Reduced Immunogenicity of Induced Pluripotent Stem Cells Derived from Sertoli Cells

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ABSTRACT

Induced pluripotent stem cells (iPS cells) resemble embryonic stem cells (ES cells) and can differentiate into all cells of our body. Thereby, iPS cells provide unique opportunities for disease modeling, drug development and regenerative medicine. Autologous iPS cells and their differentiated progenies are thought to bypass immune rejection and are therefore particularly appealing for personalized medicine. However, this assumption has remained controversial: syngeneic mouse iPS cells were found to be immunogenic and got rejected upon transplantation, while in other studies syngeneic iPS cells and their derivatives showed either “negligible” or “lack of” immunogenicity upon transplantation. All studies used fibroblasts as starting cells for iPS cell generation, which might influence iPS cell immunogenicity due to somatic memory effects.

Testicular Sertoli cells provide an immune-privileged environment for developing germ cells in testis and exhibit an immune-privileged function. Here, C57BL/6 (B6) Sertoli cells were used to generate iPS cells and then injected into syngeneic mice to test for their *in vivo* immunogenicity in teratoma assay. Teratoma assay allows assessing *in vivo* immunogenicity of iPS cells and their differentiated progeny simultaneously. We found that early-passage Sertoli iPS cells (Ser-iPS) showed reduced immunogenicity compared to iPS cells from B6 mouse embryonic fibroblast (MEF-iPS). Ser-iPS formed significantly more teratomas and showed less immune cell infiltration and tissue damage and necrosis than MEF-iPS. Embyoid bodies (EBs) of Ser-iPS cells exhibited reduced T cell activation potential compare to MEF-iPS cells and showed a reduced immunogenicity similar to syngeneic ES cells. However, teratoma formation frequency for late-passage Ser-iPS cells was similar to MEF-iPS cells. These findings indicate that early-passage iPS cells retain some somatic memory that impacts on iPS cell properties. These data suggest that immune-privileged Sertoli cells might represent a preferred target for iPS cell generation, if it comes to use of iPS cell derivatives for transplantation.

Transdifferentiation represents yet another promising method of reprogramming one cell type into another. Successful transdifferentiation of fibroblasts into several cell types has been achieved, such as neurons, cardiomyocytes, hepatocytes and blood cells. Here, I attempted to transdifferentiate MEF from Vav-iCre/ROSA26R-fGFP reporter mice (Vav-MEF) into Vav-GFP$^+$ hematopoietic cells by Oct4 and Oct4 plus Bmi1. Infection of CD45 MACS depleted Vav-MEF by Oct4 and Oct4 plus Bmi1 yielded Vav-GFP$^+$ hematopoietic cells. However, hematopoietic cells failed to show up after transfection of CD45 plus Vav-GFP FACS depleted Vav-MEF with the same methods. These findings indicate that Oct4 or Oct4 plus Bmi1 do not have the potential to transdifferentiate MEF into blood cells. Screening for further transcriptional factors, which are important for hematopoiesis, will be necessary for successful transdifferentiation into hematopoietic cells.

ZUSAMMENFASSUNG

Induzierte pluripotente Stammzellen (iPS Zellen) können ähnlich wie embryonale Stammzellen (ES Zellen) in alle Körperzellen des menschlichen Organismus differenzieren und bieten daher einzigartige Möglichkeiten bei der Erforschung von Krankheiten, der Medikamentenentwicklung und in der regenerativen Medizin. So könnten bei Verwendung von autologen iPS Zellen und daraus differenzierter Zellen Abstoßungsreaktionen vermieden werden und diese Zellen sind daher besonders interessant für die personalisierte Medizin. Allerdings wird diese Annahme bisher kontrovers diskutiert. In einigen Studien wurden isogene iPS Zellen von Mäusen nach der Transplantation abgestoßen. Andere Studien berichteten von keiner oder einer vernachlässigbaren Immunogenität solcher iPS Zellen und der aus ihnen differenzierten Zellen. Alle bisherigen Untersuchungen nutzten iPS Zellen, die aus Fibroblasten generiert wurden. Da iPS Zellen ein somatisches Gedächtnis besitzen, ist es möglich, dass die Immunogenität von iPS Zellen von den ursprünglich zur Reprogrammierung verwendeten Zellen abhängt.

Sertoli Zellen aus dem Hoden besitzen die Fähigkeit, eine vor dem Immunsystem geschützte Umgebung zu schaffen, was für die Entwicklung von Keimzellen im Hoden wichtig ist. In der vorliegenden Arbeit wurden iPS Zellen aus C57BL/6 (B6) Sertoli Zellen generiert und in isogene Mäuse injiziert, um deren Immunogenität in Teratomen zu untersuchen. Der Teratom Assay erlaubt die gleichzeitige Bewertung von Immunogenität und Differenzierungspotential von iPS Zellen *in vivo*. Es konnte gezeigt werden, dass aus Sertoli Zellen abgeleitete iPS Zellen (Ser-iPS) in frühen Passagen eine verringerte Immunogenität aufweisen und nach der Injektion in isogene Empfänger signifikant mehr Teratome bildeten als von Maus embryonalen Fibroblasten (MEF) abgeleitete iPS Zellen (MEF-iPS). Diese Teratome von Ser-iPS wiesen eine geringere Infiltration durch Immunzellen und weniger Gewebeschädigungen durch Nekrose auf als Teratome von MEF-iPS Zellen. Embryoid bodies (EB) von Ser-iPS zeigten außerdem ein reduziertes Potenzial T-Zellen zu aktivieren, im Vergleich zu MEF-iPS, und ihre Immunogenität war vergleichbar mit der von isogenen ES Zellen. Im Gegensatz dazu war die Teratomrate von Ser-iPS aus späten Passagen vergleichbar mit der von MEF-iPS. Zusammengefasst zeigen die Daten, dass das somatische Gedächtnis von iPS Zellen deren Eigenschaften beeinflusst. Daher sind immun-priviligierte Sertoli Zellen zur Generierung von iPS Zellen zu Transplantationszwecken ein bevorzugter Zelltyp.

Transdifferenzierung ist eine *in vitro* Differenzierungsmethode, die einen Zelltyp direkt in einen anderen umwandelt, ohne den Schritt der Reprogrammierung zur pluripotenten Stammzelle. Diese Art der Differenzierung wird vielfach angewendet, und es konnten erfolgreich z. B. Neurone, Herzmuskelzellen, Leberzellen und Blutzellen generiert werden. In dieser Studie sollten MEF von Vav-iCre/ROSA26R-fGFP Reporter-Mäusen (Vav-MEF) durch die Einführung von Oct4 oder Oct4 plus Bmi1 in hämatopoetische Vav-GFP^+ Zellen transdifferenziert werden. Dazu wurden MEF mittels (i) MACS oder (ii) FACS von $CD45^+$-Zellen depletiert und mit den Transkriptionsfaktoren Oct4 bzw. Oct4 plus Bmi1 infiziert. Es wurde gefunden, dass nach stringenter FACS Depletion der MEF mit Oct4 oder Oct4 plus Bmi1 keine hämatopoetischen Zellen erhalten werden konnten. Diese Ergebnisse weisen darauf hin, dass Oct4 und Bmi1 nicht ausreichen, um MEF zu hämatopoetischen Zellen zu transdifferenzieren. Nachfolgende Studien sind nötig, um weitere Transkriptionsfaktoren für eine erfolgreiche Transdifferenzierung zu finden.

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ABBREVIATIONS

Symbol	Description
ABP	Androgen-binding protein
AMH	Anti-müllerian hormone
AP	Alkaline phosphatase
APC	Antigen presenting cell
AZA	5-azacytidine
BM	Bone marrow
Bmi1	B lymphoma Mo-MLV insertion region 1 homolog
BSA	Bovine serum ovalbumin
BTB	Blood-testis barrier
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
c-Myc	v-myc myelocytomatosis viral oncogene homolog
CTL	Cytotoxic T lymphocytes
DAPI	4', 6-diamidino-2-phenylindole
DC	Dendritic cell
DFA/CD55	Decay-accelerating factor
dpc	Days post coitum
DMSO	Dimethylsulfoxide
EB	Embryoid body
ES cells	Embryonic stem cells
FASL	Fas ligand
FCS	Fetal calf serum
for	Forward primer
GFP	Green fluorescent protein
HE	Hematoxylin and eosi
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICAM1	Intercellular adhesion molecules 1
IDO	Indoleamine 2,3 dioxygenase
IFNγ	Interferon γ
IGF	Insulin-like growth factor
IL	Interleukin
iPS cells	Induced pluripotent stem cells
Klf4	Krüppel-like factor 4
LB	Lysogeny broth

LCMV	Lymphocytic choriomeningitis virus
LIF	Leukemia inhibitory factor
LN_2	Liquid nitrogen
Mbd3	Methyl-CpG binding domain protein 3
MEF	Mouse embryonic fibroblast
MET	Mesenchymal to epithelial transition
MHC	Major histocompatibility complex
miHA	Minor histocompatibility antigen
MLR	Mixed lymphocyte reaction
MSC	Mesenchymal stem cell
NEAA	Non-essential amino acid
NK	Natural killer cell
Oct4	Octamer-binding protein 4
OSKM	Oct4, Sox2, Klf4, c-Myc
PB	Polybrene
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PMA	Phorbol myristate acetate
P/S	Penicillin/streptomycin
qRT-PCR	Quantitative real-time reverse transcription PCR
RBC	Red blood cell
rev	Reverse primer
SCF	Stem cell factor
SCNT	Somatic cell nuclear transfer
SERPIN	Serine protease inhibitor
Sox2	Sex determining region Y-box 2
Sox9	Sex determining region Y-box 9
Sry	Sex determing region of the Y chromosome
TCR	T cell receptor
TGFβ	Transforming growth factor β
Treg	Regulatory T cell
wt	Wild type
Zg16	Zymogen granule protein 16

1 INTRODUCTION

1.1 Somatic cell reprogramming

Embryonic stem cells (ES cells), which exist transiently in the inner cell mass of mammalian blastocysts, represent one of the best-understood pluripotent stem cell types. Mouse ES cell lines were firstly established in 1981 from mouse pre-implantation embryos. Mouse ES cells have the ability to expand and grow without limitation while maintaining their pluripotentcy, and the ability to differentiate into cell derivatives of all three germ layers (Evans and Kaufman 1981; Martin 1981). The establishment of mouse ES cell lines stimulated the discovery of human ES cells, which were first generated by Thomson and colleagues from *in vitro* fertilization produced blastocysts (Thomson et al., 1998). Human ES cells possess similar characteristics to mouse ES cells, such as indefinite self-renewal and pluripotent differentiation potential (Smith 2001) and cell derived thereof might be useful for treatment of diseases (Thomson et al., 1998). However, ethical issues challenge the use of human embryos. One way to circumvent these issues is to generate pluripotent cells *in vitro*. The increasing understanding of pluripotency of ES cells allowed generation of pluripotent cells from somatic cells, which is referred to as reprogramming. Several methods can be used for reprogramming somatic cells into a pluripotent state, such as somatic cell nuclear transfer (SCNT; Wilmut et al., 1997) and cell fusion (Tada et al., 2001). However, due to their rather low efficiency and potential risks, these methods are not widely used.

1.1.1 Induced pluripotent stem (iPS) cells

Generation of iPS cells

Cells derived form patient-specific pluripotent stem cells have the potential to be applied for clinical treatment of diseases. Both SCNT and cell fusion can generate pluripotent cells, but they have disadvantages, which prevented their use in the clinics. Until 2006, a landmark study by Yamanaka group demonstrated that mouse embryonic or adult fibroblasts can be reprogrammed into iPS cells by ectopic expression of four transcriptional factors octamer-binding protein 4 (Oct4), sex determining region Y-box 2 (Sox2), krüppel-like factor 4 (Klf4) and v-myc myelocytomatosis viral oncogene homolog (c-Myc; OSKM; Takahashi and Yamanaka 2006). iPS cells exhibited similar properties to ES cells, including pluripotency, differentiation capacity and the potential to produce viable mice by tetraploid complementation (Zhao et al., 2009). iPS cells have been

generated from a broad rang of cell types, including terminally differentiated cells, and cells come from all three germ layers (ectoderm, mesoderm and endoderm). Additionally, iPS cells can be generated with different combination of transcriptional factors (Hanna et al., 2008; Kim et al., 2009; Liu et al., 2010). iPS cells were also generated from various species, such as human, monkey, rat and pig (Liao et al., 2009; Liu et al., 2008; Takahashi et al., 2007; Wu et al., 2009;). Surprisingly, iPS cells were also generated *in vivo*, which exhibited both embryonic and extraembryonic differentiation potential (Abad et al., 2013). This new method for iPS cell generation represents a powerful strategy for regenerative medicine and tissue reprogramming *in situ*.

Generation of iPS cells can be achieved by integration and integration-free methods. Based on the vector types being used, the induction methods are divided into four categories: virus, DNA (plasmid), protein or RNA (Mochiduki and Okita 2012). iPS cells were initially generated by ectopic expression of reprogramming factors with retroviral or lentiviral vectors (Takahashi and Yamanaka 2006; Yu et al., 2007). However, potential tumorigenic effects due to insertional mutagenic lesions of retrovirus or lentivirus question the application of iPS cells containing integrated vector DNA, although the Cre-LoxP system can be used to remove transgenes (Soldner et al., 2009). To generate iPS cells with minimal or absence of genetic modification, various non-integration methods have also been developed (Okita et al., 2008; Yu et al., 2009).

Plasmid vectors were reported to induce mouse iPS cell generation, but with low efficiency (less than 0.0002%, Okita et al., 2008). Then, an episomal plasmid system was employed to generate human iPS cells (Yu et al., 2009). However, with these DNA-based vector methods, genomic integration can also occur at a low frequency. To entirely avoid potential integration of vector DNA into chromosomal DNA, one possible strategy is protein transduction. Zhou and colleagues use several cycles of purified poly-arginine tagged OSKM proteins to successfully generate iPS cells from mouse embryonic fibroblast (MEF) (Zhou et al., 2009). By using a similar method, iPS cells were obtained from human newborn fibroblasts after several rounds of supplement with 293 cell extracts expressing poly-arginine tagged OSKM genes (Ko et al., 2009). Another promising method to generate iPS cells without genetic modification is microRNA transduction (Telpalo-Carpio et al., 2013). Human iPS cells were generated at high efficiency by the application of modified mRNA containing OSKM transcriptional factors or by miRNA (Anokye-Danso et al., 2011; Warren et al., 2010). It is also possible to generate iPS cells by highly reproducible RNA-based approach, such as using a single,

synthetic self-replicating VEE-RF RNA replicon that expresses OSKM reprogramming factors (Yoshioka et al., 2013). Additionally, human iPS cells were efficiently generated by application of Sendai virus-based vector system (Fusaki et al., 2009). Sendai virus is a negative sense, single-stranded RNA virus and belongs to *Paramyxovirdae* family (Lamb and Kolakofsky 1996). Furthermore, recombinant Sendai virus vectors do not go through a DNA stage nor integrate into the genome of the host cells (Li et al., 2000). Thus, Sendai virus-based vector system prodives a critical method for cell reprogramming without DNA integration. Although diverse strategies for iPS generation have been rapidly developed, more efficient and safe methods are still necessary for generating clinically useful iPS cells.

With the development of iPS technology, more and more details involved in the reprogramming process have been clarified. Based on gene expression during OSKM-induced MEF reprogramming, the multistep processes of iPS generation were characterized by three phases: initiation, maturation and stabilization (Samavarchi-Tehrani et al., 2010). The initiation phase marks a process referred to as mesenchymal to epithelial transition (MET), which suppressed the pro-epithelial to mesenchymal transition signals (Li et al., 2010). The stabilization phase depends on the removal of transgene expression, which is required for the maturation phase (Golipour et al., 2012). Transcription factor induced reprogramming is generally an inefficient and stochastic event due to the “rate-limiting step”, which is an unknown step in the transition from somatic cells to iPS cells (Buganim et al., 2013). Recently, Tanabe and colleagues showed that maturation, not initiation, limits direct reprogramming of human fibroblasts to pluripotency state (Tanabe et al., 2013). However, the rate-limiting step can be overcomed by depleting methyl-CpG binding domain protein 3 (Mbd3; a core member of the Mbd3/NuRD repressor complex) together with OSKM transduction. With this method, mouse and human somatic cells can be reprogrammed into iPS cells with 100% efficiency (Rais et al., 2013).

Although much progress has been made in understanding the complex reprogramming process, many questions related to the molecular mechanism during reprogramming remain unsolved. Addressing these unsolved problems will be required for deeper understanding of the reprogramming process and further application of iPS cells in clinical therapy.

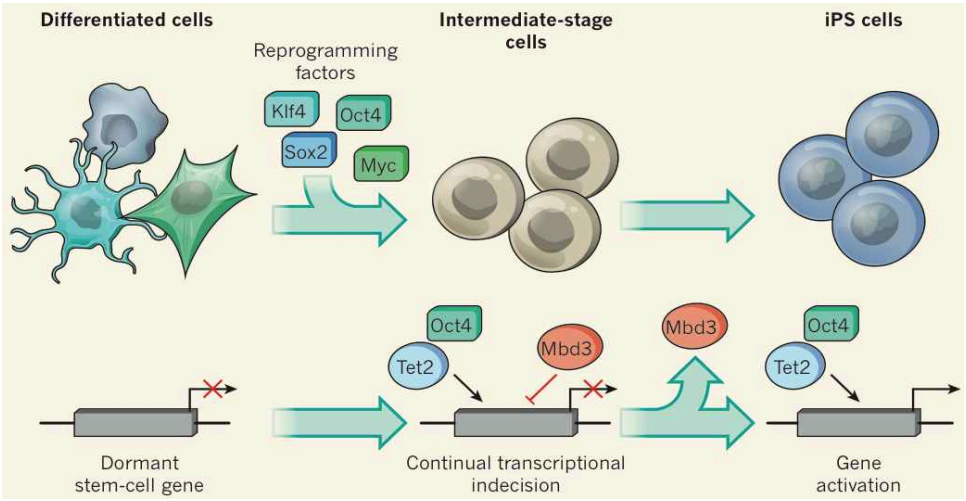

Figure 1.1. The battle of pluripotency.

Elimination of Mbd3 enabled efficient activation of dormant stem-cell genes, allowing almost all starting cells to be converted into pluripotent state (Loh and Lim 2013).

Differentiation of iPS cells

Three-germ layer differentiation potential of iPS cells is another important property of iPS cells further to their unlimited self-renewal potential. Frequently, differentiation potential of iPS cells is examined in embryoid body (EB) assay *in vitro* and teratoma assay in immune deficient mice *in vivo*. iPS cells differentiate into a variety of cell types, such as lung and airway progenitors, cardiomyocytes and hepatic progenitor cells (Mou et al., 2012; Kawamura et al., 2012; Yanagida et al., 2013). For *in vitro* analysis or *in vivo* transplantation, specifical differentiated cell type in large quantities from iPS cells is a precondition. However, the development of methods favoring the efficient differentiation of iPS cells into one specfic cell type has been challenged. Knockdown of Sox2 can accelerate differentiation of pluripotent stem cells (De et al., 2014). Small molecules, such as ascorbic acid, are attractive agents to direct specific cell differentiation, (Cao et al., 2012). Interestingly, iPS cells have been differentiated *in vivo* into human hematopoietic cells in teratoma assays. These hematopoietic cells were transplantable and reconstituted the human immune system (Amabile et al., 2013). This study provides a new concept to obtain specific differentiated cells from iPS cells.

While there is progress in differentiation protocols for iPS cells, some limitations still remain. The potential tumorigenic risk, due to contaminating undifferentiated cells in differentiated products, is one of the biggest roadblocks for their clinical application (Tang and Drukker 2011). Immunodepletion with antibodies against human iPS cells and with two additional pluripotency surface markers have proved feasible to deplete

undifferentiated cells (Tang et al., 2011). Additionally, both low differentiation efficiency and the immature phenotype of differentiated cells are critical limitations of current differentiation protocols (Oshima et al., 2010). The long-standing question is to generate mature and functional cells with high efficiency and purity, which is important for therapeutic transplantation, *in vitro* drug screening and disease research.

Epigenetic memory in iPS cells

iPS cells have been obtained from numerous somatic cell types, including fibroblasts, keratinocytes, B-lymphocytes, hepatocytes and stomach cells (Stadtfeld et al., 2008; Aasen et al., 2008; Hanna et al., 2008; Aoi et al., 2008). An interesting question is whether there are differences among iPS cell lines derived from different cell origins. Gene expression profiles of different human iPS cell lines showed that iPS cells retained significant donor cell gene expression (Chin et al., 2009; Marchetto et al., 2009; Ghosh et al., 2010; Bar-Nur et al., 2011). More interestingly, when iPS cells derived from human cells or from the same mouse but different cell types, resulting iPS cells cluster together according to their cell of origin by DNA methylation analysis (Polo et al., 2010; Kim et al., 2010; Lister et al., 2010; Ohi et al., 2011; Kim et al., 2011b). Furthermore, these iPS cells were more likely to re-differentiate into the somatic cell lineage used for reprogramming than unrelated lineages (Polo et al., 2010; Kim et al., 2010; Pfaff et al., 2012). Epigenetic memory that favors iPS cell re-differentiation into the cell type of origin has been reported in human iPS cells derived from different somatic cell types (Hu et al., 2010; Bar-Nur et al., 2011; Kim et al., 2011b). *In vivo* experiments indicated that, the teratoma-forming potential of neurospheres has marked differences depending on the tissue origin of iPS cells (Miura et al., 2009). The mechanisms of different teratoma formation potential are still elusive, but could be related to the somatic memory in iPS cells from distinct tissues that affects the differentiation propensity of resulting iPS cells (Bilic and Izpisua Belmonte 2012).

It is important to note that, in some of these studies, early-passage mouse iPS cells (p4-6) or human iPS cells (p<20) were used for analysis (Polo et al., 2010; Kim et al., 2010; Ohi et al., 2011). However, prolonged passaging of iPS cells removed the differences in gene expression profiles. These late-passage iPS cells failed to cluster together according to their cell of origin (Chin et al., 2009; Chin et al., 2010; Polo et al., 2010). In addition, treatment with chemical reagent 5-azacytidine (AZA) or using several differentiation-reprogramming periods into the desired direction, erased the

differentiation propensity ability of iPS cells (Kim et al., 2010).

It seems that fully reprogramming takes longer, and requires several passages even after the emergence of ES cell-like morphology and expression of pluripotency markers (Bilic and Izpisua Belmonte 2012). It is still unclear if epigenetic reprogramming continues during extended culturing, or if fully reprogrammed cells are selected during passaging, or if exogenous reprogramming factors get silenced with time (Papp and Plath 2011).

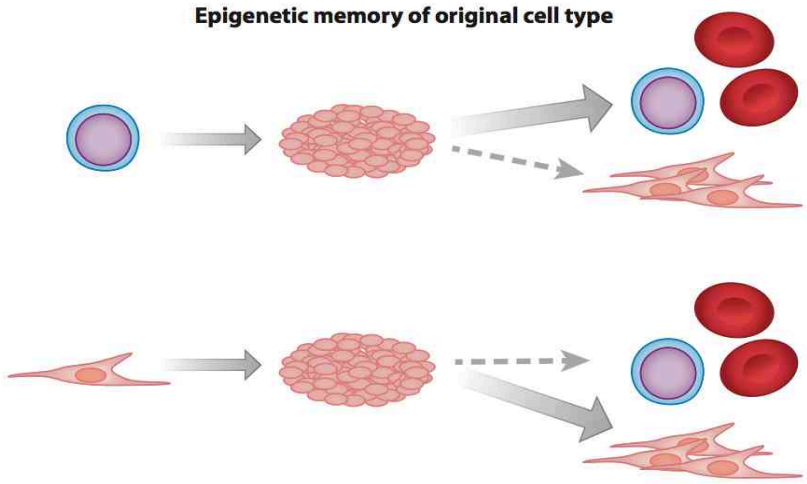

Figure 1.2. iPS cells have different differentiation capacity.

iPS cells often have more potential to differentiate towards cell types related to the lineage they come from (wide arrows) over towards the unrelated cell types (dotted arrows; Cherry and Daley 2013).

