD and CHIR concentrations were reduced to 20% of the original level. Studies to systematically seeking the best concentrations should be designed and conducted in the future. Nevertheless, ciPSCs formed embryoid bodies and differentiated into cells of all three germ layers. In addition, ciPSCs incorporated into the chimeric chickens, potentially the gonad, which is the most stringent criterion to prove ciPSCs have reached a high level of pluripotency.

Until recently, most iPSC have been generated by using integrating retroviral or lentiviral vectors leading to both integration of the viral construct and the transgenes. The Minicircle vectors we used in the study avoid lentiviral backbones or bacterial plasmid backbone elements that would not be permitted for commercial livestock applications [68]. However, two human reprogramming genes from minicircle DNA were ultimately found to have integrated into the genome of ciPSCs. As these ciPSCs may be mixed populations with different genetic characteristics, it would be helpful if ciPSCs were clonally isolated by FACS and expanded to obtain clonal populations. In this case, genomic PCR screening can distinguish those clones free of genomic integration easily. More recent strategies have focused on DNA free approaches, such as mRNA, protein or microRNA based methods in iPSCs generation. Although these methods suffer from lower reprogramming efficiency compared to plasmid mediated methods, iPSCs can be truly free of transgene integration [73, 75-76].

Several applications await ciPSCs in the future. First, the signals that support chicken stem cell pluripotency are not well defined as is evident by the difficulties in maintaining authentic stable cESC lines [13]. ciPSCs provide a screening platform for candidate factors that maintain both proliferation and pluripotency of chicken pluripotent stem cells. Second, they may

be converted into animals by using them as donors for somatic cloning with the prospect of significantly increasing cloning efficiency compared to conventional donors [206-207]. Third, ciPSCs would facilitate the precise genetic engineering of farm chickens for improved production traits and biopharming. Beyond these agricultural and pharmaceutical applications, ciPSCs would help to provide animal models for diseases for which mouse models are not suitable, such as an avian-borne virus, like new castle virus, complementing research currently carried out with other laboratory animals.

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