Although iPS cells continue to offer great prospects for therapeutic applications, substantial research and development are still needed before the application of iPS cell-based cell therapies. On the other hand, differentiation bias of iPS cells may be advantageous in generation of specific cell types that currently are difficult to produce *in vitro* for certain research and therapeutic application.

Application of iPS cells

iPS cells are an attractive source for disease research when a sufficient number of primary cells from patient are difficult to obtain. One way that iPS cells will influence modern medicine is the generation of iPS cells from individual patient with recognized disease characters. Indeed, disease-specific iPS cells are available for a broad range of diseases, such as Parkinson disease (Soldner et al., 2009), diabetes type I (Maehr et al., 2009) and cystic fibrosis (Somers et al., 2010). Disease-based iPS cells are expected to

contribute to disease research in two aspects: (i) as the basis for drug screening that may be used as therapeutics and (ii) as tools for disease mechanism studies (Cherry and Daley 2013). The aim to create disease-specific iPS cell is to identify the disease phenotype and correct the defective gene(s) for further use in the patient (Unternaehrer and Daley 2011). Despite considerable progress made in disease-specific iPS cells, utilization of these cells for understanding disease mechanisms and developing therapies is just at the beginning.

1.1.2 Transdifferentiation

In the stem cell field, the possibility of direct cell fate conversion has attracted great interest for patient-specific cell therapy. Although larger quantity of pluripotent stem cells and easy genetic correction can be achieved by iPS technology, the complexity for iPS generation and their high risk of tumorigenesis are challenges for their clinical application. An alternative method for reprogramming is transdifferentiation, which can convert one cell type directly into another, without a detour via pluripotent state.

Whether different cell fates are interchangeable have already been addressed decades ago. Lassar and colleagues discovered for the first time that exogenous expression of transcription factor myoblast determination induced muscle-specific properties in fibroblast (Davis et al., 1987). Subsequently, studies for successful cell fate conversion increase at a remarkable speed. Transdifferentiation of mouse B-lymphocytes into macrophages, mouse pancreatic acinar cells to beta-islet cells have already been proved feasible (Xie et al., 2004; Zhou et al., 2008). All these previous studies induced transdifferentiation only between lineages within the same germ layer. However, whether cell fate conversion between different germ layers is possible remained to be investigated. It was not until 2010 that Wernig and colleagues achieved the conversion from mouse fibroblasts (mesoderm) to neurons (ectoderm), showing for the first time that cell fate conversion across germ layers is feasible (Vierbuchen et al., 2010). These experiments were performed on mouse cells.

Recently, a number of reports have made great progress in directing human fibroblasts to neural cells/specific neuronal subtypes (Ambasudhan et al., 2011; Son et al., 2011; Pfisterer et al., 2011; Caiazzo et al., 2011). Furthermore, fibroblasts and Sertoli cells can also be transdifferentiated into neural stem/progenitor cells by the combination of different transcriptional factors or even by sox2 alone (Sheng et al., 2011; Kim et al., 2011a; Lujan et al., 2011; Han et al., 2012; Thier et al., 2012; Ring et al., 2012).

Transdifferentiation into neurons was even achieved *in vivo* (Torper et al., 2013; Rouaux and Arlotta 2013; Niu et al., 2013). Additionally, transdifferentiation into other cell types is also possible, such as transdifferentiation into cardiomyocyte/cardiac progenitors, hepatocytes, embryonic Sertoli-liked cells and multilineage blood progenitors/hemogenic cells (Ieda et al., 2010; Szabo et al., 2010; Sekiya and Suzuki 2011; Huang et al., 2011; Efe et al., 2011; Islas et al., 2012; Buganim et al., 2012; Pereira et al., 2013). More importantly, transdifferentiation of human mesenchymal stem cells (MSC) and fibroblasts to hematopoietic cells was achieved by treating with chemical reagent AZA and growth factors (Harris et al., 2011). Moreover, hepatocytes derived from human fibroblasts were reported to reconstruct mouse livers (Zhu et al., 2014). Transdifferentiation was also possible in other species, such as porcine and rat (Zhu et al., 2012; Yang et al., 2013). Like iPS cell technology, transdifferentiation can be used for examining disease mechanisms. Induced neural cells from patient's fibroblasts reproduced the disease phenotypes observed in endogenous neurons, thus validating the use of induced neural cells for disease research (Qiang et al., 2011; Chanda et al., 2013).

Transdifferentiation can be used to turn one cell type directly to another cell type in a shorter time in a relative simple procedure. Transdifferentiation represents a valuable method for cell replacement therapy. However, generation of specialized cell type with sufficient quantity remains a great challenge. Considering the common concerns for cell reprogramming, the existence of somatic memory in transdifferentiated cells is possible. Overall, considerable work is still required to find a most appropriate method for production of specific cell types with therapeutic application.

1.2 Hematopoietic cells

Hematopoietic stem cells (HSC) derived from blood and bone marrow (BM) are responsible for constant self-renewal and differentiate to a variety of specialized blood cells. Currently, no other stem cells have been best-characterized like HSC and actually used in therapy. The existence of HSC is first discoved by Till and McCulloch in 1961 (Till and Mc 1961). Since then, studies about phenotype and functional characteristics of HSC have rapidly developed. However, challenges for HSC assay and application are how to identify HSC and keep them proliferating *in vitro*.

1.2.1 Generation of hematopoietic cells *in vitro*

Generation of hematopoietic cells from pluripotent stem cells

To discover how hematopoietic cells emerge during embryonic development is a good start for better understanding of hematopoietic cells' properties. Embryos can differentiate into cells of all three germ layers, including hematopoietic cells. In 1985, it was shown for first time that hematopoietic precursors can be obtained during EB differentiation of mouse ES cells (Doetschman et al., 1985). Generation of hematopoietic cells from human ES cells has also been reported, as evidenced by the production of gamma-globin, a blood protein (Itskovitz-Eldor et al., 2000). In vitro differentiation of ES cells recapitulate early hematopoietic development and follow-up studies about hematopoietic cell generation have been described.

After development of iPS technology it is feasible to generate hematopoietic cells from iPS cells. iPS technology has been applied to cure hematopoietic diseases (sickle cell anemia) in humanized mouse model (Hanna et al., 2007). Although pluripotent stem cells have advantages in clinical application, problems remain. For example, human hematopoietic cells derived from pluripotent stem cells *in vitro* failed to reconstitute the entire hematopoietic system (Wang et al., 2005;Doulatov et al., 2013). However, a recent study showed that human hematopoietic stem/progenitor cells could be generated *in vivo* by teratoma assay in immunocompromised mice. Furthermore, these hematopoietic stem/progenitor cells could reconstitute human immune system in immunodeficient mice (Amabile et al., 2013). This method is particularly appealing for those cell types that are normally difficult to obtain from pluripotent stem cells *in vitro*. Yet, the method bears the problem of possible tumorigenesis and requirement of sufficient quantities for clinical application.

Generation of hematopoietic cells from somatic cells

With the development of transdifferentiation technology, numerous successful transdifferentiation experiments have been reported. Moreover, successful transdifferentiation into multilineage blood progenitors has been achieved by ectopic expression of Oct4 together with hematopoietic cytokines in human fibroblasts (Szabo et al., 2010). Such hematopoietic cells gave rise to granulocytic, monocytic, megakaryocytic and erythroid lineages, and possessed engraftment capacity *in vivo.* More recently, mouse fibroblasts transfected with other transcriptional factors (Gata2, Gfi1b, cFos and Etv6) can be efficiently induced to hematopoietic progenitors (Pereira et al., 2013). Furthermore, human MSC and skin fibroblasts can be transformed to hematopoietic cells by treating exclusively with demethylating reagent AZA and growth

factors (Harris et al., 2011). These findings indicate an alternative approach to obtain hematopoietic cells, which are normally difficult to get from pluripotent stem cells *in vitro* for autologous cell replacement therapies.

1.2.2 Gene regulation during hematopoiesis

Self-renewal and differentiation of HSC have been tightly controlled by a number of transcriptional factors (McKinney-Freeman et al., 2012). Several transcription factors play important roles during hematopoiesis. Polycomb group proteins, which control embryonic development by inhibiting homeotic gene expression, were initially discovered in *Drosphila* (Muyrers-Chen et al., 2004). Polycomb repressive complex 1, one of the polycomb group protein complexes, has been well described. B lymphoma Mo-MLV insertion region 1 homolog (Bmi1), a key component of polycomb repressive complex 1, is selectively expressed in various kinds of adult stem cells, such as HSC and neural stem cells (Park et al., 2003; Molofsky et al., 2005). The role of Bmi1 in the maintaining adult stem cell pool is mainly by repression of Ink4a/Arf locus, which encodes inhibitors of cell cycle kinase p16Ink4a and p19Arf (Akala et al., 2008).

Function of Bmi1 in HSC was also investigated in Bmi1 deficient ($Bmi1^{-/-}$) mice and competitive transplantation experiments (Lessard and Sauvageau 2003; Park et al., 2003). Accordingly, overexpression of Bmi1 increases self-renewal of HSC in both the mouse and the human system (Iwama et al., 2004; Rizo et al., 2008). Furthermore, potential of Bmi1 in promoting hematopoietic cell development from ES cells has been reported (Ding et al., 2012). Increasing evidence of Bmi1 function in maintaining self-renewal of adult stem cells and enhancing hematopoietic cell development from ES cells indicates that Bmi1 plays a major role during hematopoiesis. However, whether Bmi1 is crucial for transdifferentiation somatic cells into hematopoietic cells has so far not been studied.

1.3 Sertoli cells

Sertoli cells are first discovered by Sertoli in 1865. Sertoli cells, together with different stages of sperm cells and peritubular myoid cells, form the seminiferous tubules of testis. They extend from the basement membrane, which is formed by myoid cells, to the lumen of seminiferous tubules, completely surrounding the developing germ cells. Sertoli cells provide nutrients and immune-protective factors to support the development of germ cells (Griswold 1998) and Sertoli cells are crucial for spermotogenesis and adult fertility.

1.3.1 Sertoli cell development

During mouse development the gonads were morphologically indistinguishable until at about 12.5 days post coitum (dpc), when testis cords formed in male about 48 hours after sex determining region of Y chromosome (Sry) expression (Gubbay et al., 1990). Sertoli cells, the primary structural composition of testicular seminiferous tubules, epithelialize and enclose germ cells and are surrounded by peritubular myoid cells to form a tubular structure. Sertoli cells play a key role in sex determination. They were regarded to be the first somatic cell type to differentiate in the testis (Karl and Capel 1998). Moreover, they were the only cell type in testis required to possess a Y chromosome and direct other testicular somatic cells to male pathway (Burgoyne 1988).

To better understand how Sertoli cells direct testis development, it is essential to know from where they emerge. Previously, Sertoli cells have been proposed to derive from coelomic epithelium, mesonephros, or both (Byskov 1986). Subsequent studies indicated that cell types from mesonephros that migrated into male gonad were peritubular myoid cells and endothelial cells (Merchant-Larios et al., 1993), while Sertoli cells derived from coelomic epithelial between tail somite 15 and 17 stages (Karl and Capel 1998).

Many cell types are considered as terminally differentiated cells, including Sertoli cells (Chaudhary et al., 2005). Sertoli cells proliferate more rapidly before birth and keep on proliferating for another 2 weeks (mice) to 3 weeks (rats) after birth (Orth 1982; Vergouwen et al., 1991). During puberty, Sertoli cells progressively cease proliferation, fix their morphology and physiology and become terminally differentiated cells (Jegou 1992). Thereafter, nucleus of Sertoli cells enlarges and nucleolus becomes more prominent. Furthermore, tight junctions form between adjacent Sertoli cell membranes, which are termed blood-testis barrier (BTB), to create unique adluminal compartments (Sharpe et al., 2003). However, recently it has been shown that Sertoli cells from adult mouse and human have the potential to proliferate during *in vitro* culture (Ahmed et al., 2009). In addition, adult Sertoli cells from hamsters regained their proliferative capacity *in vivo* (Tarulli et al., 2006).

1.3.2 Sertoli cell function

Immune-privileged sites are places where autoantigens or foreign antigens are tolerated for extended periods of time without raising detrimental immune responses (Barker and Billingham 1977). Testis is an immunologically privileged site that not only protects auto-

immmuogenic male germ cells but also provides tolerogenic environment for the survival of foreign tissues without any immunosuppressive drugs (Barker and Billingham 1977). Sertoli cells have been identified as key players for creating immune-privileged testicular environment (Whitmore et al., 1985). A number of mechanisms contribute to this immune-privileged function of Sertoli cells.

Blood-testis barrier

The BTB, formed between adjacent Sertoli cell membranes, provides the physical immune protective role for germ cells. BTB prevents auto-immunogenic germ cells from rejecting by systematic circulation (Cheng and Mruk 2002). However, BTB is only partially responsible for the immune-privileged function of Sertoli cells. Because spermatogonia cells, which locate outside BTB and are immunogenic, yet are not attacked by immune system (Yule et al., 1988). This suggests that, not only BTB, but also factors secreted by Sertoli cells are important for establishment and maintenance of the testicular immune-privileged environment.

Induction of peripheral tolerance

Immune-modulatory factors secreted by Sertoli cells facilitate the formation of testical immune-privileged environment and avoid immune rejection. Immune responses involve immune cells, such as T cell, B cell and macrophage. *In vitro* experiments showed that Sertoli cells inhibit the proliferation of B cells and T cells (Wyatt et al., 1988; Selawry et al., 1991; De Cesaris et al., 1992). Inhibition of T cell proliferation by Sertoli cells was through arresting T cells in G1 phase of the cell cycle (Wyatt et al., 1988; Selawry et al., 1991; De Cesaris et al., 1992). This related to the decreased production of interleukin-2 (IL-2) and could not be compensated by addition of exogenous IL-2 (Selawry et al., 1991; De Cesaris et al., 1992).

Evidences from islet cell allogeneic transplantation into mouse testis indicated that T cells underwent apoptosis after entering into testicular environment and graft antigen-specific regulatory T cells (Tregs) increased (Dai et al., 2005; Nasr et al., 2005). Consistently, Sertoli cells exhibited the capacity to accelerate Treg generation ($CD4^+CD25^+Foxp3^+$; Dal Secco et al., 2008). These studies indicated that T cells in testicular environment could be switched from a type 1 response, which is related to cell-mediated immunity and rejection, to a type 2 response that is mainly associated with immune modulation and tolerogenic (Meinhardt and Hedger 2011). This was confirmed

by co-transplanting Sertoli cells and islets into non-obese diabetic mice (Suarez-Pinzon et al., 2000). In addition, most of the testicular macrophages showed decreased production of proinflammatory cytokines and increased immune-regulatory functions, which are the characteristics of type 2 phenotype (Hedger 2002; Maresz et al., 2008). Functions of other testicular immune cells, such as natural killer (NK) cell, dendritic cell (DC), remain largely unexplored.

Inhibition of complement activation and membrane-associated cell lysis

The complement system is an enzyme cascade that “complements” the ability of antibodies and phagocytic cells to clear pathogens from an organism. Activation of the complement cascade leads to hyperacute, acute and chronic graft rejection. The immune-privileged function of Sertoli cells was shown to involve resistance of complement activation (Doyle et al., 2012). Classical pathway-mediated activation of the complement system results in cell lysis by formation of membrane attach complexes (Mital et al., 2010). Sertoli cells exhibit resistance to such killing mechanism as evidenced by their survival after xenogeneic transplantation and human antibody/complement-mediated killing assay (Gores et al., 2003; Dufour et al., 2005). Moreover, factors associated with the resistance of complement activation are highly expressed in Sertoli cells, such as serine protease inhibitor G1 (SERPING1) and decay-accelerating factor (DAF/CD55; Murray-Rust et al., 2009; Esposito et al., 2010). Additionally, clusterin expressed in Sertoli cells is reported to block membrane attach complex-mediated cell lysis (Jenne and Tschopp 1989). These findings indicate that resistance of the complement mechanism is important for the immune-privileged function of Sertoli cells.

Inhibition of apoptosis through the granzyme-mediated or Fas-Fas ligand (FasL) pathways also contributes to the immune-privileged function of Sertoli cells. CD8 T cell and NK cell mediated cell killing was via Fas-FasL or granule-mediated pathways (Chavez-Galan et al., 2009). Several studies indicated that Sertoli cells express SERPINA3N and protease inhibitor-9 (SERPINB9, PI9), which are granzyme B inhibitors (Sipione et al., 2006; Bladergroen et al., 2001; Hirst et al., 2001). Furthermore, PI9 has been shown to inhibit Fas-FasL induced apoptosis (Cunningham et al., 2007). FasL, a member of the tumor necrosis factor family, initiates caspase activation and apoptotic cell death by binding to Fas receptor. FasL-expressing Sertoli cells are hypothesized to interact with Fas-bearing lymphocytes and result in the death of lymphocytes through

apoptosis pathway (Mital et al., 2010). It is found that Fas and FasL also exist in soluble forms, sFas and sFasL, which makes their function in testis even more complicated.

Expression of multiple immune-regulating factors

Sertoli cells secrete multiple cytokines, which play important roles in their immune-privileged function. Most notable cytokines are members of the transforming growth factor β (TGFβ) family. It has already been reported that TGFβ1 supported islet beta-cell survival during co-transplantation (Suarez-Pinzon et al., 2000), while TGFβ3 regulated the disruption of tight junction, which involves in the protective function of BTB (Lui et al., 2003). Thus, functions of the TGFβ family in maintaining testicular immune-privileged milieu seem to be complex. Inhibiting the expression of indoleamine 2,3 dioxygenase (IDO), a tryptophan-metabolising enzyme, abrogates the capability of porcine Sertoli cells to protect islet allograft in mice model (Fallarino et al., 2009). Interleukin 6 and leukemia inhibitory factor (LIF) secreted by Sertoli cells, provide an immune-privileged function by inducing Treg generation and directing type 1 to type 2 immune response (Diehl and Rincon 2002; Zenclussen et al., 2006; Doyle et al., 2012). Other molecules involved in immune-privileged function of Sertoli cells should be further investigated. It is most likely that the interaction of multiple immune-protective factors contributes to the immune-privileged function of Sertoli cells.

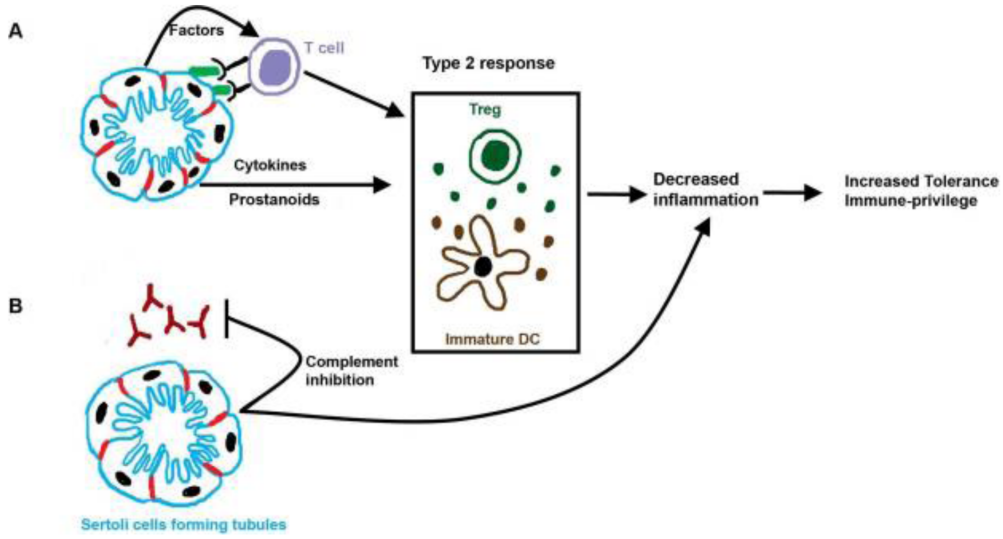

Figure 1.3. Schematic representation of Sertoli cell immune function.

Sertoli cells form tubule structures with tight junctions (thick red lines) and increase adhesion to lymphocytes, thereby limiting leukocyte migration.
A: Factors (e.g., cytokines and prostanoids) secreted by Sertoli cells convert the cell-mediated immune response to a type 2 response and suppress inflammation, therefore resulting in immune tolerance and immune privilege. Green line, adhesion molecules.
B: Humorally, Sertoli cells express complement inhibitors that inhibit activation of the complement cascade and cell lysis, decrease leukocyte recruitment and inflammation, and thus contribute to

immune privilege. Green and brown dots, anti-inflammatory molecules secreted by Tregs and immature DC; Y, immunoglobulin (Doyle et al., 2012).

Taken together, Sertoli cells provide immune-privileged testicular environment by attenuating immune response: increased interactions between cells to decrease leukocyte migration; inhibition of complement reaction and inducing a type 2 immune response, all of which support an anti-inflammatory response (Doyle et al., 2012).

1.3.3 Sertoli cell application

The unique immune protective function of Sertoli cells has been used in co-transplantation studies, to protect other grafts from immune rejection. Evidence from the co-transplantation of Sertoli cells with pancreatic islets showed that Sertoli cells significantly prolonged the survival of pancreatic islets (Dufour et al., 2003). Furthermore, Sertoli cells have been shown to improve survival of other grafts, such as xenogeneic neurons (Willing et al., 1999), xenogeneic liver cells (Rahman et al., 2005), allogeneic heart grafts (Lim et al., 2009). In addition, co-tranplantation of porcine Sertoli cells and islets into patients with type I diabetes was also feasible (Valdes-Gonzalez et al., 2005; Valdes-Gonzalez et al., 2007).

During co-transplantation, Sertoli cells themselves also survive for long periods while their protective function for the grafts is lost with time. The long-term survival of Sertoli cells indicates that they are useful vehicles for cell-based therapeutic product delivery. This idea was first tested by transplanting Sertoli cells expressing green fluorescent protein (GFP) reporter gene into kidney capsule and such cells survived for at least 60 days (Dufour et al., 2004). Afterwards, transplantation of genetically engineered therapeutic Sertoli cells carrying human trophic factor, neurotrophin-3 has been studied. However, neurotrophin-3 production was only for 3 days (Trivedi et al., 2006). More recently, engineered insulin-expressing Sertoli cells have been transplanted into diabetic mice. Yet, the decrease of glucose level was transient and mice returned to the diabetic state within 8 days (Halley et al., 2010). Although Sertoli cells can be used as potential tools for delivery of therapeutic proteins, further studies are needed to improve the potential of engineered Sertoli cells.

1.4 Immunogenicity of pluripotent stem cells

Pluripotent stem cells can differentiate into all cells of our body and therefore have great potential for regenerative medicine (Robinton and Daley 2012). Intensive investigations

focus on using functional cells derived from pluripotent stem cells for treating degenerative diseases. However, potential immune responses induced by these cells are often neglected. At present, immunological restrictions during transplantation have not been well addressed and remain a major obstacle for cell replacement therapy.

1.4.1 Immunogenicity of embryonic stem cells

Major histocompatibility complex and costimulatory molecules expression

Major histocompatibility complex (MHC) has been identified as one of the major issues during transplantation. Incompatibility of MHC between organ donors and recipients results in graft rejection (Bradley et al., 2002). It was originally thought that ES cells possessed immune privilege characteristics partially due to their low to undectable expression of MHC class I and complete absence of MHC class II antigens and costimulatory molecules (CD80 and CD86; Magliocca et al., 2006; Drukker et al., 2002; Li et al., 2004). However, with the differentiation of ES cells into EBs and teratomas, expression of MHC class I gene increases 4 and 10 fold, respectively (Drukker et al., 2002). Immune responses induced by ES cells increase over time after allotransplantation (Swijnenburg et al., 2005). However, MHC class II molecule is neither expressed nor upregulated on the surface of undifferentiated or differentiated cells, as well as after stimulated with interferon γ (IFNγ; Drukker et al., 2002). Similarly, CD80 and CD86 expression is undetected either during differentiation or treatment with IFNγ (Li et al., 2004; Drukker et al., 2006).

Considering the low to absent expression of MHC class I and absence of MHC class II expression on mouse ES cells, they fail to stimulate T cell proliferation in mixed lymphocyte reaction (MLR) (Bonde and Zavazava 2006). Furthermore, mouse ES cells are resistanted to lysis by allogeneic cytotoxic T lymphocytes (CTL) and are only lysed by CTL at a moderately level after treatment with IFNγ (Bonde and Zavazava 2006). Even lymphocytic choriomeningitis virus (LCMV) infected mouse ES cells or EBs showed resistence to LCMV specific CTL lysis (Abdullah et al., 2007). In contrast, mouse ES cells pulsed with ovalbumin peptide were efficiently lysed by ovalbumin-specific T cell receptor (TCR) transgenic T cells (Dressel et al., 2009).

NK cells, one of the components of innate immunity, represent the first line of defense. It has been reported that rat ES cells were killed by NK cells due to their low expression of MHC class I molecules and their expression of ligands for the activating NK receptor NKG2D (Dressel et al., 2008). However, mouse ES cells showed no or low potential of

being killed by NK cells (Koch et al., 2008). As allogeneic human ES cells were immune-rejected by recipient (Boyd et al., 2012), most recently Rong et al exhibited a new approach to avoid immune responses. Human ES cells were engineered to constitutively express cytotoxic T lymphocyte antigen 4-immunoglobulin fusion protein and programmed death ligand-1, and such cells were immune protected by inhibiting T cell activities. Cells derived from these engineered human ES cells showed immune tolerance in humanlized mice (Rong et al., 2014). However, this approach also increases the risk of oncogenic transformation or might cause an accumulation of pathogenic viruses, due to the immune-privileged characteristics of these engineered cells (Willinger and Flavell 2014). In summary, this new strategy facilitates the application of allogeneic human ES cells in a clinical setting. Yet, a more safe and effective method to exploit allogeneic human ES cells and their derivatives warrants further investigation.

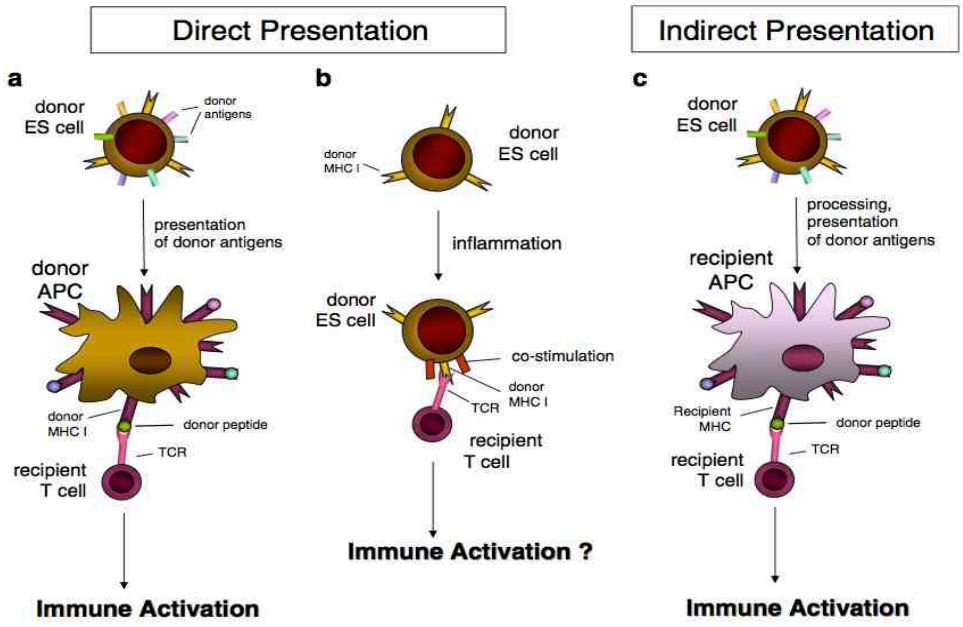

Figure 1.4. Possible antigen presentation pathways in allograft rejection.

a: In the direct pathway donor APC present alloantigens, which are recognized by recipient T cells.
b: Recognition of allo-MHC molecules directly by recipient T cells.
c: After transplantation alloantigens are recognized by recipient APC and presented to recipient T cells (Kadereit and Trounson 2011).

Immune protective molecules expression

Further to low expression of MHC antigens and costimulatory molcules, immune-modulatory molecules secreted by ES cells represent another contribution for their reduced immunogenicity. It has been reported that serine protease inhibitor 6 (serpin-6), an endogenous inhibitor of granzyme B, was highly expressed in mouse ES cells. Serpin-6 showed a protection of ES cells from lysing by CTL (Abdullah et al., 2007). Anti-

inflammation cytokines were found to be highly expressed in mouse ES cells and showed an inhibition of T cell proliferation and antigen presenting cell (APC) maturation (Koch et al., 2008). The impaction of ES cells on T cell activation and polarization was likely through negatively regulating the protein kinase C-θ (Mohib et al., 2012). Other possible immune-modulatory molecules and their mechanisms related to the low immunogenicity of ES cells still need to be investigated.

The expression of other immune-related factors

Minor histocompatibility antigens (miHA) are peptides derived from normal cellular proteins that show polymorphism among individuals. miHA is sufficiently antigenic to be recognized by CTL (Wallny and Rammensee 1990). Mouse ES cells showed vigorous rejection upon transplantion into MHC-matched mice, simply due to the different expression of miHA (Robertson et al., 2007). Further to expression of MHC and miHA proteins, culture conditions can also contribute to immune responses. It has been shown that human ES cells cultured with animal-derived feeder cells or serum induced immune responses, because of incorporation of animal sialic acid residues or serum components (Martin et al., 2005; Hisamatsu-Sakamoto et al., 2008). However, to what extent the miHA, culture conditions or other potential factors will affect the immunogenicity of ES cells remain to be determined.

1.4.2 Immunogenicity of iPS cells and their derivatives

Autologous iPS cells are believed to be particularly appealing as a cell source for personalized regenerative therapies due to their expected ability to bypass immune rejection (Nishikawa et al., 2008). However, recent studies challenged this view by showing immune rejection of mouse fibroblast derived iPS cells upon syngeneic transplantation as measured by teratoma formation and lymphocytic infiltration (de Almeida et al., 2014; Zhao et al., 2011). These findings are in contrast with the results from Guha et al. where syngeneic iPS cells showed 100% graft survival (Guha et al., 2013).

Follow-up studies evaluated the immunogenicity of specific differentiated cells derived from iPS cells in a syngeneic setting (Araki et al., 2013; Guha et al., 2013;de Almeida et al., 2014). Cardiomyocytes derived from iPS cells *in vitro* cause significant levels of T cell infiltration after syngeneic transplantation (Araki et al., 2013). However, other studies indicated that syngeneic iPS cell-derived cells elicited only minimal immune responses or induced tolerogenic immune responses following transplantation (Araki et al., 2013;

Guha et al., 2013;de Almeida et al., 2014). However, skin and BM cells used for transplantation developed in iPS cell-derived chimeric mice (Araki et al., 2013), where immunogenic or abnormal cells might get eliminated or induced to acquire immune tolerance (Fu 2014; Scheiner et al., 2014). Guha et al. transplanted terminally differentiated cells of all three germ layers into syngeneic hosts and there was no immune response as measured by T cell infiltration and graft survival (Guha et al., 2013). However, in this study the transplantation site (subcapsular renal space) is prone to induce immune tolerance due to the presence of immature DC (Coates et al., 2004; Fu 2014). In addition, so far only three cell types (endothelial cells, hepatocytes and neuronal cells) differentiated from iPS cells were investigated and it is currently unclear, which cell type contains leftover of the reprogramming process and thus immunogenicity (Cao et al., 2014). Furthermore, Morizane et al used nonhuman primate as a model to test the immunogenicity of neural cells derived from autologous and allogeneic iPS cells (Morizane et al., 2013). They found that neural cells derived from autologous iPS cells elicited only a minimal immune response compared to allogeneic iPS cells.

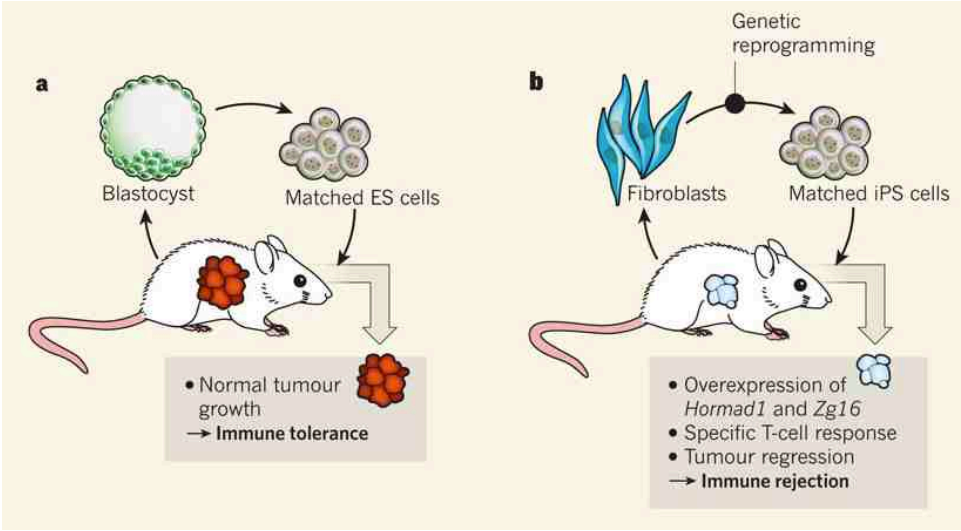

Figure 1.5. Immunogenicity of autologous iPS cells.

a: Syngeneic ES cells grow into teratomas following transplantation into mice and cause immune tolerance.
b: Autologous iPS cells derived from fetal fibroblasts elicit an unexpected immune rejection (Apostolou and Hochedlinger 2011).

An interesting question is what might cause immunogenicity of iPS cells and their differentiated progeny? The somatic cell type used for reprogramming might impact on the immunogenicity of iPS cells (Boyd et al., 2012). Human umbilical cord mesenchymal cells were used for iPS cell generation, as mesenchymal cells exhibit immune-modulatory properties (Liu et al., 2013). It was found that neural progenitors derived from

these iPS cells showed lower immunogenicity compared to those from fibroblast-derived iPS cells. This study indicates that both iPS cells and their differentiated progenies retain some epigenetic memory after cell reprogramming and subsequent differentiation. However, in this study the immunogenicity of iPS cells and their derived cells has only been investigated *in vitro* and several questions remained: (i) *in vivo* immunogenicity of these iPS cells and their derived cells; (ii) the effect of passage number on the immunogenicity of iPS cells and their derivatives; (iii) the effect of other immune-privileged somatic cell types on the immunogenicity of iPS cells and their derivatives.

1.5 Objectives and aims

The first establishment of iPS cells by introduction of four reprogramming factors (OSKM) stimulated great prospects for regenerative medicine. Most of the studies focus on the mechanisms, methods and efficiency for iPS cell generation, differentiation ability of iPS cells and their potential application for disease modelling. However, recent studies indicated that undifferentiated iPS cells were immune rejection upon syngeneic transplantation, which challenged the application of autologous iPS cells and their differentiated progeny for personalized medicine. Although follow up studies support the safety of syngeneic iPS cells and their differentiated derivatives, we should be alert about their possible immunogenicity. Several factors might influence the immunogenicity of iPS cells, such as the somatic cell type used for iPS cell generation (Boyd et al., 2012), *in vitro* culture conditions (Scheiner et al., 2014), cell types used for transplantation and the transplantation sites (Cao et al., 2014; Scheiner et al., 2014). It has been reported that somatic memory affects the characteristics and functions of iPS cells to some extent (Polo et al., 2010; Bar-Nur et al., 2011), thus indicating the importance of selecting the most appropriate starting cell population for iPS cell generation.

The objective of this study is to determine the influence of the parental somatic cell type on the immunogenicity of resulting iPS cells. To answer this question, immune-privileged Sertoli cells were used to generate iPS cells (Ser-iPS cells) and compared their immunogenicity with mouse embryonic fibroblast derived iPS cells (MEF-iPS cells). The immunogenicity of Ser-iPS cells was examined by both *in vivo* syngeneic transplantation and *in vitro* co-culture with T cells. Immune response was investigated by flow cytometry, qRT-PCR and immunohistochemistry.

As iPS cells have potential risk of tumorigenesis, researchers investigated alternative reprogramming methods, such as transdifferentiation. Here MEF from Vav-iCre/ROSA26R-fGFP reporter mice (Vav-MEF) were used for transdifferentiation into hematopoietic cells by overexpression of Oct4 and Oct4 combined with Bmi1. Flow cytometry analysis was used to investigate the expression of hematopoietic cell markers.

2 MATERIALS

2.1 Instruments

Instruments	Manufactures
Centrifuges	Heraeus (Hanaw, Germany); Eppendorf Centrifuge 5424; Sigma 2K15
Flow cytometer	BD FACS Canto II
Incubator	Heraeus (Hanaw, Germany)
Polymerase chain reaction (PCR)	Thermocycler (Eppendorf, Hamburg, Germany)
Quantitative real-time RT-PCR (qRT-PCR)	StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA)
Microscopes	Axiovert 200; AMG EVOS®fl; Leica DMIL; Leica DMRX
Gel documentation system	Biorad Gel doc X systerm (Bio-Rad, Hercules, CA, USA)
CASY cell counting and analysis system	Schärfe Systems (Schärfe Systems, Reutlingen, Germany)
Gentle MACS Dissociator	Miltenyi Biotec
Nanodrop Spectrophotometer	Thermo Scientific
Haemacytometer	Brand

Computers	Software
MAC Mini	Word, Excel, Power Point, Adobe Reader, Tierbase, FlowJo 7.6.5 for Mac
Mac PC	Endnote X6, FlowJo 7.6.5 and 8.7.2 for Mac, Adobe Photoshop, Microsoft Office 2011 for Mac, GraphicConverter X, GraphPad Prism 5, MeV_4_8, Origin Pro 8.0, StepOne™ software (version 2.1)

2.2 Mouse strains

	Strain name	Source
BL6 wild type (wt)	C57BL/6 (B6)	In-house breeding
Rosa26R-fGFP	Rosa26R-CAG-farnesylted-eGFP	A kind gift of Emma L. Rawlins, Gurdon Institute, Cambridge, UK (Rawlins et al., 2009)
Vav-iCre	Vav-iCre	A kind gift of Dimitris Kioussis, London, UK and Meinrad Busslinger, IMP, Vienna, Austria (de Boer et al., 2003; Kwon et al., 2008)
Vav-iCre/Rosa26R-fGFP	B6-tg (Vav-iCre)(Rosa26R-fGFP)	In-house breeding
NOD-Scid	NOD-(scid)(IL2Rg) tm	In-house breeding
CD1		In-house breeding

2.3 Antibodies

FACS antibodies

Target protein	Clone	Label	Company
CD4	GK1.5	PE-conjugated	eBioscience
CD44	IM7	eFluor450-conjugated	eBioscience
CD4	RM4-5	FITC-conjugated	eBioscience
CD25	PC61.5	APC-conjugated	eBioscience
Foxp3	FJK-16s	PE-conjugated	eBioscience
CD45	30-F11	APC-Cy7-conjugated	BD
F4/80	CI:A3-1	Pacific Blue-conjugated	Abcam
F4/80	BM8	APC-conjugated	eBioscience
CD19	MB19-1	PE-Cy5-conjugated	eBioscience
CD11b	M1/70	eFluor450-conjugated	eBioscience
CD8	53-6.7	PE-Cy7-conjugated	eBioscience

Immunofluorescence antibodies

Primary antibodies			
Target protein	Clone	Dilution	Company
Oct4	C-10	1:200	Santa Cruz Biotechnology
SSEA1	480	1:200	Santa Cruz Biotechnology

Secondy antibodies	Dilution	Company
Alexa594 goat anti-mouse Ig (H+L)	1:300	Life Technologies

2.4 Vector and Plasmid Constructs

Backbone	Constructs	Source
pMX	Oct4 (mouse)	Add Gene
	Sox2 (mouse)	Add Gene
	Klf4 (mouse)	Add Gene
	c-Myc (mouse)	Add Gene
	GFP (mouse)	Add Gene
pMSCV	Bmi1 (mouse)	From our lab

2.5 Chemicals and solutions

If not stated otherwise, all chemicals were purchased from Sigma-Aldrich, Merck or Fluka in analytical grade quality.

Chemicals	Manufactures
Anti-CD4 MicroBeads	Miltenyi Biotec
4',6-Diamidino-2-Phenylindole (DAPI)	Vector Laboratories
Mounting solution	Dako
DNA ladder (Gene Ruler 1kb and 100bp ladder plus)	Fermentas

2.6 Consumable materials

Materials	Manufactures
Cell strainer (70 µm, 40 µm)	BD Falcon™
MACS separation columns (MS)	Miltenyi Biotec
Gentle MACS M tubes	Miltenyi Biotec
FACS tubes	Sarstedt
qRT-PCR plate	Applied Biosystems

Other cell culture dishes, disposable pipettes and centrifuge tubes were purchased from Eppendorf, Brand, Nunc, TPP or BD Falcon™.

2.7 Kits

The following kits were used in molecular and biochemical assay:

Kits	Manufactures
NucleoSpin tissue kit	Macherey-Nagel
NucleoSpin RNA II kit	Macherey-Nagel
NucleoBond Xtra Maxi kit	Macherey-Nagel
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Fast SYBR Green master mix	Applied Biosystems
Alkaline phosphates kit	Stemgent
Mouse Regulatory T Cell Staining Kit	eBioscience
VECTASTAIN® ABC KIT	Vector Laboratories Ltd

2.8 Primers for PCR

Primers for genotyping

Gene	Sequences information (5' ➔ 3')	Annealing temp.	Cycle no.
Cre for	GCCTGCCCTCCCTGTGGATGCCACCT	65°C	36
Cre rev	GTGGCAGAAGGGGCAGCCACACCATT		
Rosa26 for	CACTTGCTCTCCCAAAGTCG	60°C	30
Rosa26 rev	TAGTCTAACTCGCGACACTG		
CAG	GTTATGTAACGCGGAACTCC		

for: forward primer; rev: reverse primer

Primers for RT-PCR

Gene	Sequences information (5' ➔ 3')	References
Dazl for	GCACTCAGTCTTCATCAGCAAC	(Kinarm Ko et al., 2009)
Dazl rev	CTATCTTCTGCACATCCACGTC	
Vasa for	CTTGCAGAGATGTTCAGCAGAC	(Ko et al., 2009)
Vasa rev	CTCCAAGAGCTTGCTCTCTCTC	
Hsd3β6 for	TGGACAAGTTCTTCAGACCAGA	
Hsd3β6 rev	TCTCCTTCCAACACTGTCACC	

TGFβ1 for	TGAGTGGCTGTCTTTTGACG	(Dufour et al., 2008)
TGFβ1 rev	TTGGTATCCAGGGCTCTCC	
Gata4 for	CCCTACCCAGCCTACATGG	(Kyrönlahti et al., 2011)
Gata4 rev	GAGCTGGCCTGCGATGTCTGAGTG	
Rex1 for	CAGCAGCTCCTGCACACAGA	
Rex1 rev	GATCCGCAAACACCTGCTTT	
Rex3 for	GAGCGGAGCAGGTCTGAGAA	
Rex3 rev	GACTCCATTACTCCTGGGCCT	
Dppa4 for	CATTGGGGGTAGGAACACGGAAGG	
Dppa4 rev	TGCCCCAAGTGTGTTCATAA	
Sox2 for	F-TAGAGCTAGACTCCGGGCGATGA	
Sox2 rev	R- TTGCCTTAAACAAGACCACGAAA	
GAPDH for	GGGGTGAGGCCGGTGCTGAGTAT	
GAPDH rev	CATTGGGGGTAGGAACACGGAAGG	

Primers for qRT-PCR

Gene	Sequences information (5' ➔ 3')	References
Oct4 for	TCTTTCCACCAGGCCCCCGGCTC	(Takahashi et
Oct4 rev	TGCGGGCGGACATGGGGAGATCC	al., 2006)
Flk1 for	CTTCTGGCTCCTTCTTGTCATTG	
Flk1 rev	TCATTGGCCCGCTTAACG	
Nanog for	TCTGGGAACGCCTCATCAAT	
Nanog rev	GGAGAGGCAGCCTCTGTGC	
MHCα for	CATGGCATCACTCTTCTCTACCTATGC	
MHCα rev	TACCACTGACACCGGTATCA	
AFP for	TCAGCGAGGAGAAATGGTCC	
AFP rev	ATGAAAATGTCGGCCATTCC	
β-actin for	CACCAGTTCGCCATGGATG	
β-actin rev	GCCGTTGTCGACGACCAG	
Nestin for	AGAGTCAGATCGCTCAGATCC	
Nestin rev	GCAGAGTCCTGTATGTAGCCAC	
CD3 for	TCGTCACTGTCTAGAGGGCA	
CD3 rev	CCTCCTAGCTGTTGGCACTT	
CD4 for	CAAGCGCCTAAGAGAGATGG	
CD4 rev	CACCTGTGCAAGAAGCAGAG	
CD8b for	GATGGTCTTTGGGACAGGG	(Rui et al.,

CD8b rev	ATGCCAGCAGAAGCAGGAT	2012)
CD11c for	CTGGATAGCCTTTCTTCTGCTG	(Shaul et al.,
CD11c rev	GCACACTGTGTCCGAACTC	2010)
B200 for	AAGAGTTGTGAGGCTGGCAC	
B220 rev	GCTCAAACTTCTGGCCTTTG	
Mac1 for	GTTTGTTGAAGGCATTTCCC	
Mac1 rev	ATTCGGTGATCCCTTGGATT	
Gr1 for	TGCCCCTTCTCTGATGGATT	(Doloff et al.,
Gr1 rev	TGCTCTTGACTTTGCTTCTGTGA	2012)
Zg16 for	CATCACCGCCTTCCGTAT	(Zhao et al.,
Zg16 rev	CGTTGAAACTTGTGCCTGA	2011)
Hormad1 for	CCAGATTACCAACCACCAG	(Zhao et al.,
Hormad1 rev	TGAAAAGGTGTTGGGACT	2011)
Sox9 for	AGTACCCGCATCTGCACAAC	(Svingen et
Sox9 rev	TACTTGTAATCGGGGTGGTCT	al., 2007)
AMH for	GGTGGAGGCTCTTGGAACTT	
AMH rev	GGGAGACTGGAGAACAGCAG	
ABP for	CTCAGGGCCTGGTCTATGTC	
ABP rev	TCTCTCCCTTGGGGCTTTAC	
ArginaseI for	AACACGGCAGTGGCTTTAACC	Kropf et al.,
ArginaseI rev	GGTTTTCATGTGGCGCATTC	2005
CD3-ζ chain for	TCAATCTAGGGCGAAGAGAGG	
CD3-ζ chain rev	GGGCATCACAGGTGTCCTT	
Foxp3 for	CGAAAGTGGCAGAGAGGTATTGA	(Mohib et al.,
Foxp3 rev	ACTGTCTTCCAAGTCTCGTCTGAA	2012)
IFNγ for	GAAAATCCTGCAGAGCCAGA	(Mohib et al.,
IFNγ rev	TGAGCTCATTGAATGCTTGG	2012)
IL4 for	CGAGCTCACTCTCTGTGGTG	
IL4 rev	TGAACGAGGTCACAGGAGAA	
MHC class I for	CCAGACGAAAATATCTCAGCG	
MHC class I rev	GGGTTGATGATGCTCCTGTT	
MHC class II for	CAACCGTGACTATTCCTTCC	Biswas et al.,
MHC class II rev	CCACAGTCTCTGTCAGCTC	2012
CD80 for	GGCAAGGCAGCAATACCTTA	Shaul et al.,
CD80 rev	CTCTTTGTGCTGCTGATTCG	2010
CD86 for	TCTCCACGGAAACAGCATCT	Shaul et al.,

CD86 rev	CTTACGGAAGCACCCATGAT	2010

2.9 Reagents, cytokines and growth factors for cell culture

Reagents	Manufactures
DMEM (4.5g/L high Glucose)	GIBCO® Invitrogen Cell Culture
RPMI-1640	GIBCO® Invitrogen Cell Culture
DMEM/F12 (1:1)	GIBCO® Invitrogen Cell Culture
Phosphate buffered saline (PBS)	GIBCO® Invitrogen Cell Culture
Penicillin-streptomycin (P/S)	GIBCO® Invitrogen Cell Culture
L-Glutamin	GIBCO® Invitrogen Cell Culture
Non-essential amino acid (NEAA)	GIBCO® Invitrogen Cell Culture
β-mercaptoethanol	GIBCO® Invitrogen Cell Culture
HEPES	GIBCO® Invitrogen Cell Culture
Trypsin-EDTA	GIBCO® Invitrogen Cell Culture
Collagenase, Type IV	GIBCO® Invitrogen Cell Culture
Fetal calf serum (FCS) for ES/iPS cells	Lonza, Cologne, Germany
FCS for cell lines	Invitrogen Life Technologies
FCS for HSC	Invitrogen Life Technologies

Cytokines and growth factors	
Stem cell factor (SCF)	Recombinant mouse SCF or 1% supernatant of SCF producing CHO KLS C6 cells
Hyper-interleukin 6 (IL-6)	A kind gift of S. Rose-John, Kiel University, Kiel, Germany
Flt3 ligand	Purchased from Peprotech
Insulin-like growth factor 1 (IGF1, long range, human)	Sigma-Aldrich
Human LIF	Peprotech
Matrigel™	BD Bioscience

2.10 Medium for cell culture

Cells	Medium
Sertoli cells	DMEM/F12 (1:1); 2 mM L-Glutamine; 100 U/ml P/S; 10% FCS (for cell lines)
MEF	DMEM (4.5g/L high Glucose); 2 mM L-Glutamine; 100 U/ml P/S; 10% FCS (for cell lines)
293T cells	DMEM (4.5g/L high Glucose); 2 mM L-Glutamine; 100 U/ml P/S; 10% FCS (for cell lines)
T cells	RPMI-1640; 0.05 mM β-mercaptoethanol; 2 mM L-Glutamine; 100 U/ml P/S; 0.1 mM NEAA; 10% (heat-inactivated) FCS for HSC
ES/iPS cells	DMEM (4.5g/L high Glucose); 0.1 mM β-mercaptoethanol; 2 mM L-Glutamine; 100 U/ml P/S; 0.1 mM NEAA; 25 mM HEPES; 15% (heat-inactivated) FCS for ES cells
HSC	RPMI-1640; 0.05 mM β-mercaptoethanol; 2 mM L-Glutamine; 100 U/ml P/S; 10% (heat-inactivated) FCS for HSC
Freezing medium for ES/iPS	90% FCS (Lonza); 10% dimethyl sulfoxide (DMSO)
Freezing medium for cell lines	90% FCS (for cell lines); 10% DMSO

2.11 Buffers

Buffer	Recipes	Buffer	Recipes
Red blood cell (RBC) lysis buffer	150 mM NH_4Cl 100 mM $NaHCO_3$ 10 mM EDTA in ddH_2O pH adjusted to 7.4	10×TBE	0.89 M Tris/HCl 0.89 M boric acid 20 mM EDTA
4% paraformaldehyde (PFA),	4% paraformaldehyde in PBS pH 7.3	2 M $CaCl_2$	$CaCl_2$: 184.9 g H_2O: 100 ml
Flow cytomentry buffer	1% bovine serum albumin (BSA,	Hepes stock	Hepes: 11.92 g H_2O: 50 ml

	Fraction V, Sigma) in PBS	solution	
MACS buffer	0.5% FBS EDTA (0.5 M): 5 ml PBS: 500 ml	2×HBS	NaCl (280 mM): 1.64 g Hepes: 5 ml stock solution Na_2HPO_4: 0.021 g pH 7.08
Carboxyfluorescein succinimidyl ester (CFSE) labeling buffer	5% FCS in PBS	0.05% PBST	0.05% Tween20 in PBS

3 METHODS

3.1 Mice

Vav-iCre mice, Rosa26R-fGFP mice and B6 wt mice are bred at the Central Animal Facility of RWTH Aachen University Hospital. Heterozygote mice for Rosa26R-fGFP were crossed with transgenic mice expressing Cre under the control of Vav promoter (Vav-iCre × Rosa26R-fGFP$^{+/-}$) to generate homozygote (Vav-iCre/Rosa26R-fGFP$^{+/+}$) offspring (see below 4.6.1). All mice were maintained under specific pathogen free conditions in the Central Animal Facility of RWTH Aachen University Hospital. All animal experiments were approved by the local authorities (Bezirksregierung Köln) in compliance with the German animal protection law.

3.2 MEF preparation

Primary MEF were prepared from day 13.5 pregnant Vav-iCre/Rosa26R-fGFP mice and B6 mice. Briefly, day 13.5 pregnant mice were sacrificed. The uterus was removed and opened to extract the fetal bag, which contains embryo and placenta. The following steps were performed under sterile conditions. Fetal bags were put into a Petri dish and embryos were picked out. Embryos were washed twice with 1× P/S and 1×PBS, respectively. The head, liver and inner organs were removed with forceps. The carcasses were washed once with 1×PBS and 0.05% trysin/EDTA solution, respectively, and then finely minced with scissors and incubated with 0.05% trypsin at 37°C for 10 min. Pipetted up and down several times to homogenize the suspension. MEF medium was added to terminate the digestion. The cells were centrifuged at 1000 rpm for 5 min and plated at 2 ×10^6 cells per 10 cm dish. MEF medium was refreshed every 2-3 days and cells were split when confluent. Cells were frozen either as vital cells or after γ-ray inactivation.

3.3 Sertoli cell preparation

Testis of B6 mice (age: 7-10 days) was de-capsulated and primary Sertoli cells were isolated as described before (Yang and Han 2010) with modifications. Briefly, testis was de-capsulated with forceps, washed once with PBS and dispersed in 0.1% collagenase (type IV) at 37°C for 5 min. The tubules were collected and centrifuged at 200 g for 5 min followed by two washes with PBS to remove testicular interstitial cells and blood cells. Collected tubules were then treated with 0.05% trypsin-EDTA (0.5 ml/testis) for another 5

min at 37°C and pipetted up and down to disperse the testicular cells. Subsequently, fetal bovine serum was added to a final concentration of 10% (v/v) to terminate the digestion. Fragments were filtered through a 40 μm cell strainer and washed twice with DMEM/F12. The cell pellet was re-suspended in DMEM/F12 supplemented with 10% (v/v) FCS, 2 mM L-Glutamine, 100 U/ml P/S and plated onto tissue culture dishes. After overnight culturing, floating cells were removed by refreshing medium. Obtained Sertoli cells were frozen in liquid nitrogen (LN_2) for later use when cells were 90-95% confluent.

3.4 Plasmid preparation

Competent E.coli cells were prepared by $CaCl_2$ method and stored at -80°C. Strain DH5α was used for amplifying plasmids. About 100 μl competent cells were thawed on ice and mixed with plasmid DNA (50 ng), then incubated on ice for 30 min. The cells were heat shocked at 42°C for 90 sec followed by incubating another 2 min on ice. Then, the cells were added with 800 μl lysogeny broth (LB) medium and incubated at 37°C for 1 hour in the shaker. Proper amount of the cells (about 200 μl) were plated on pre-warmed LB-agar plates with antibiotics. The plates were incubated at 37°C for overnight. Single colony was manually picked and amplified by culturing for plasmid preparation.

3.5 Cell transfection

293T cells

293T cells were grown in DMEM medium supplemented with 10% FCS, 2 mM L-Glutamine and 100 U/ml P/S and incubated at 37°C with 5% CO_2. 293T cells were split before they were 80% confluent. To split 293T cells, cells were washed once with PBS and incubated with 0.05% trypsin-EDTA for 3 min. Trypsin treatment was terminated with 293T medium and appropriate numbers of cells were plated in cell culture dishes.

Retrovirus preparation

One day prior to transfection, 293T cells were plated at 3.5×10^6 cells per 10 cm dish and evenly distributed. Medium was refreshed one hour before transfection. Transfection was carried out using calcium phosphate/DNA co-precipitation method. Plasmid DNA, calcium chloride and 2×HBS buffer were prepared as below.

DNA solution was added dropwise into HBS buffer with continuously bubbling. The HBS/DNA mixture was added dropwise onto the cells and distributed evenly. Medium

was changed 24 hours after infection. Retrovirus was harvested 72 hours post-transfection and the supernatant was passed through 0.45 µm filter for further use.

Retrovirus preparation	Volume
Target construct (12 µg) Envelope pVPack-Eco (8 µg) Gag, pol pVPack GP (8 µg) 2 M $CaCl_2$ 62.5 µl Water up to 500 µl	500 µl
2×HBS	500 µl

3.6 Reprogramming somatic cells to hematopoietic/iPS cells

3.6.1 Transdifferentiation Vav-MEF to hematopoietic cells

Purify Vav-MEF with MACS or FACS sorting

Vav-MEF were prepared from Vav-iCre/Rosa26R-fGFP reporter mice, which expressing GFP reporter gene under the control of Vav promoter (see 3.1 and below 4.6.1). Vav-MEF were isolated as described before (see 3.2) and passaged once. Single cells were collected by treating with 0.05% trypsin. For MACS selection, Vav-MEF were passed through MACS MS column (CD45 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) and depletion of $CD45^+$ cells was used to remove hematopoietic cells. For FACS sorting, Vav-MEF were selected as $CD45^-GFP^-$ cells to remove hematopoietic cells (FACSAria, BD Biosciences). Then, an aliquot of purified cells were tested for purity by flow cytometry (FACSCanto II). Purified Vav-MEF were frozen in LN_2 for further use.

Transdifferentiation Vav-MEF to hematopoietic cells

One day before transfection, MACS or FACS sorted Vav-MEF were seeded into 6-well plates at a density of 1×10^5 cells per well. For transdifferentiation, pMX based retrovirus expressing reprogramming factor Oct4 and pMSCV based retrovirus expressing Bmi1 were used. pMX-GFP retrovirus was used as a control to assess virus titer. Retrovirus suspension was added onto Vav-MEF in the presence of 8 µg/ml polybrene (PB, Sigma-Aldrich). MEF medium was refreshed 2 days after infection and then changed to HSC medium with the addition of cytokines 4 days after infection. The following cytokines were used for transdifferentiation:

SCF	30 U/ml	1% supernatant of stably transfected CHO KLS C6 cells
Hyper-IL-6	5 ng/ml	A kind gift of S. Rose-John (Kiel University, Kiel, Germany)
Flt3 ligand	25 ng/ml	Peprotech
IGF-1 long range (human)	40 ng/ml	Sigma

Medium was changed every 2 days. When non-adherent cells emerged, the supernatant was collected, centrifuged, re-suspended with fresh HSC medium and then put back into the Petri dish.

3.6.2 Reprogramming Sertoli cells and MEF into iPS cells

One day before reprogramming, B6 Sertoli cells and B6 MEF were seeded into 6-well plates at a density of 1×10^5 cells per well. pMX retroviral vectors expressing reprogramming factors OSKM (Kim et al., 2008) were used for iPS cell generation. Sertoli cells and MEF were infected with retrovirus expressing either four factors (OSKM) or three factors (OSK) in the presence of 8 μg/ml PB. Two days after infection, medium was changed with Sertoli cell and MEF medium, respectively. Five days later, medium was replaced with ES medium in the presence of LIF (hereafter referred to as complete ES medium). Colony-liked cells emerged at around day 10 after retrovirus transduction and were manually picked at day 14 and transferred into 96-well plates. Single cells were obtained by treating with 0.05% trypsin and further cultured in 24-well plates and then in 6-well plates on MEF feeder cells with complete ES medium.

3.7 ES and iPS cell differentiation in EB assays

ES cells and iPS cells were grown in complete ES medium. For spontaneous differentiation *in vitro*, undifferentiated ES cells and iPS cells were subjected to EB assays. Briefly, ES cells and iPS cells were trypsinized into single cells and plated onto 0.1% gelatin-coated Petri dishes for 40 min to remove feeder cells. ES cells and iPS cells were collected and counted by hemocytometer measurement. Cells were suspended with appropriate volume of ES medium without LIF. EBs were generated by hanging drop with 500 cells per 20 μl drops in an inverted bacterial Petri dish. Three days later, EBs were collected and cultured in bacterial Petri dishes for another 3 days. Subsequently, EBs were put onto 0.1% gelatin-coated Petri dish for further differentiation.

3.8 Alkaline phosphatase and immunofluorescence staining

For alkaline phosphatase (AP) staining, ES cells and iPS cells were seeded in 6-well plates one day in advance. Then cells were washed once with PBS and performed AP staining (see 2.7; Stemgent) according to the manufacturer's instruction. Cells were covered with PBS and images were acquired with Leica microscopy.

For immunofluorescence staining, ES cells and iPS cells were grown on gelatin-coated coverslips and fixed with 4% PFA (20 min, room temperature, RT). For Oct4 staining cells were permeabilized with 0.1% TX-100 (30 min, RT). Cells were washed twice with 0.5% BSA/PBS buffer and incubated with primary antibodies (14-16 hours, 4°C). After washing, secondary antibody, Alexa594 goat anti-mouse Ig (H+L) was used (see 2.3; 1:300, 1 hour, RT; Life Technologies, Carlsbad, CA). DAPI (0.1 µg/mL, 30 min) was used to stain nuclei. Samples were mounted with mounting solution (Dako, Glostrup, Denmark) and images were acquired with Axiovert 200 microscopy (Carl Zeiss, Jena, Germany). The following primary antibodies were used: Oct4 and SSEA1 (see 2.3; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA).

3.9 Teratoma assay

For teratoma assay, ES cells and iPS cells were trypsinized to single cells and feeder cells were removed by attaching to gelatin-coated Petri dishes for 40 min. 1×10^6 cells were injected into the rear thigh of NOD-Scid mice and B6 male mice. Four weeks later, recipient mice were sacrificed and teratomas were excised. Half of the teraotoma was fixed in 4% PFA, embedded in paraffin and sectioned for histological analysis. The other half of the teratoma was frozen in -80°C for RNA isolation. Frequency of teratoma formation was assessed as number of injections relative to teratomas formed.

3.10 HE and immunohistochemical staining

Hematoxylin and eosin (HE) staining and immunohistochemical staining of teratoma sections were performed at IZKF Immunohistochemistry Core Facility (RWTH Aachen University Hospital, Aachen, Germany). Briefly, paraffin-embedded teratoma sections were cut, de-waxed and rehydrated. Some of the sections were directly used for HE staining.

For immunohistochemical staining, the primary antibody employed was rabbit polyclonal anti-CD3 antibody (1:200 dilution; Abcam, Cambridge, UK). Then, teratoma sections

were incubated with diluted biotinylated secondary antibody solution (30 min) followed by 30 min incubation with VECTASTAIN® ABC Reagent (Vector Laboratories, Peterborough, UK). Subsequently, sections were incubated in peroxidase substrate solution until desired staining intensity developed. With each set of staining process, spleen was included as positive control.

3.11 Preparing DNA and RNA

Isolation of mouse tail genomic DNA, bacterial plasmid, total RNA were all performed with commercial kits (see 2.7). To get RNA from teratomas, tissues were first homogenized by gentleMACS Dissociator using specific gentleMACS M tubes. Homogenized samples were centrifuged (2000 g, 1 min), proceeded with RNA isolation according to manufacture's instruction (NucleoSpin RAN II kit, Macherey-Nagel). The quality and concentration of RNA were determined with Nanodrop 1000 (Thermo Scientific, Wilmington, DE).

3.12 Polymerase chain reaction

Reverse transcription

To evaluate the level of gene expression, cDNA was synthesized by reverse transcription. mRNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's instruction. Briefly, 1 μg of total RNA was used as template, together with the reaction mixture from the kit to perform the reverse transcription. The standard thermal cycle was 25°C for 10 min, 37°C for 2 hours and 75°C for 5 min.

PCR reaction

PCR is used to amplify a specific region of DNA strand. Primers were designed containing sequence complementary to the target region. A typical PCR reaction mixture was:

cDNA sample	50 ng in 2 μl
10×buffer $(NH_4)_2SO_4$	2 μl
$MgCl_2$ (25 mM)	2 μl
for primer (10 mM)	0.5 μl
rev primer (10 mM)	0.5 μl
dNTP (10 mM each)	0.5 μl

Recombinant Taq DNA Polymerase (Fermentas)	0.5 µl
(dd) water	12 µl
Total volume	20 µl

The PCR reaction was performed on thermal cycling machine (Eppendorf, Hamburg, Germany). A standard reaction scheme was:

Initiation	95°C	5 min	
Denaturation	95°C	30 sec	
Annealing	Gene-specific annealing temperature	1 min	Gene-specific cycle numbers
Extension	72°C	1 min	
Final extension	72°C	5 min	
Termination	4°C	∞	

Quantitative real-time RT-PCR

qRT-PCR enables quantification for one or more specific sequences in a cDNA sample which corresponds to the transcription level of target genes. We performed qRT-PCR reaction on StepOne™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA). All the reagents and plastic plates for qRT-PCR were purchased from Applied Biosystems. Data are represented in heatmap format (MultiExperiment Viewer MeV_4_8, http://www.tm4.org.) with fold change in gene expression normalized to β-actin. The typical recipe for qRT-PCR reaction was:

Fast SYBR Green Master mix	10 µl
for primer (10 mM)	0.5 µl
rev primer (10 mM)	0.5 µl
cDNA sample	50 ng (5 µl after 1:5 dilution)
(dd) water	4 µl
Total volume	20 µl

The qRT-PCR program was carried out as below:

	Temperature	Time	Number of cycles
Enzyme activation	95°C	20 sec	1
Denaturation	95°C	3 sec	40
Annealing and extension	60°C	30 sec	

3.13 Mouse splenic CD4 T cell isolation

B6 mouse spleens (age: 6-10 weeks) were removed aseptically and washed once with 1×P/S and PBS, respectively. Then, spleens were gently homogenized with the stamp of a 1 ml syringe and passed through 40 µm cell strainer. Cells were then washed with PBS, stained with CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany; 15 min, 4°C) and put into MS column (Miltenyi Biotec) for MACS selection. CD4 T cells were collected and cultured in T cell medium for further use. The purity of CD4 T cells was determined by flow cytometry (FACSCanto II).

3.14 Flow cytometry

Cells were collected, washed once with flow cytometry buffer and incubated with specific antibodies (1:100 dilution, 30 min, 4°C). Then, cells were washed and re-suspended with proper volumn of flow cytometry buffer. The following antibodies were used: APC-Cy7-conjugated CD45, APC-conjugated F4/80, PE-conjugated CD4, FITC-conjugated CD4, APC-conjugated CD25 and PE-conjugated Foxp3. Results were analyzed using Flowjo (Tree Star Inc., Ashland, OR, USA). FACS sorting was performed with FACSAria (BD Bioscience) at RWTH Aachen University Hospital (kindly operated by Christiane Becker, Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University, Aachen, Germany).

3.15 T cell proliferation assay

CD4 T cells were resuspended in CFSE labeling buffer (5% FCS in PBS) and labeled with 2.5 uM cell tracking dye CFSE (10 min, RT). After washing, 5×10^4 CD4 T cells were cultured in triplicates in 96-well U-bottom microtiter cell plates (Costar) with or without phorbol myristate acetate (PMA, 25 ng/ml) and ionomycin (0.02 mM). Co-culture with T cells was performed with undifferentiated cells (day 0-5) or EBs (day 12-17 of differentiation) from iPS cells or ES cells at 2:1 ratio. After 5 days of co-culture, proliferation of CD4 T cells was analyzed by assessing the dilution of CFSE signal using flow cytometry (FACSCanto II). The assay was carried out in duplicates for n=3 independent experiments.

3.16 Regulatory T cell assay

CD4 splenic T cells were isolated from B6 mice and pretreated with PMA (25 ng/ml) and ionomycin (0.02 mM) to active T cells (16 hours). Then, T cells were collected and co-

cultured with undifferentiated iPS cells or with EBs of iPS cells (day 12-17 of differentiation) at 2:1 ratio. After 5 days of co-culture, T cells were harvested and stained for surface markers CD4 and CD25 (30 min, 4°C). Subsequently, cells were fixed and permeabilized using the Foxp3 fixation/permeabilization buffer and intracellular Foxp3 (eBioscience) staining was carried out according to manufacturer's instruction. FACS analysis was performed with FACSCanto II.

3.17 Statistic analysis

Results are given as mean ± standard derivation. Statistical analysis was performed using unpaired Student's t-test. P values below 0.05 were considered to be statistically significant.

4 RESULTS

4.1 Generation and characteristics of Ser-iPS cells

To generate iPS cells, Sertoli cells were isolated from B6 mice, and transfected with pMX retroviral vectors expressing either four factors OSKM or three factors OSK. iPS cells derived from B6 MEF (MEF-iPS cells) were generated accordingly and used as control. Sertoli cell derived iPS cells (Ser-iPS cells) were injected into B6 mice to test their *in vivo* immunogenicity in teratoma assay. B6 MEF-iPS cells and B6 ES cells (JM8, kindly provided by Konstantinos Anastassiadis, BIOTEC, Technical University Dresden) were taken as controls (Figure 4.1).

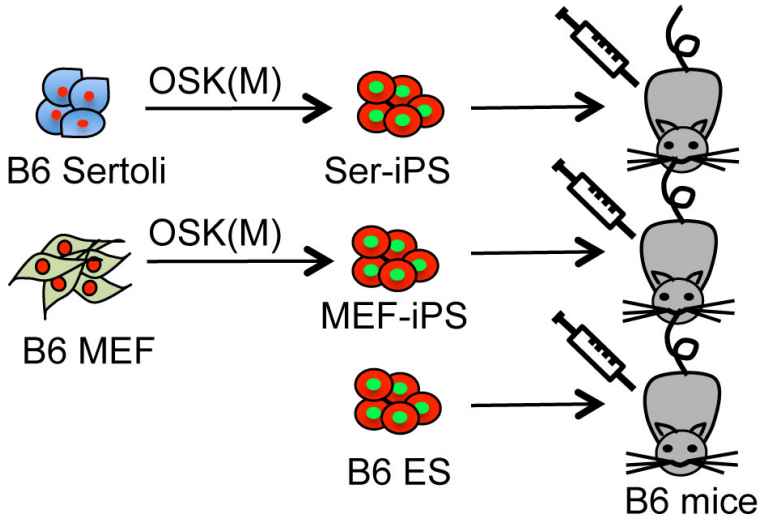

Figure 4.1. Schematic representation of Ser-iPS cell generation and teratoma assay in B6 mice.

Sertoli cells were isolated from day 7-10 immature B6 male mice. MEF were isolated from day 13.5 pregnant B6 mice. iPS cells were generated by transfection with retrovirus expressing either four factors OSKM or three factors OSK, hereafter referred to as Ser-iPS cells and MEF-iPS cells, respectively. B6 Ser-iPS cells were injected into syngeneic B6 male mice to test their teratoma formation ability. B6 MEF-iPS cells and B6 ES cells are shown as controls. OSKM: Oct4, Sox2, Klf4 and c-Myc; OSK: Oct4, Sox2 and Klf4.

4.1.1 Sertoli cell isolation and culture

Sertoli cells were isolated from day 7-10 newborn B6 male mice as described above (Methods 3.3). Immature animals are chosen as the source of Sertoli cells, because they produce Sertoli cells with relatively high cell yield and purity, and they are also well-differentiated cell type (Nicholls et al., 2011). Furthermore, Sertoli cells from immature animals are well-characterized model for *in vitro* culture of primary Sertoli cells (Nicholls et al., 2011).

Primary Sertoli cells were cultured in Sertoli cell medium and became confluence 2-3 days later. In culture, primary Sertoli cells were similar to fibroblast in morphology (Figure 4.2A). After one passage, Sertoli cells stretched out, formed a monolayer, showed more cytoplasm compared to peritubular cells and exhibited an irregular shape (Figure 4.2A; Ahmed et al., 2009) with much lower growth rate compared to primary Sertoli cells.

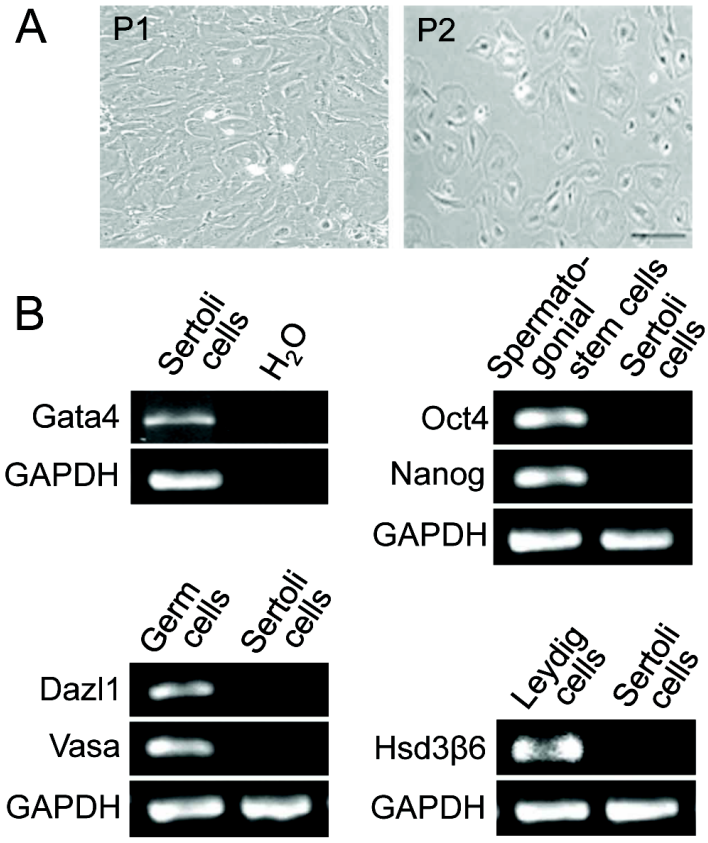

Figure 4.2. Isolation of Sertoli cells from immature B6 mice.

A: Sertoli cells were isolated from day 7-10 B6 mice and were split once when they were confluent. P0: passage 0; P1: passage 1. Scale bar represents 800 μm.
B: RT-PCR analysis for the purity of *in vitro* cultured Sertoli cells. Sertoli cells were collected at passage 1 after becoming confluent. Germ cells and Leydig cells, which were freshly isolated from adult B6 mice, were used as positive controls. B6 ES cells were used as a positive control. GAPDH were served as loading control.

Purity of *in vitro* cultured Sertoli cells was analyzed by RT-PCR. Sertoli cells expressed Gata4, a specific marker for Sertoli cells (Doyle et al., 2012). They were negative for Oct4 and Nanog, two markers expressed in spermatogonial stem cells (Guan et al., 2006), as well as Dazl1 (Lifschitz-Mercer et al., 2002) and Vasa (Toyooka et al., 2000), two specific markers for germ cells. Sertoli cells failed to express Hsd3β6 (Ma et al., 2004), a specific marker for Leydig cells (Figure 4.2B). Since spermatogonial stem cells are difficult to isolate and culture *in vitro*, mouse B6 ES cells, which express Oct4 and

Nanog, were used as a positive control. These data indicate that Sertoli cells isolated from immature B6 male mice can survive and proliferate during *in vitro* culture with a much higher purification.

4.1.2 Generation and characteristics of Ser-iPS cells

Generation of Ser-iPS cells

iPS cells were generated by transfection with traditional Yamanaka vectors (Takahashi and Yamanaka 2006; Kim et al., 2008). Sertoli cells were prepared for transfection as described above and infected with pMX retroviral vectors expressing either four factors OSKM or three factors OSK (Methods 3.6.2). MEF-iPS cells were generated from B6 MEF accordingly and used as a control. pMX retroviral vector expressing GFP reporter gene was used as a positive control to monitor the transfection efficiency. Two days after infection, GFP fluorescence was observed with microscopy. Lower efficiency of Sertoli cell infection was observed compared to MEF according to the GFP fluorescence (Figure 4.3). This may be due to the slower proliferation efficiency of Sertoli cells after one passage than MEF during *in vitro* culture. Because retroviral vectors used here require actively dividing cells during transfection (Cooray et al., 2012).

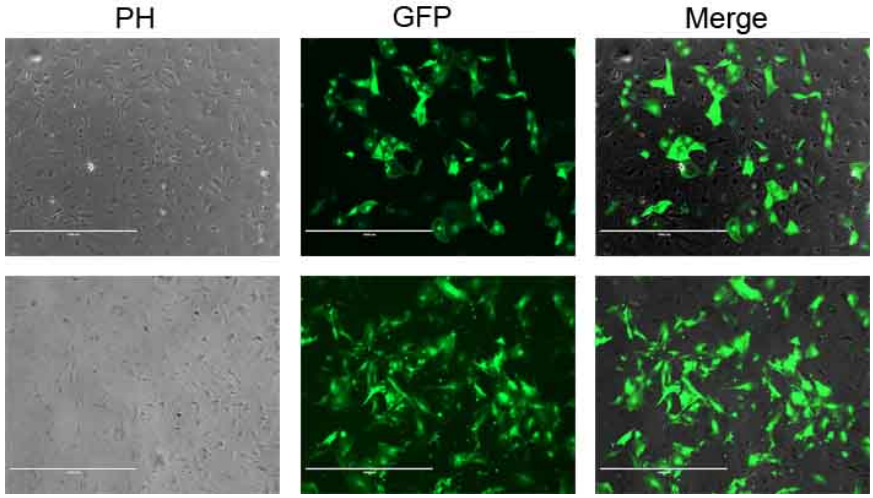

Figure 4.3. Transfection efficiency of Sertoli cells with retroviral vectors.

Sertoli cells were infected with GFP-expressing retrovirus to determine the efficiency of transfection (upper panel). Corresponding B6 MEF are shown as control (lower panel). Images were taken 2 days after infection. PH: phase contrast. Scale bar represents 1000 μm.

After 2-3 days of infection, Sertoli cell medium was replaced with complete ES medium. About 8 days after infection, clusters of cells were observed with a higher nuclear-to-cytoplasmic ratio and an increased proliferation rate. These clusters kept proliferating and clustering. About 10 days after retrovirus induction, multilayered compact Ser-iPS

cell clones with clearly marked borders, which were similar to mouse ES cell clones, emerged (Figure 4.4). About 12 days after induction, Ser-iPS cell clones were manually picked, trypsinized into single cells and kept in culture on feeder cells with complete ES medium.

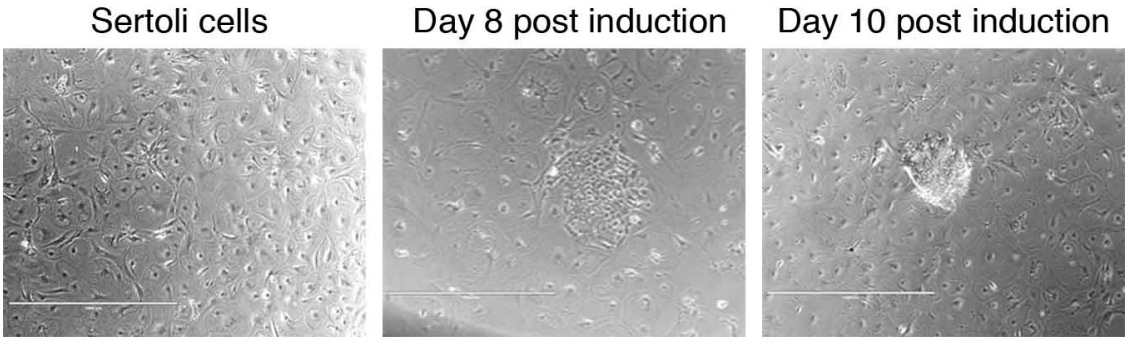

Figure 4.4. Bright-field images depicting reprogramming of mouse Sertoli cells into iPS cells.

Sertoli cells clustered together with an increased proliferation rate and altered morphology after transfection with retrovirus and finally became Ser-iPS cell clones. Scale bar represents 1000 μm.

Pluripotency of Ser-iPS cells

Ser-iPS cell clones (86 clones) and MEF-iPS cell clones (65 clones) were picked and cultured on feeder cells. Good clones were selected for expanding according to their proliferation rate and morphology during *in vitro* culture (Ser-iPS cells: 19 clones; MEF-iPS cells: 17 clones). Among these clones, three representative Ser-iPS cell clones and two representative MEF-iPS cell clones, which exhibited proliferation and differentiation potential similar to ES cells, were selected for further analysis (Table 1). For selected three Ser-iPS cell clones, Ser-iPS 1 refers to 4F iPS cells (OSKM, clone 1) and Ser-iPS 2 and 3 refer to 3F iPS cells (OSK, clones 2 and 3, respectively); for selected two MEF-iPS clones, MEF-iPS 1 and 2 refer to 4F iPS cells (OSKM, clone 1) and 3F iPS cells (OSK, clone 2), respectively.

Table 4.1. Numbers of Ser-iPS cell and MEF-iPS cell clones picked, expanded and selected for further analysis

	Picked clones	Expanding clones	Clones used for further analysis
Ser-iPS (OSKM)	80	16	1
Ser-iPS (OSK)	6	3	2
MEF-iPS (OSKM)	60	14	1
MEF-iPS (OSK)	5	3	1

In vitro cultured Ser-iPS cells showed typical ES cell morphology. They are presented as homogenous clusters of small, tightly packed cells, bright at the edge, and have similar grown rates to ES cells *in vitro* (Figure 4.5A). These clones showed a high level of AP expression, as determined by AP staining (Figure 4.5B).

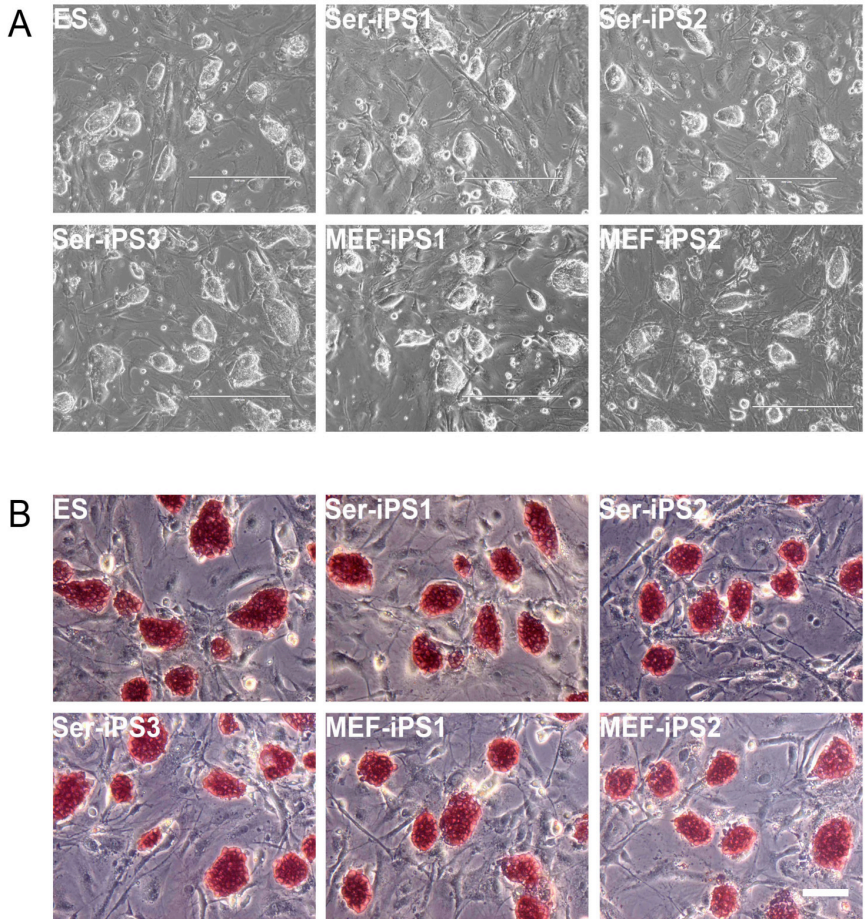

Figure 4.5. Ser-iPS cells *in vitro* culture and AP staining.

A: *In vitro* cultured Ser-iPS cells. MEF-iPS cells and ES cells were used as controls. Representative phase contrast images are shown. Scale bar represents 400 µm.
B: AP expression in Ser-iPS cells. Ser-iPS cells were grown on MEF feeder cells. AP staining was performed one day after passage. Cells were fixed and stained with substrate to AP according to the manufacturer's instruction. MEF-iPS cells and ES cells, controls as in (A). All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Scale bar represents 200 µm.

Furthermore, immunofluorescence staining was performed to test the expression of pluripotency genes, Oct4 (nuclear localization) and SSEA1 (cell surface localization). These two pluripotency markers were highly expressed in Ser-iPS cells, similar to MEF-iPS cells and ES cells (Figure 4.6A, B). Pluripotency gene expression in Ser-iPS cells was further analyzed by RT-PCR. The endogenous Oct4, Sox2, Nanog, Rex1, Rex3 and Dppa4 genes were activated and expressed at a similar level to those of MEF-iPS cells and ES cells (Figure 4.7).

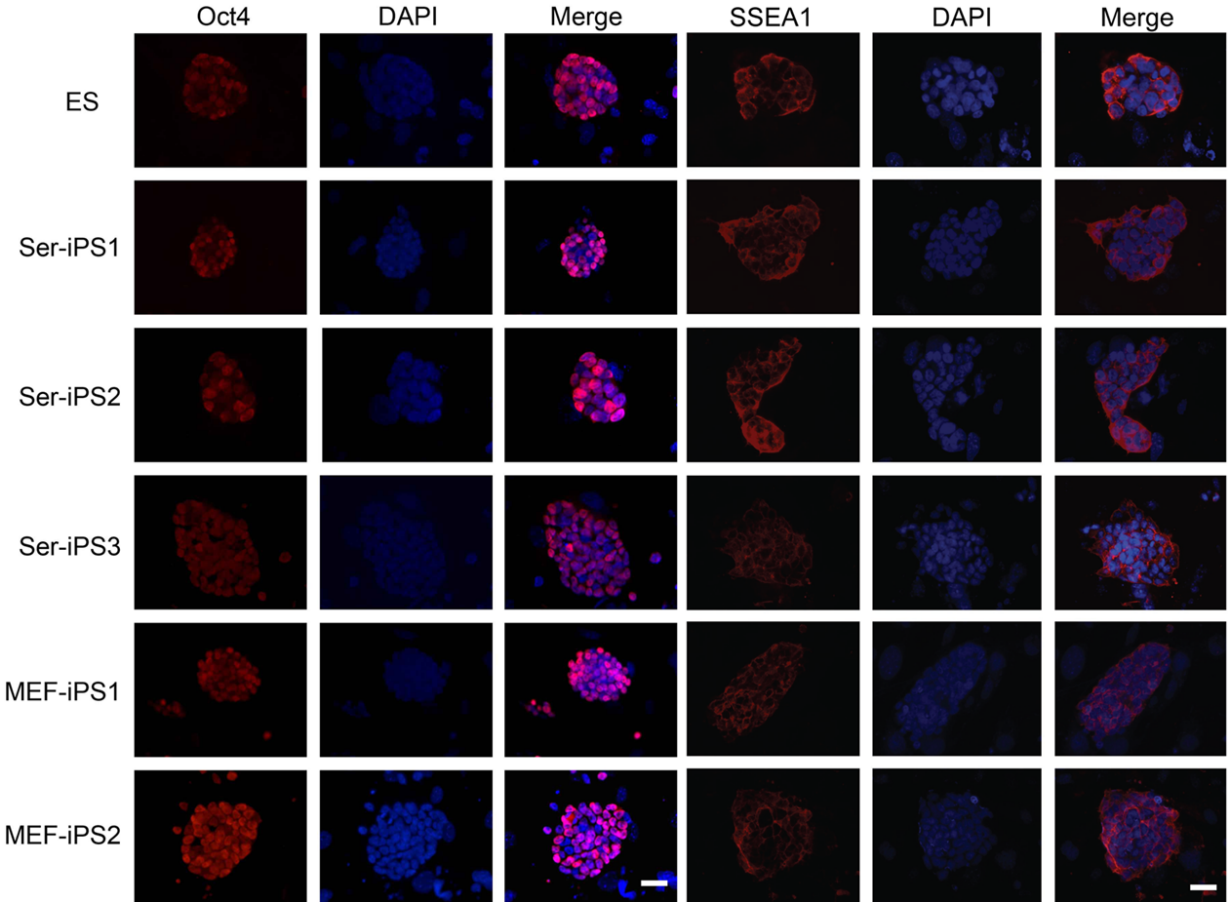

Figure 4.6. Immunofluorescence staining of pluripotency gene markers in Ser-iPS cells.

A-B: Oct4 and SSEA1 expression in undifferentiated Ser-iPS cells. Oct4 and SSEA1 staining, red color. DAPI (blue) is used for the nuclei staining. Ser-iPS cells organized in tight compact clones similar to clones of undifferentiated MEF-iPS cells and ES cells. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Scale bar represents 35 μm.

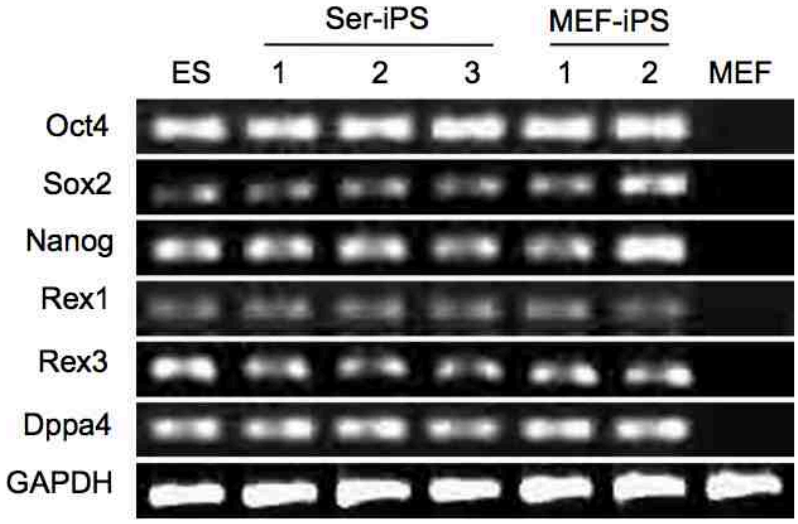

Figure 4.7. Pluripotency gene expression in Ser-iPS cells by RT-PCR.

RNA was isolated from Ser-iPS cells. RT-PCR was performed to examine the expression of pluripotency genes Oct4, Sox2, Nanog, Rex1, Rex3 and Dppa4 in these Ser-iPS cells. MEF-iPS

cells and ES cells were taken as positive controls. MEF were used as negative control. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). GAPDH was served as a loading control.

All together, Ser-iPS cells displayed pluripotent characteristics similar to MEF-iPS cells and ES cells, such as similar morphology, AP activity and expression of endogenous pluripotency genes. Thus, Ser-iPS cells represent bona fide pluripotent cells.

Differentiation of Ser-iPS cells

Although Ser-iPS cells are very similar to ES cells in pluripotency, another important criteria to evaluate iPS cells is their differentiation capability. The classical methods to assess iPS cell differentiation potential are EB assay *in vitro* and teratoma assay in immunodeficient mice *in vivo*. For *in vitro* differentiation, EB assay was performed as described before (Methods 3.7). Day 14 EBs were collected, RNA was isolated and employed to qRT-PCR. Undifferentiated iPS cells and ES cells were used as controls.

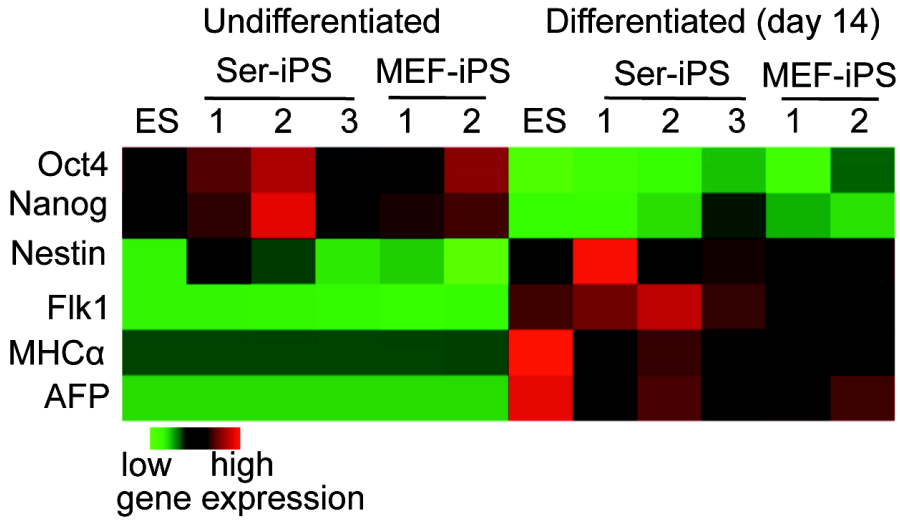

Figure 4.8. EB assay of Ser-iPS cells.

EB assay of Ser-iPS cells was performed by hanging drop. Their differentiation potential was examined by qRT-PCR with both undifferentiated cells and day 14 differentiated EBs. The results are presented in heatmap format according to their fold change normalized to β-actin. Green represents low expression and red represents high expression. The mRNA level in undifferentiated ES cells was arbitrarily set to 1. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage).

The expression of several pluripotency and differentiation genes was tested, such as Oct4, Nanog (pluripotency gene markers), Nestin, Flk1, MHCα and AFP (differentiation gene markers). The qRT-PCR result was presented in heatmap format (Figure 4.8). After 14 days of differentiation, Ser-iPS cells downregulated endogenous pluripotency gene expression, Oct4 and Nanog, compared to undifferentiated Ser-iPS cells. The expression

of differentiation gene markers, such as Nestin (neural cell marker), Flk1 (early mesoderm marker), MHCα (cardiomyocyte marker) and AFP (endoderm marker) was all upregulated compared to undifferentiated Ser-iPS cells. Thus, Ser-iPS cells have the ability to differentiate into all three germ layers *in vitro*, which was similar to that of MEF-iPS cells and ES cells.

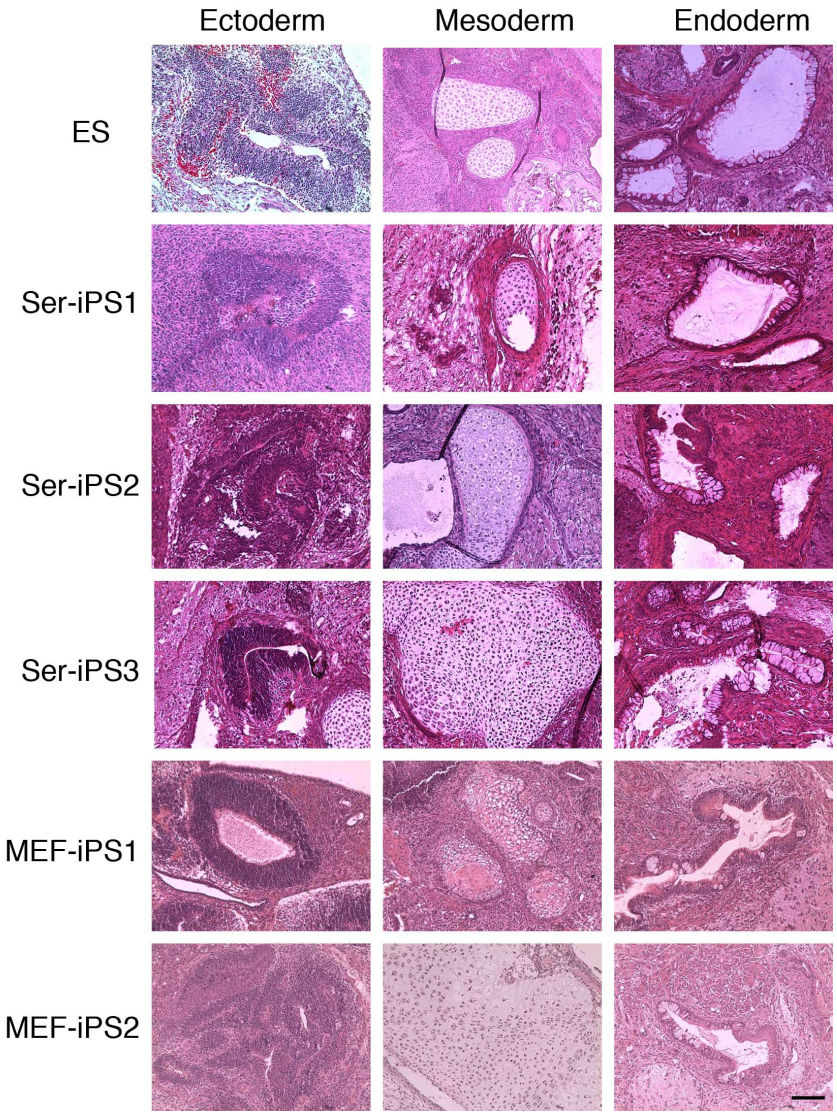

Figure 4.9. Teratoma assay in NOD-Scid mice.

1×10^6 Ser-iPS cells were injected into NOD-Scid mice subcutaneously. One mouse with two injection sites was used for each clone. MEF-iPS cells and ES cells were used as controls. Four weeks later, mice were sacrificed and teratomas were excised. The samples were fixed in 4% PFA and stained with HE. Representative images of ectoderm, mesoderm and endoderm are shown. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). HE: haematoxylin and eosin. Scale bar represents 200 μm.

For iPS cell *in vivo* differentiation, teratoma assay is a standard method. After injection into immunodeficient mice, pluripotent stem cells form teratomas with cell derivatives of

all three germ layers. To examine the differentiation potential of Ser-iPS cells, teratoma assay was performed in NOD-Scid mice. 1×10^6 undifferentiated Ser-iPS cells in 100 µl DMEM in the presence of Matrigel (BD Biosciences; DMEM/Matrigel: 4/1) were injected into the rear thigh of NOD-Scid mice. B6 MEF-iPS cells and ES cells were injected accordingly and taken as controls. Teratomas were collected 4 weeks after injection, fixed in 4% PFA, embedded in paraffin and sectioned for HE staining. The result indicates that Ser-iPS cells can form teratomas contained cell derivatives of all three germ layers, including neural rosette (ectoderm), cartilage (mesoderm) and glandular epithelium (endoderm) (Figure 4.9).

Overall, all these data indicate successful reprogramming of Sertoli cells into pluripotent state and Ser-iPS cells exhibit pluripotent differentiation potential. These results suggest that Sertoli cells were successfully reprogrammed into iPS cells by overexpressing reprogramming factors. Obtained Ser-iPS cells display pluripotent characteristics, such as typical ES-liked morphology and growth *in vitro*, high AP expression, endogenous pluripotency genes, differentiation into all three germ layers by EB assay *in vitro* and by teratoma assay in NOD-Scid mice. Thus, these Ser-iPS cells are qualified as bona fide iPS cells and can be used for further analysis.

4.2 Teratoma assay in B6 mice

Immunogenicity of iPS cells and iPS cell-derived cells is detected *in vivo* in teratoma assay (Koch et al., 2008; Zhao et al., 2011). Teratomas comprise a broad spectrum of differentiated cells of all three germ layers and thus allow assessing *in vivo* immunogenicity of iPS cells and of their differentiated progeny simultaneously. Thus, to investigate whether iPS cells derived from immune-privileged Sertoli cells show reduced immunogenicity, undifferentiated Ser-iPS cells were syngeneically injected into B6 mice for teratoma assay. The frequency of teratoma formation served as a measure of immunogenicity of iPS cells and iPS cell-derived cells: high teratoma formation reflects low immunogenicity and low or no teratoma formation indicates high immunogenicity and consequently immune rejection.

4.2.1 Teratoma assay in B6 mice

To investigate the immunogenicity of Ser-iPS cells, 1×10^6 B6 Ser-iPS cells were injected into male B6 mice. B6 MEF-iPS cells and B6 ES cells were injected accordingly and used as controls. For each iPS cell clone, 10 mice with 20 injection sites were used for

teratoma assay. Four weeks after injection, mice were sacrificed and teratomas were excised. Half of the teratoma was fixed in 4% PFA, embedded in paraffin and sectioned for further analysis. The other half of the teratoma was frozen in -80°C for RNA isolation. Upon syngeneic transplantation, Ser-iPS cells showed the potential to form teratomas of different sizes (Figure 4.10A) with cell derivatives of all three germ layers, including neural rosettes (ectoderm), cartilage (mesoderm) and glandular epithelium (endoderm), similar to those of MEF-iPS cells and ES cells (Figure 4.10B).

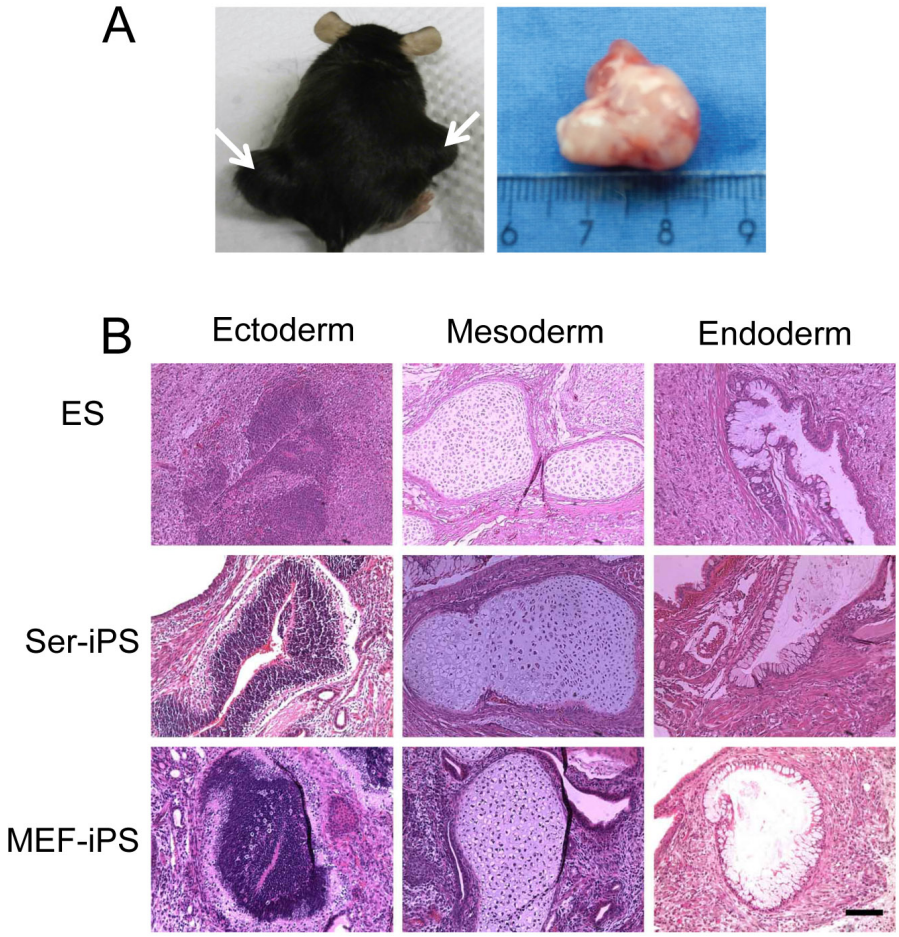

Figure 4.10. Ser-iPS cell teratoma formation in B6 mice.

A: Ser-iPS cell teratomas in B6 mice. 1×10^6 Ser-iPS cells were injected into B6 mice subcutaneously and teratomas were collected four weeks after injection. Representative images of B6 mice with two teratomas at rear thigh (left panel) and the corresponding teratoma collected from B6 mice (right panel) are shown. Teratomas from Ser-iPS cells (OSKM, clone 1) are shown. White arrows indicate the position of teratomas. Ruler is used to measure the size of obtained teratomas.
B: Tissue structures of Ser-iPS cell teratomas from B6 mice. MEF-iPS cell and ES cell teratomas were used as controls. Tissue structures of all three germ layers were observed in teratomas from B6 mice. Representative images of ectoderm, mesoderm and endoderm from Ser-iPS cell (OSKM, clone 1), MEF-iPS cell (OSK, clone 2) and ES cell teratomas are shown. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Scale bar represents 200 µm.

The efficiency of teratoma formation by iPS cells and ES cells in B6 mice is shown in Table 4.2. Ser-iPS cells formed teratomas at an efficiency of 80%, while ES cells had 90% teratoma formation frequency. But the incidence of teratoma formation for MEF-iPS cells was only about 20% (Table 4.2). Frequency of teratomas formed in B6 mice is also shown as histogram. Ser-iPS cells have a much higher incidence of teratoma formation (80%) than MEF-iPS cells (20%) and the incidence of teratoma formation for Ser-iPS cells is comparable to ES cells (90%; Figure 4.11A). There was no significant difference in the size of teratomas formed by Ser-iPS cells and MEF-iPS cells, while the size of teratomas formed by ES cells was larger (Figure 4.11B). The average size of teratomas formed in B6 mice was about 1-1.5 cm.

Table 4.2 Efficiency of teratoma formation in B6 mice

Clones	Nr. Teratomas	Nr. Injection sites	Frequency of teratoma formation
ES	18	20	90%
Ser-iPS	48	60	80%
MEF-iPS	8	20	20%

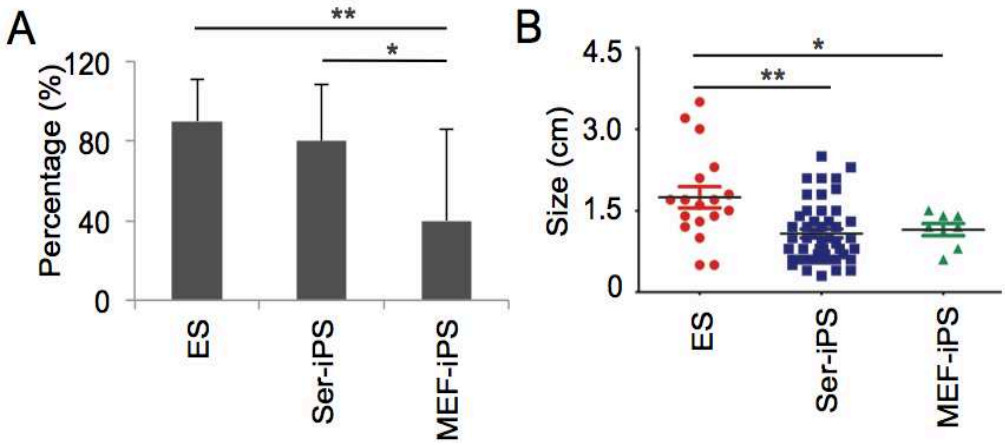

Figure 4.11. Number and size of teratomas formed by Ser-iPS cells in B6 mice.

A: Numbers of teratomas formed by Ser-iPS cells are summarized as histogram. Teratomas from MEF-iPS cells and ES cells were used as controls. For Ser-iPS cells and MEF-iPS cells, 30 and 20 mice, respectively, were used, and for ES cells, 10 mice were used.
B: Size of teratomas formed by Ser-iPS cells. Teratomas from MEF-iPS cells and ES cells were used as controls in (A). Ser-iPS cells and MEF-iPS cells in (A) and (B) refer to average values of all three Ser-iPS cell clones and two MEF-iPS cell clones. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Unpaired Student's t-test was used to analyze the statistical significance. * indicates $P < 0.05$, ** indicates $P < 0.01$. Bars represent mean ± standard deviation.

Collectively, iPS cells or ES cells form teratomas with cell derivatives of all three germ layers upon syngeneic injection into B6 immunocompetent mice. In addition, Ser-iPS

cells form more teratomas upon syngeneic transplantation than MEF-iPS cells. These data suggest that iPS cells derived from immune-privileged Sertoli cells elicit less immune responses and thus permit more efficient teratoma formation *in vivo*.

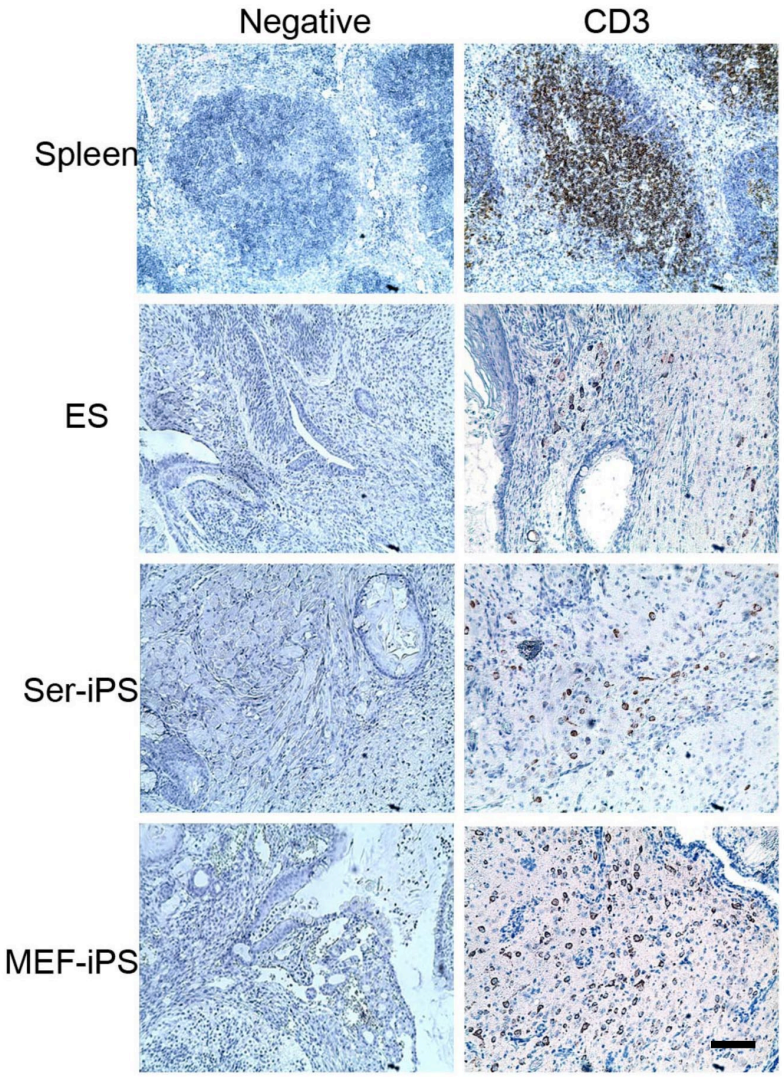

Figure 4.12. CD3 T cell infiltration in Ser-iPS cell teratomas formed in B6 mice.

Immunohistochemical staining for CD3 T cells was performed in Ser-iPS cell teratomas. Teratomas of MEF-iPS cells and ES cells are shown as controls. CD3 staining in spleen was taken as a positive control. Brown cells represent CD3 positive T cells. Representative images of CD3 T cell infiltration in teratomas from Ser-iPS cells (OSKM, clone 1), MEF-iPS cells (OSK, clone 2) and ES cells are shown. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Scale bar represents 200 μm.

4.2.2 T cell infiltration and tissue damage and necrosis in teratomas

Although both iPS cells and ES cells formed teratomas in B6 mice, the incidence of teratoma formation was very different. To further investigate the *in vivo* immunogenicity of Ser-iPS cells, immunohistochemical staining of B6 teratoma sections was performed.

Teratoma sections were prepared as described before (Methods 3.9), and analyzed for CD3 T cell infiltration. CD3 staining was performed at IZKF Immunohistochemistry Core Facility as described in Methods 3.10. As expected, Ser-iPS cell teratomas showed lower CD3 T cell infiltration than those of MEF-iPS cells. Low CD3 T cell infiltration in Ser-iPS cell teratomas was similar to those of ES cell teratomas (Figure 4.12). Furthermore, Ser-iPS cell teratomas showed less tissue damage and necrosis compared to MEF-iPS cell teratomas by HE staining. The reduced tissue damage and necrosis in Ser-iPS cell teratomas was similar to ES cell teratomas (Figure 4.13).

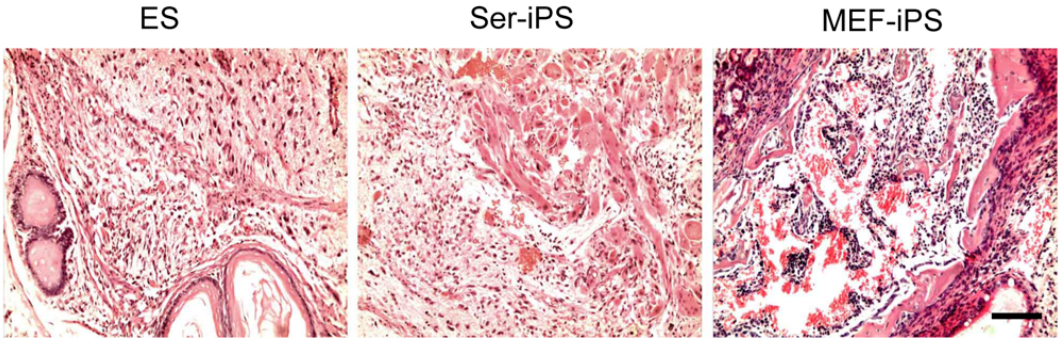

Figure 4.13. Tissue damage and necrosis in teratomas from B6 mice.

Tissue damage and necrosis was detected in teratomas formed by both iPS cells and ES cells in B6 male mice. Representative images of tissue damage and necrosis in teratomas from Ser-iPS cells (OSKM, clone 1), MEF-iPS cells (OSK, clone 2) and ES cells are shown. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Scale bar represents 200 μm.

4.2.3 Gene expression of different immune cell types in teratomas

Different immune cell types are involved in immune responses upon syngeneic transplantation. CD4 T helper cells recognize antigens presented by MHC class II molecules and produce cytokines that help the activities of other immune cells. Cytotoxic CD8 T cells are activated by antigens presented by MHC class I molecules and are very efficient in lysis of target cells bearing these antigens. B cells, another main type of lymphocytes, work mainly by secreting antibodies into the body's fluids. DC are antigen-presenting cells, which present processed antigens on the surface to other immune cells. Macrophages are generated from differentiated monocytes and protect the body by ingesting and destructing harmful cells.

By immunohistochemical staining, low CD3 T cell infiltration in Ser-iPS cell teratomas was observed. Other immune cell infiltration in teratomas was further examined by qRT-PCR, such as for T cells (CD3, CD4 and CD8), DC (CD11c), B cells (B220), macrophages (Mac1) and granulocytes (Gr1). Ser-iPS cell teratomas exhibited much lower CD3, CD11c and B220 gene expression than MEF-iPS cells (Figure 4.14). The low

expression of these genes in Ser-iPS cell teratomas was comparable to that in ES cell teratomas. CD3 gene expression analyzed by qRT-PCR was consistent with the result of CD3 immunohistochemical staining. Expression of CD4 and CD8 T cell markers was also lower in Ser-iPS cell teratomas compared to that of MEF-iPS cells, but this did not reach statistical significance. However, there was no significant difference in the expression of macrophage and granulocyte markers (Mac1 and Gr1, respectively).

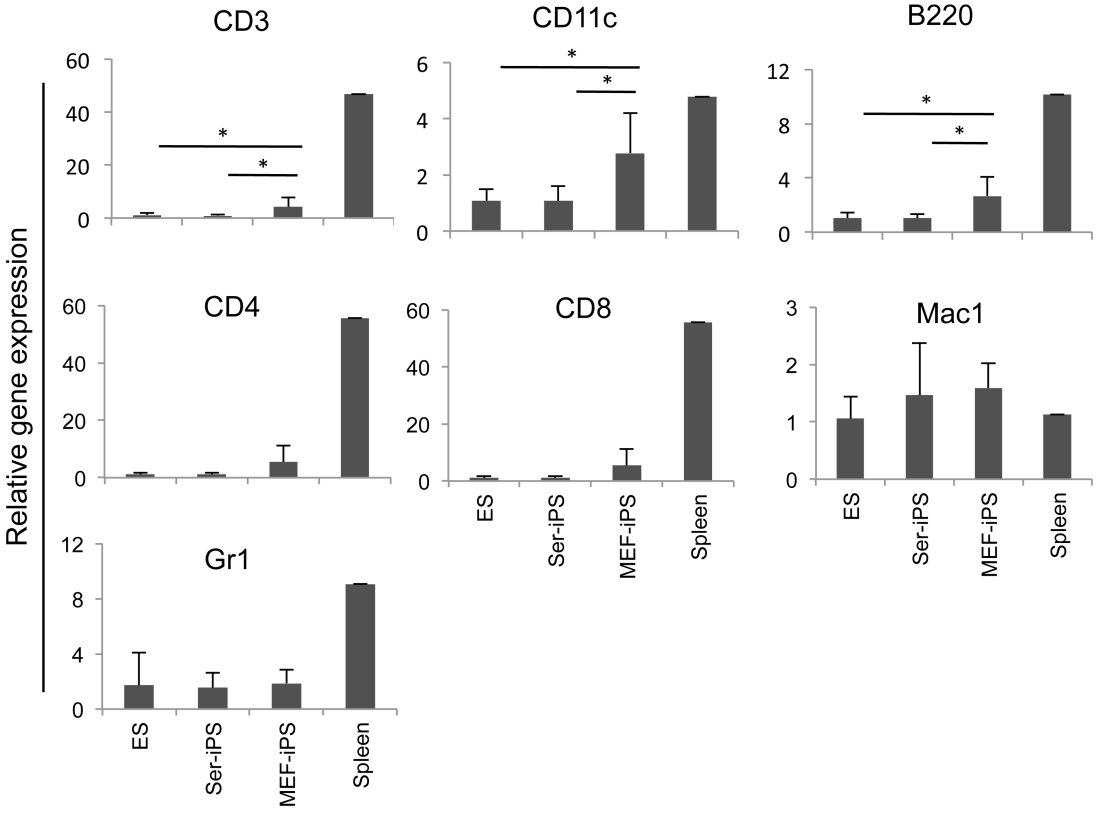

Figure 4.14. Gene expression of different immune cells in Ser-iPS cell teratomas.

Ser-iPS cell teratomas were first cut into small pieces and homogenized by gentleMACS Dissociator using specific gentleMACS M tubes. Then RNA was isolated and qRT-PCR was performed. Teratomas from MEF-iPS cells and ES cells were taken as controls. Relative gene expression was normalized to β-actin. Average mRNA level in ES cell teratomas was arbitrarily set to 1. The mRNA level in spleen was used as a positive control. The number of B6 teratomas analyzed: Ser-iPS cells, n=19; MEF-iPS cells, n=8; ES cells, n=10. Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). * indicates $P < 0.05$ (unpaired Student's t-test). Bars represent mean ± standard deviation.

Overall, these data indicate that syngeneic Ser-iPS cells have a higher incidence to form teratomas with less tissue damage and necrosis than syngeneic MEF-iPS cells. Furthermore, Ser-iPS cell teratomas exhibit lower T cell, DC and B cell infiltration than those of MEF-iPS cells. Thus, syngeneic Ser-iPS cells have reduced immunogenicity *in vivo* compared to MEF-iPS cells and showed low immune cell infiltration, which is

consistent with less tissue damage and necrosis.

4.3 T cell *in vitro* co-culture

Tissue destruction in teratomas is T cell dependent (Zhao et al., 2011) and Ser-iPS cell teratomas showed low T cell infiltration and tissue damage and necrosis. In addition, CD4 T lymphocytes play a more important role than CD8 T lymphocytes during immune rejection (Swijnenburg et al., 2008). Thus, the impact of Ser-iPS cells on CD4 T cell proliferation and Treg profile was examined *in vitro*. EB assay represents the *in vitro* equivalent of teratoma formation *in vivo*. Therefore, Ser-iPS cells were subjected to EB assays (12 days), dissociated and co-cultured with CD4 T cells in T cell medium.

4.3.1 T cell proliferation assay

For T cell proliferation assay *in vitro*, CD4 MACS selected B6 splenocytes were labeled with CFSE and co-cultured with undifferentiated Ser-iPS cells and EBs of Ser-iPS cells (day 12-17 of differentiation). The proliferation of CD4 T cells was analyzed by assessing the dilution of CFSE signal 5 days after co-culture by flow cytometry (FACSCanto II; Figure 4.15). Proliferation of CD4 T cells was calculated as the percentage of cells that divided at least once.

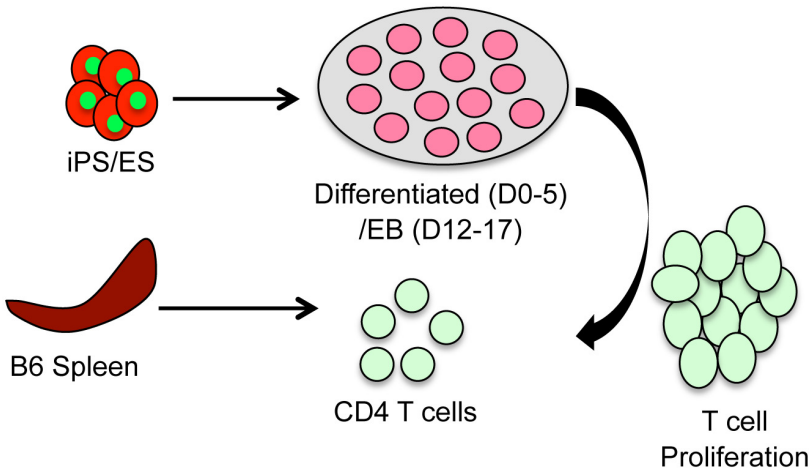

Figure 4.15. Schematic representation of CD4 T cell proliferation assay *in vitro*.

Splenic CD4 T cells from B6 mice were acquired by purification with MACS. EBs of Ser-iPS cells obtained by hanging drop were kept in suspension culture for 6 days followed by another 6 days of adherent culture in gelatin-coated Petri dishes. Both undifferentiated and EBs (day 12-17 of differentiation) of Ser-iPS cells were trypsinized into single cells, filtered through 40 µm cell strainer and co-cultured with CFSE labeled CD4 T cells at a ratio of 1:2. After 5 days of co-culture, proliferation of CD4 T cells was examined by assessment the dilution of CFSE signal using flow cytometry.

Purified CD4 T cells were obtained by MACS with MS tubes and their purity was immediately examined by flow cytometry. The purity of CD4 T cells after MACS selection was about 95.5% (Figure 4.16).

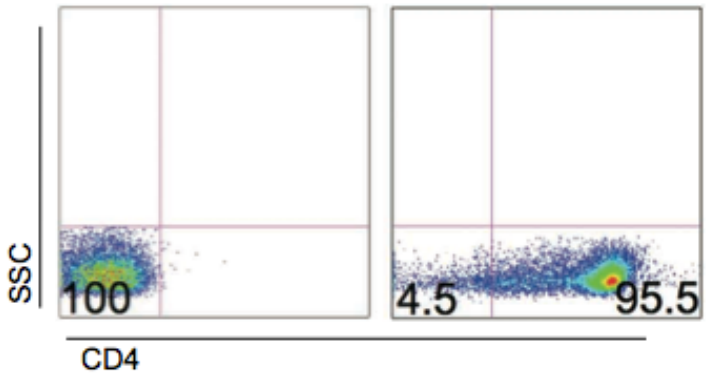

Figure 4.16. Purity of CD4 T cells after MACS selection.

CD4 T cells were purified by MACS and stained with PE-conjugated CD4 for 30 min. Purity of freshly isolated CD4 T cells was examined by flow cytometry. Unstained CD4 T cells were used as control. SSC: Side Scatter.

For CD4 T cell co-culture experiments, Ser-iPS cells were not inactivated, to mimic pluripotent stem cell differentiation *in vivo* in teratoma assays. For undifferentiated cells, there was no significant difference in the percentage of dividing T cells between Ser-iPS cells and MEF-iPS cells, as well as ES cells (all about 15-20%; Figure 4.17A). About 70% of T cell proliferated in positive control (CD4 T cells stimulated with PMA and ionomycin). However, when co-cultured with EBs of Ser-iPS cells, MEF-iPS cells and ES cells (day 12-17 of differentiation), the proliferation of CD4 T cell was very different. The percentage of dividing CD4 T cells co-cultured with EBs of Ser-iPS cells (day 12-17 of differentiation) was about 20%, similar to that of ES cells. However, the percentage of dividing T cells was about 50% after co-culture with EBs of MEF-iPS cells. T cell proliferation was low when co-cultured with Sertoli cells and MEF (Figure 4.17B). The results shown in Figure 4.17B were summarized as histogram (n=3). Significantly, lower T cell proliferation was observed after co-culture with EBs of Ser-iPS cells than MEF-iPS cells. However, no significant difference of T cell proliferation was observed between EBs of Ser-iPS cells and ES cells (Figure 4.17C).

Alltogether, no significant difference is obsevered in undifferentiated iPS cells and ES cells for their CD4 T cell stimulation potential. However, EBs of Ser-iPS cells exhibit significantly lower T cell stimulation potential *in vitro* compared to MEF-iPS cells, which very much relates to the reduced immunogenicity observed in teratoma assays.

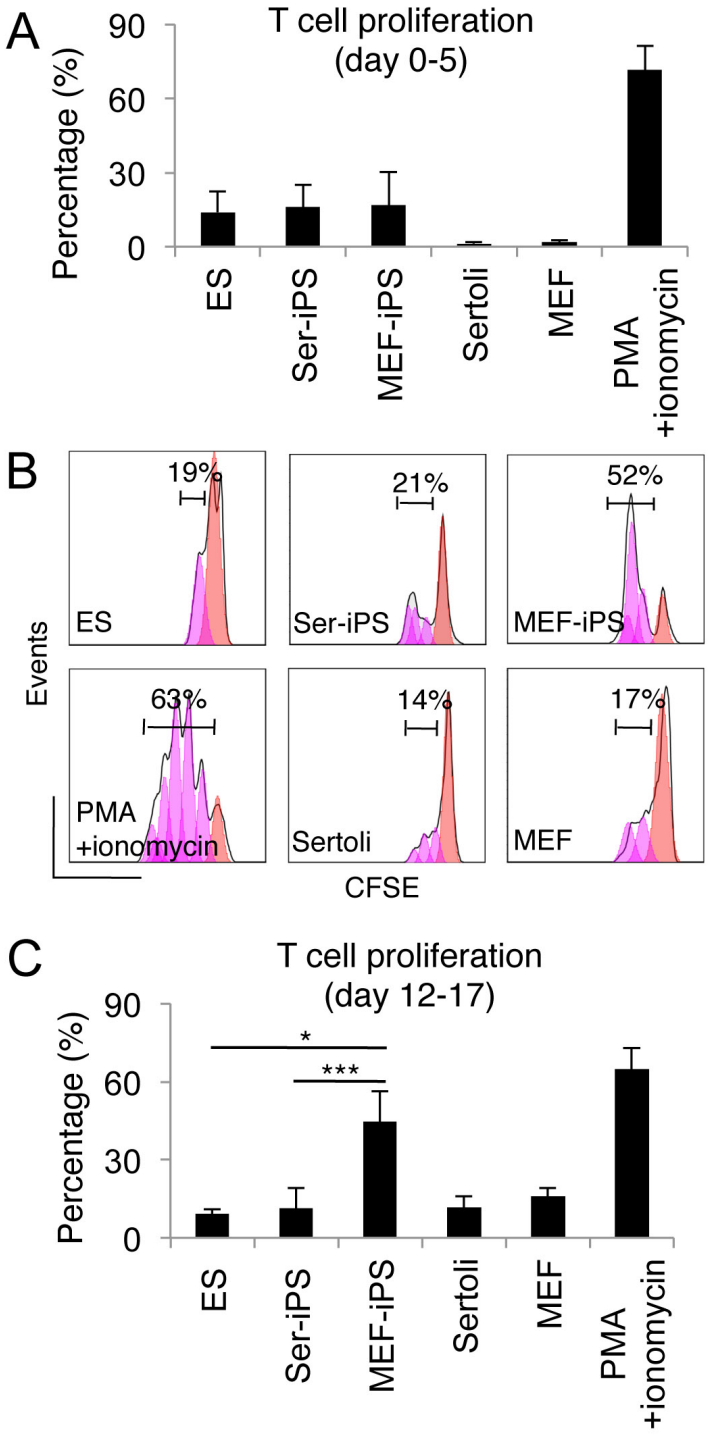

Figure 4.17. CD4 T cell proliferation after co-culture with undifferentiated and EBs of Ser-iPS cells (day 12-17 of differentiation).

A: CD4 T cells co-cultured with undifferentiated Ser-iPS cells in T cell medium. MEF-iPS cells, ES cells, Sertoli cells and MEF were used as controls. T cells activated with PMA and ionomycin were employed as positive control. T cell proliferation refers to the percentage of dividing T cells after 5 days of co-culture. The results are summarized as histogram for n=3 independent experiments. Bars represent mean ± standard deviation.
B: CD4 T cells co-cultured with EBs of Ser-iPS cells (day 12-17 of differentiation) in T cell medium. Representative images of T cell proliferation analyzed by flow cytometry are shown. Ser-iPS cells (OSKM, clone 1), MEF-iPS cells (OSKM, clone 1). The first peak on the right in each image

indicates undivided CD4 T cells with strong CFSE fluorescence signal and the other peaks on the left note the proliferating T cells as demonstrated by the decline of CFSE fluorescence intensity compared to the peak of non-dividing cells. The number in each image indicates the percentage of dividing cells. T cells activated with PMA and ionomycin, positive control as in (A).
C: Summary of CD4 T cell proliferation showed in (B). Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A) and (C) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). The proliferation assay was carried out in duplicates for n=3 independent experiments. T cells activated with PMA and ionomycin, positive control as in (A). * indicates $P < 0.05$; *** indicates $P < 0.001$. Bars represent mean ± standard deviation.

4.3.2 Regulatory T cell assay

Activation by PMA and ionomycin was first examined by measuring the T cell activation markers, CD25 and CD44. After one day of activation, T cells expressing CD25 and CD44 were 40% and 70%, respectively, and 2 days after activation, 50% T cells expressed these T cell activation markers. Afterwards, the percentage of activated T cells declined to 30% (Figure 4.18). This result suggests that CD4 T cells can be effectively activated by PMA and ionomycin after treatment for one day.

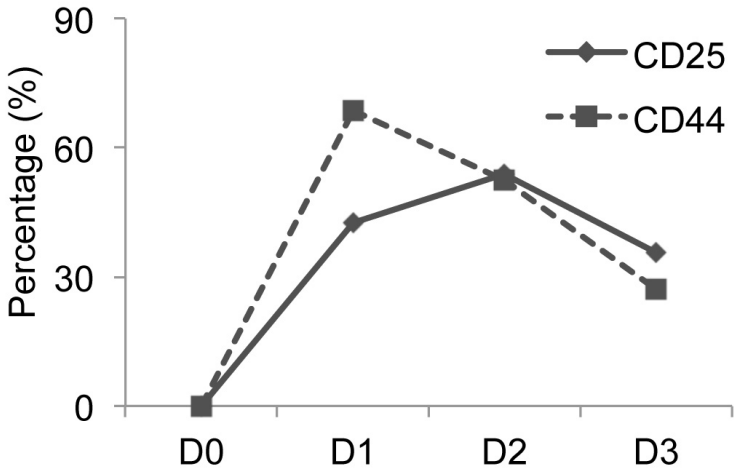

Figure 4.18. Activation of CD4 T cells with PMA and ionomycin.

CD4 T cells were activated with PMA and ionomycin for 3 days. The percentage of activated T cells was examined every day by flow cytometry for the T cell activation markers CD25 and CD44. Image summarizes the results of flow cytometry analysis.

Regulatory T cells (Tregs) play an essential role in maintenance of immunologic tolerance by suppressing aggressive T cell response, preventing autoimmunity and delaying allograft rejection in several animal models (Shevach 2002; Asano et al., 1996; Kingsley et al., 2002). Sertoli cells can skew T cells towards a Treg profile and this represents one way how Sertoli cells generate an immune-privileged testicular environment (Dal Secco et al., 2008; Doyle et al., 2012). Therefore, the frequency of Tregs in co-cultures of CD4 T cells with Ser-iPS cells was examined by measuring the expression of CD4, CD25 and Foxp3 by flow cytometry. MEF-iPS cells and ES cells

were used as controls. Sertoli cells were taken as a positive control, since they skew T cells towards a Treg profile (Dal Secco et al., 2008). To avoid any influence of chemical

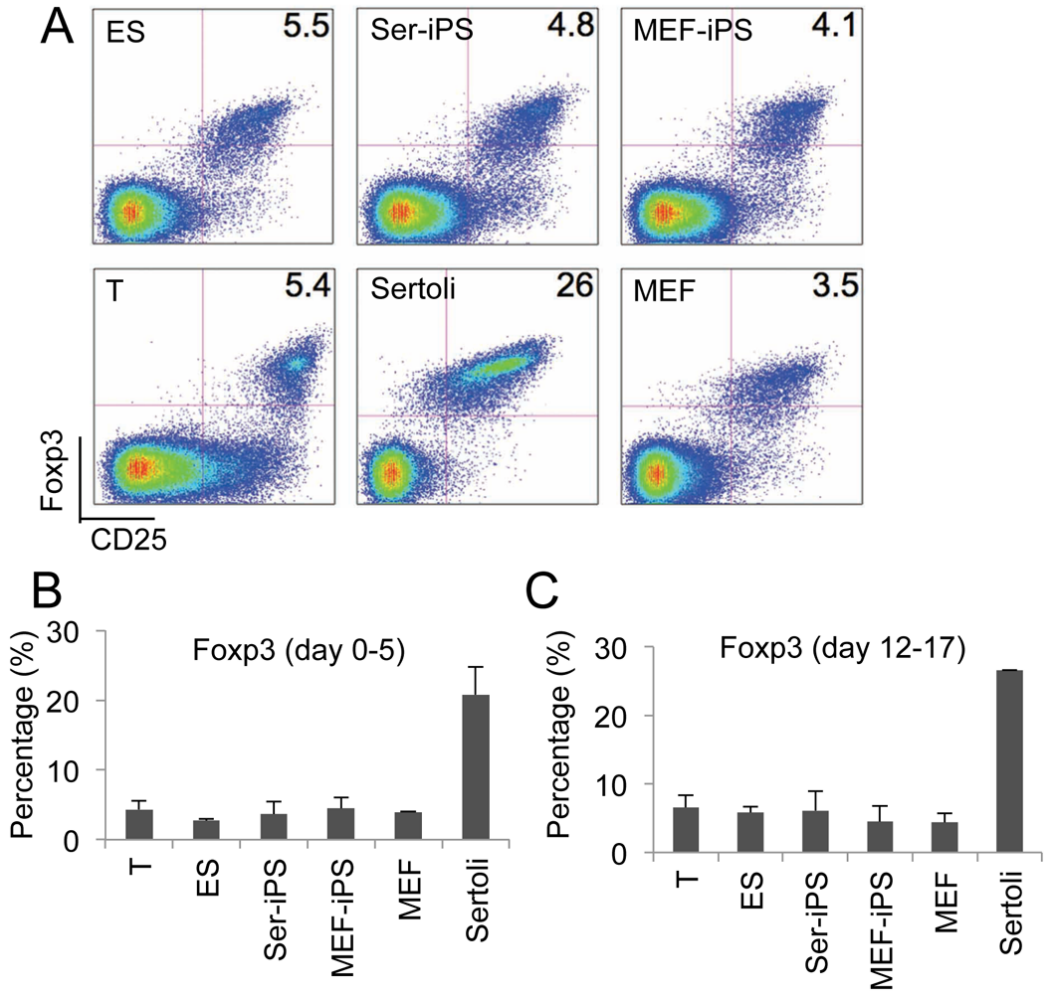

Figure 4.19. Frequency of Tregs after co-culture with undifferentiated or EBs of Ser-iPS cells (day 12-17 of differentiation).

A: Percentage of Tregs after co-culture with EBs of Ser-iPS cells (day 12-17 of differentiation). MEF-iPS cells, ES cells, Sertoli cells and MEF were used as controls. Activated T cells with PMA and ionomycin were taken as a further control. Representative images of Treg expression after co-culture with EBs of Ser-iPS cells are shown. Ser-iPS cells (OSK, clone 2), MEF-iPS cells (OSK, clone 2). The gate was set on $CD4^+$ cells followed by $CD25^+$ cells. T: activated T cells with PMA and ionomycin. Numbers depict the percentage of corresponding populations.
B-C: Summarized results of flow cytometry analysis. Treg profile after co-culture with undifferentiated Ser-iPS cells (B) or with EBs of Ser-iPS cells (C) in T cell medium. MEF-iPS cells, ES cells, Sertoli cells, MEF and actived T cells with PMA and ionomycin were used as controls as in (A). T: activated T cells with PMA and ionomycin. Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (B) and (C) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Bars represent mean ± standard deviation. Experiments was carried out in duplicates for n=2 independent experiments.

reagents (PMA and ionomycin) on the properties of Ser-iPS cells, CD4 T cells were activated one day before co-culture. Then these compounds were removed by washing with PBS and T cell medium. Indeed, Sertoli cells showed an increase in Foxp3

expressing cells (20-26%), while T cells after co-culture with EBs of Ser-iPS cells, MEF-iPS cells and ES cells showed Treg frequencies of about 6% (Figure 4.19A). Summarized results of flow cytometry analysis were further shown as histogram. No significant differences were observed for undifferentiated Ser-iPS cells, MEF-iPS cells and ES cells (Figure 4.19B) and also for EB-derived differentiated Ser-iPS cells, MEF-iPS cells and ES cells (Figure 4.19C). Thus, Ser-iPS cells apparently do not possess the potential to convert T cells into a Treg phenotype.

4.4 Generation of teratomas from late-passage Ser-iPS cells

All results obtained so far are based on early-passage iPS cells (p9-15), at which stage somatic memory most likely still exists (Polo et al., 2010). Early-passage Ser-iPS cells showed reduced immunogenicity in following aspects: form teratoma at high frequency upon syngeneic transplantation, have lower T cell, DC and B cell infiltration in teratomas, exhibit less tissues damage and necrosis and less T cell stimulation potential at the EB stage. Thus, Ser-iPS cells appear to retain some somatic memory from immune-privileged Sertoli cells, which might contribute to their reduced immunogenicity.

Previous studies indicate that somatic memory will be erased upon extended passaging *in vitro* (Chin et al., 2009; Polo et al., 2010). Thus, the effect of extended culture periods on the immunogenicity of Ser-iPS cells was investigated. Late-passage Ser-iPS cells (p35-38) were injected into syngeneic mice to test their *in vivo* immunogenicity in teratomas assay. For each iPS and ES cell clone, 3 mice were used. Four weeks later teratomas were collected as before. Surprisingly, teratoma formation efficiency by late-passage Ser-iPS cells was only about 50%, which was comparable to that of MEF-iPS cells (58.3%), but the incidence of teratoma formation by corresponding ES cells was 100% (Table 4.3). No significant difference was observed for the number of teratomas formed by late-passage Ser-iPS cells and MEF-iPS cells. However, teratoma formation frequency for late-passage ES cells was much higher than those of iPS cells (Figure 4.20A). In addition, given the size of teratomas formed by late-passage iPS cells and ES cells, no significant difference was observed (Figure 4.20B). For Ser-iPS cells, teratoma incidence of their late-passage (50%) was much lower than their early-passage counterparts (80%). While teratoma formation frequency of late (58.3%) and early-passage (20%) MEF-iPS cells were reversed compare to Ser-iPS cells. However, no significantly different teratoma formation efficiency was found between late (100%) and early-passage (90%) ES cells (Figure 4.20C).

Table 4.3 Teratoma formation in B6 mice by late-passage Ser-iPS cells

Clones	Nr. Teratomas	Nr. Injection sites	Frequency of teratoma formation
ES	6	6	100%
Ser-iPS	9	18	50%
MEF-iPS	7	12	58.3%

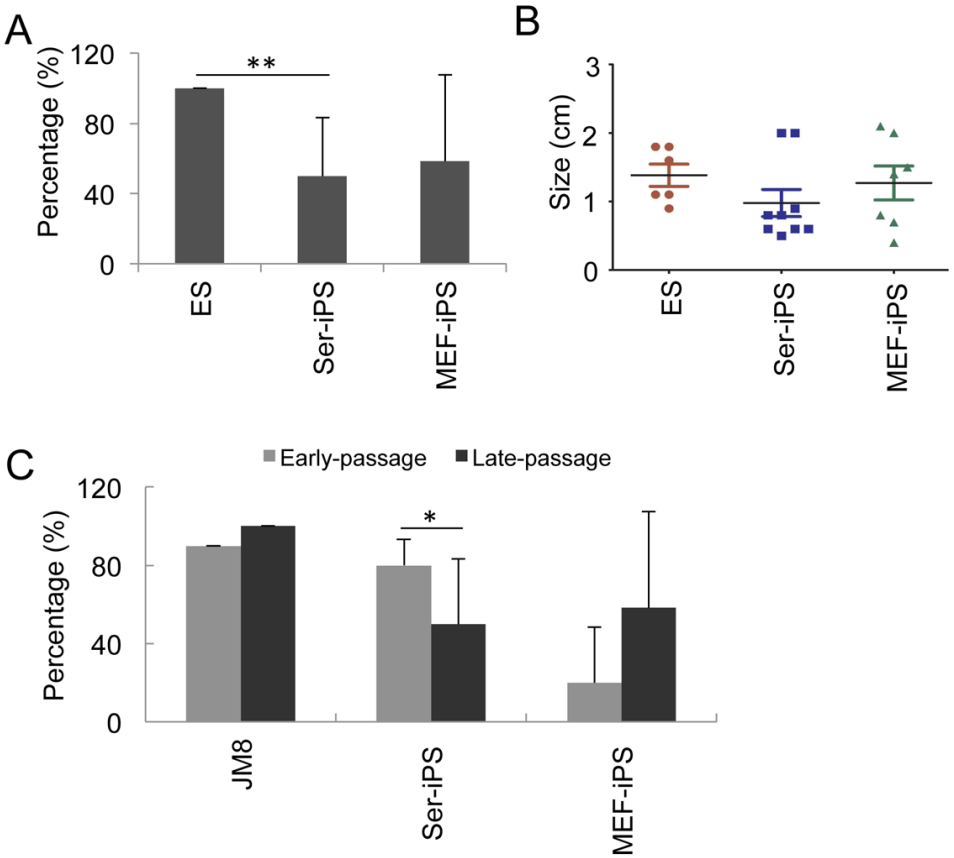

Figure 4.20. Teratoma number and size formed by late-passage Ser-iPS cells in B6 mice.

A: Number of teratomas formed by late-passage Ser-iPS cells was summarized as histogram. Late-passage MEF-iPS cells and ES cells were taken as controls. For Ser-iPS cells and MEF-iPS cells, 9 and 6 mice, respectively, were used; for ES cells, 3 mice were used. ** indicates $P < 0.01$. Bars represent mean ± standard deviation.
B: Size of teratomas formed by late-passage Ser-iPS cells. Late-passage MEF-iPS cells and ES cells were taken as controls as in (A). Bars represent mean ± standard deviation.
C: Comparison of teratoma formation frequency by early-passage (p9-15) and late-passage (p35-38) Ser-iPS cells. Corresponding MEF-iPS cells and ES cells were taken as controls as in (A). Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A), (B) and (C) are as in Figure 4.11. Late-passage refers to passage 35-38. * indicates $P < 0.05$. Bars represent mean ± standard deviation.

Taken together, late-passage Ser-iPS cells exhibit reduced teratoma formation efficiency compared to their early counterparts. This result suggests that the potential somatic memory in Ser-iPS cells might get lost during extended passaging *in vitro.* However,

teratoma formation efficiency of ES cells are independent of their passage number. Because ES cells come from inner cell mass of blastocyst they do not contain somatic memory. Thus, extended *in vitro* culture of Ser-iPS cells appears to impact on their immunogenicity.

4.5 Possible molecules related to the reduced immunogenicity of Ser-iPS cells

Late-passage Ser-iPS cells were found to lose their potential to form more teratomas upon syngeneic transplantation, which might be because their somatic memory gets lost and this leads to increased immunogenicity. Considering the reduced immunogenicity of early-passage Ser-iPS cells, two possibilities exist: one possibility is, that early-passage Ser-iPS cells preferentially differentiate into immune-privileged Sertoli cells. Another possibility is the early-passage Ser-iPS cells preserved some of Sertoli cell immune function and produced some immune-protective molecules to prevent immune rejection.

4.5.1 Ser-iPS cells failed to re-differentiate into Sertoli cells

To investigate whether early-passage Ser-iPS cells are more likely to re-differentiate into Sertoli cells, qRT-PCR analysis was performed. EBs (day 14 of differentiation) and teratomas of Ser-iPS cells were analyzed for Sertoli cell gene expression, such as sex determining region Y (Sry) box containing gene 9 (Sox9), anti-Müllerian hormone (AMH) and androgen-binding protein (ABP). MEF was used as negative control and Sertoli cells were taken as positive control.

For the expression of Sox9 and AMH in both EBs and teratomas, no significant difference was observed between Ser-iPS cells and MEF-iPS cells, as well as ES cells, while these genes were highly expressed in Sertoli cells (Figure 4.21A, B). ABP was more abundantly expressed in EBs and teratomas of Ser-iPS cells, than in EBs and teratomas of MEF-iPS cells and ES cells. Whether this result suggests a preferred differention of Ser-iPS cells into Sertoli cells needs to be further investigated.

4.5.2 Molecules related to immune response

MHC and costimulatory molecules expression

During teratoma formation in B6 mice, many immune-related molecules might be involved in the immune responses. MHC class I protein, which contributed to the low immunogenicity of ES cells, is expressed at a very low level on ES cells and moderately

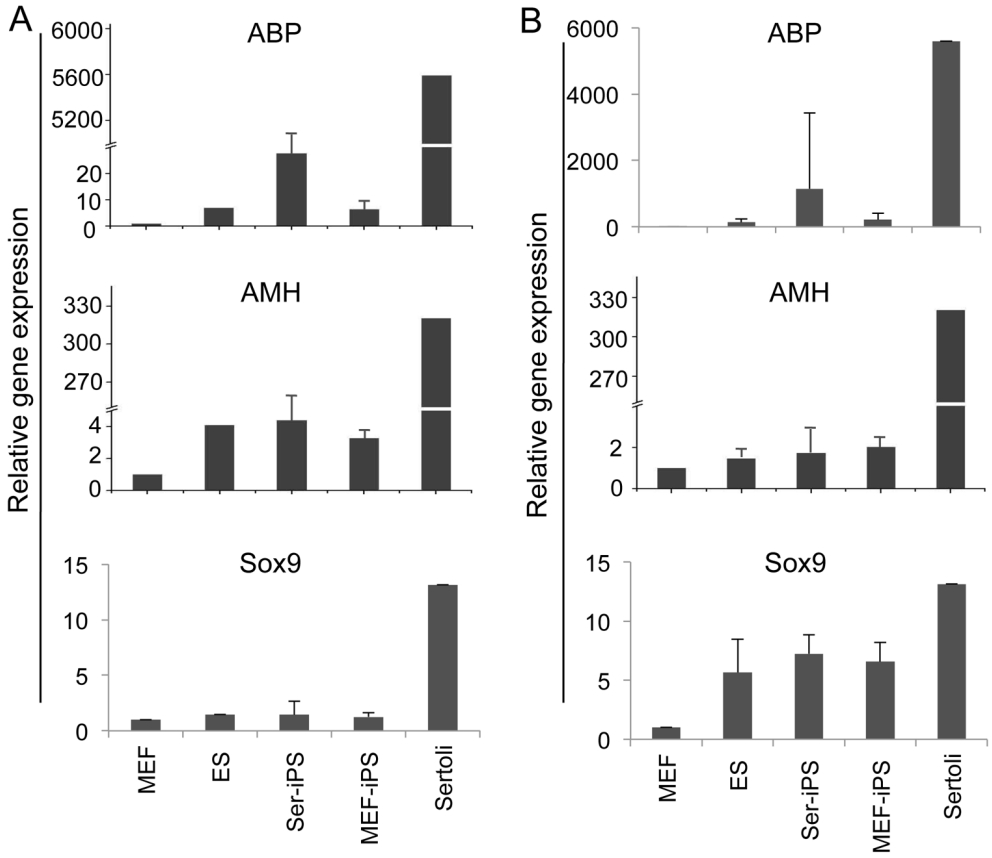

Figure 4.21. Differentiation potential of EBs and teratomas from Ser-iPS cells.

A-B: Expression of Sertoli cell markers in Ser-iPS cell EBs (left panel) and teratomas (right panel). B6 MEF were used as negative control and B6 Sertoli cells were taken as positive control. Relative gene expression was normalized to β-actin. The mRNA level in MEF was arbitrarily set to 1. Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A) and (B) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Bars represent mean ± standard deviation.

increased on their differentiated derivatives (Drukker et al., 2002). To investigate whether MHC or costimulatory molecule expression (CD80 and CD86) is responsible for the reduced immunogenicity of Ser-iPS cells, their expression on both undifferentiated and EBs of Ser-iPS cells was examined. From the qRT-PCR result, no significant difference was observed for the expression of MHC class I, MHC class II, CD80 and CD86 genes between Ser-iPS cells and MEF-iPS cells for both undifferentiated Ser-iPS cells (Figure 4.22A) and EBs of Ser-iPS cells (day 14 of differentiation; Figure 4.22B). The expression of these molecules on iPS cells was similar to the expression on ES cells. These data suggest that levels of MHC and costimulatory molecules expression do not contribute to the different immunogenicity of Ser-iPS cells and MEF-iPS cells.

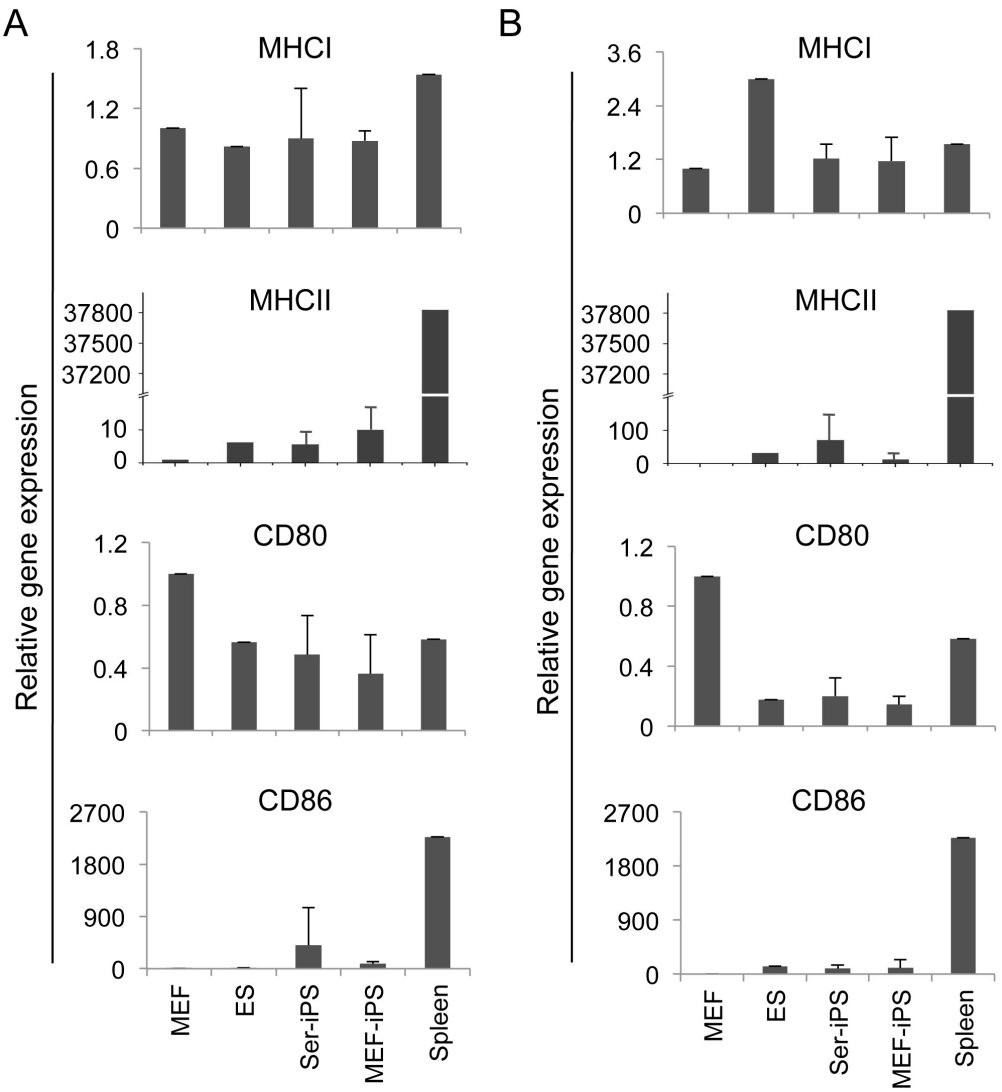

Figure 4.22. MHC and costimulatory molecules expression in undifferentiated and EBs (day 14 of differentiation) of Ser-iPS cells.

A-B: Expression of MHC class I and II (MHC I and MHC II, respectively), costimulatory molecules CD80 and CD86 on undifferentiated (A) and EBs (day 14, B) of Ser-iPS cells. B6 MEF were used as negative control, and B6 splenocytes were taken as positive control. Relative gene expression was normalized to β-actin. The mRNA level in MEF was arbitrarily set to 1. Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A) and (B) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Bars represent mean ± standard deviation.

Expression of molecules related to immunogenicity of pluripotent stem cells

Expression of Hormad1 and zymogen granule protein 16 (Zg16) genes has been reported to be associated with the immunogenicity of iPS cells (Zhao et al., 2011). They were highly expressed in shrinking iPS teratomas upon syngeneic transplantation (Zhao et al., 2011), however this finding remains controversial. Araki et al. indicated that the expression of Hormad1 and Zg16 genes was not at an elevated level in regressing teratomas (Araki et al., 2013). Furthermore, Hormad1 and Zg16 expression was similar

among undifferentiated pluripotent stem cells or their tissue-specific differentiated three germ layer cells (endothelial cells, hepatocytes and neuronal cells, Guha et al., 2013).

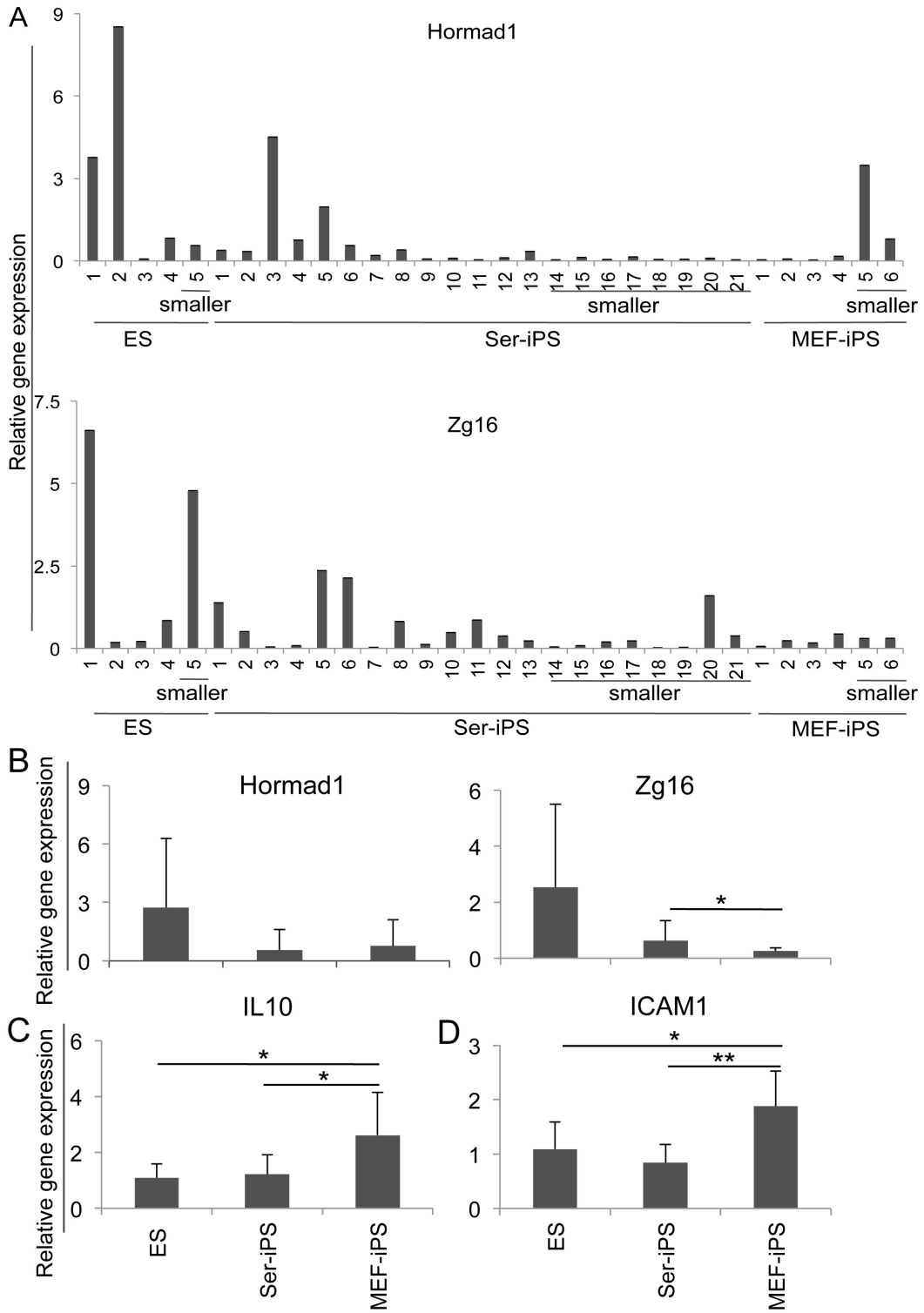

Figure 4.23. Immune-related gene expression in Ser-iPS cell teratomas.

A: Expression of Hormad1 and Zg16 genes in Ser-iPS cell teratomas from B6 mice. Teratomas from MEF-iPS cells and ES cells were used as controls. Relative gene expression was normalized to β-actin. The average mRNA level in ES cell teratomas was arbitrarily set to 1.

B: Summary of Hormad1 and Zg16 gene expression in both larger and smaller teratomas from Ser-iPS cells. Teratomas from MEF-iPS cells and ES cells were controls as in (A). The average mRNA level in ES cell teratomas was arbitrarily set to 1. Ser-iPS cells: n=21; MEF-iPS cells: n=6; ES cells: n=5. Bars represent mean ± standard deviation.
C-D: IL10 and ICAM1 gene expression in teratomas from Ser-iPS cells. Teratomas from MEF-iPS cells and ES cells were used as controls in (A). The average mRNA level in ES cell teratomas was arbitrarily set to 1. For IL10 gene expression, Ser-iPS cells: n=19; MEF-iPS cells: n=8; ES cells: n=10. For ICAM1 gene expression, Ser-iPS cells: n=13; MEF-iPS cells: n=8; ES cells: n=10. Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A), (B), (C) and (D) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). * indicates $P < 0.05$. ** indicates $P < 0.01$. Bars represent mean ± standard deviation.

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To investigate the expression of Hormad1 and Zg16 genes in Ser-iPS cell teratomas, both larger and smaller teratomas from syngeneic B6 mice were examined by qRT-PCR. No significantly different expression of Hormad1 and Zg16 genes was observed between larger and smaller teratomas, and most of the teratomas showed low expression of these two genes (Figure 4.23A). Surprisingly, the expression of Zg16 gene was much higher in teratomas from Ser-iPS cells than that of MEF-iPS cells, which seems contradictory to the reduced immunogenicity of Ser-iPS cells. For the expression of Hormad1 gene, no significant difference was found between Ser-iPS cell and MEF-iPS cell teratomas, as well as ES cell teratomas. Furthermore, both Ser-iPS cell and MEF-iPS cell teratomas showed lower expression level of Hormad1 and Zg16 than ES cell teratomas (Figure 4.23B). Thus, expression of Hormad1 and Zg16 genes is apparently unrelated to teratoma regression and iPS cell immunogenicity.

Further investigation of other genes that might be related to Ser-iPS cell immunogenicity was performed by qRT-PCR. The expression of anti-inflammatory cytokine interleukin 10 (IL10) and intercellular adhesion molecule 1 (ICAM1) genes in teratomas was analyzed. Unexpectedly, expression of IL10 gene was significantly lower in teratomas from Ser-iPS cells than in those of MEF-iPS cells (Figure 4.23C). Considering the higher frequency of

teratomas formed by Ser-iPS cells, IL10 expression appears to more strongly correlated to the immune rejection of iPS cells than their survival, which is consistent with a previous study (Robertson et al., 2007). ICAM1, which is upregulated by inflammatory cytokines, plays an important role for leukocyte travel and adhesion during immune response (Long 2011). Expression of ICAM1 gene in Ser-iPS cell teratomas was lower than in MEF-iPS cell and ES cell teratomas (Figure 4.23D). This finding indicates that ICAM1 gene expression might partially contribute to the different immune cell infiltration in teratomas from Ser-iPS cells and MEF-iPS cells.

Expression of molecules involved in immune-privileged function of Sertoli cells

Given the reduced immunogenicity of Ser-iPS cells, some of the immune-protective molecules, which contribute to the immune-privileged function of Sertoli cells, might be preserved in Ser-iPS cells. Therefore, the molecules related to the immune-privileged function of Sertoli cells were examined. EBs and teratomas from Ser-iPS cells were analyzed by qRT-PCR. Expression of arginase 1 and prostaglandin E2 (PGE2) genes are shown here. Arginase 1, expressed in Sertoli cells, hydrolyzes arginine into urea and ornithine (Guo et al., 2007). It is also expressed in human and mouse ES cells, inhibits T cell responses, which results in downregulation of TCR CD3-ζ chain and attenuated T cell activation. This was further confirmed by *in vivo* experiments that T cell infiltrating teratomas derived from mouse ES cells showed significantly lower CD3-ζ chain expression (Yachimovich-Cohen et al., 2010).

Sertoli cells express arginase 1, which is involved in immune suppression, and thus the question is whether arginase 1 expression in Sertoli cells contributes to the reduced immunogenicity of Ser-iPS cells. Arginase 1 expression in both EBs (day 14 of differentiation) and teratomas of Ser-iPS cells was analyzed by qRT-PCR. Indeed, higher expression of arginase 1 in Sertoli cells than MEF was found. Furthermore, much higher expression of arginase 1 was observed in EBs of Ser-iPS cells than those of MEF-iPS cells. While no significant difference of arginase 1 expression was found in Ser-iPS cell and MEF-iPS cell teratomas (Figure 4.24A). Arginine depletion by arginase 1 causes a downregulation of CD3-ζ chain (Rodriguez et al., 2002; Baniyash 2004). Thus, the expression of CD3-ζ chain in Ser-iPS cell teratomas was analyzed. No significantly different expression of CD3-ζ chain was observed between teratomas from Ser-iPS cells and MEF-iPS cells, as well as teratomas from ES cells (Figure 4.24B). This is consistent with the expression of arginase 1 in teratomas. Another immunosuppressive factor,

PGE2, was higher expressed in Sertoli cells than MEF, as well as higher expressed in Ser-iPS cell teratomas than MEF-iPS cell teratomas (Figure 4.24C).

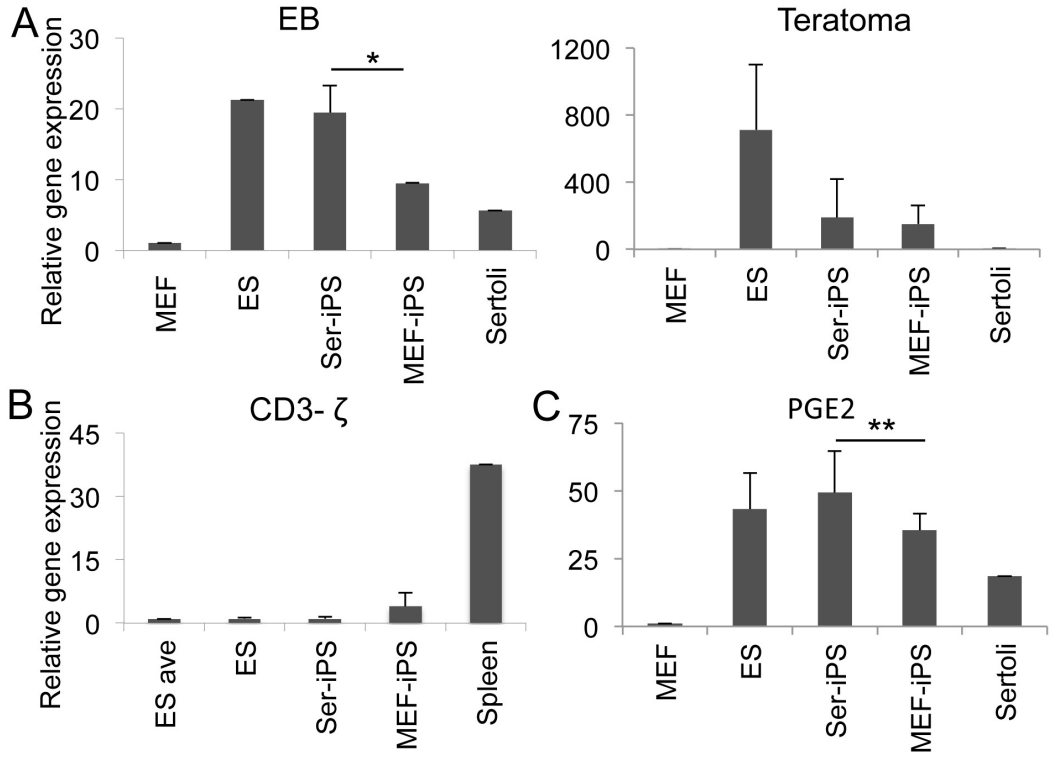

Figure 4.24. Expression of molecules related to the immune-privileged function of Sertoli cells in EBs and teratomas from Ser-iPS cells.

A: Arginase 1 expression in EBs (left panel) and teratomas (right panel) of Ser-iPS cells was examined by qRT-PCR. Corresponding MEF-iPS cells and ES cells were taken as controls. Sertoli cells and MEF were used as positive and negative control, respectively. mRNA level in MEF was arbitrarily set to 1. Replication for the expression of arginase 1 in teratomas: Ser-iPS cells: n=14; MEF-iPS cells: n=3; ES cells: n=3. * indicates $P<0.05$. Bars represent mean ± standard deviation.
B: Expression of CD3-ζ chain in Ser-iPS cell teratomas by qRT-PCR. Teratomas from MEF-iPS cells and ES cells were taken as controls. Spleen was used as a positive control. The average mRNA level in ES cell teratomas was arbitrarily set to 1. Replication for the expression of CD3-ζ chain in teratomas: Ser-iPS cells: n=12; MEF-iPS cells: n=5; ES cells: n=5. Bars represent mean ± standard deviation. ES ave: ES average.
C: Expression of PGE2 in Ser-iPS cell teratomas. Corresponding MEF-iPS cells and ES cells were taken as controls. Sertoli cells and MEF were used as positive and negative control, respectively. mRNA level in MEF was arbitrarily set to 1. Replication for the expression of PGE2 in teratomas: Ser-iPS cells: n=13; MEF-iPS cells: n=8; ES cells: n=10. Average values of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A), (B) and (C) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). ** indicates $P<0.01$. Bars represent mean ± standard deviation.

By allogeneic transplantation of primary Sertoli cells, Doyle and colleagues suggested that the mechanisms responsible for Sertoli cell immune-privileged function are favoring a type 2 immune response, immature DC and Tregs (Doyle et al., 2012). To address if Ser-iPS cells retain these functions of Sertoli cells, the frequency of Tregs and gene

expression representing type 1 and type 2 immune responses were examined by qRT-PCR. No significant difference of Foxp3 expression was observed between teratomas from Ser-iPS cells and MEF-iPS cells, as well as from ES cells (Figure 4.25A). IFNγ and IL-4 genes are representative markers for type 1 (destructive) and type 2 (protective) immune responses, respectively. The expression of IFNγ gene in Ser-iPS cell teratomas was lower compared to that of MEF-iPS cells, however this did not reach statistical significance. The expression of IL-4 gene was similar in teratomas of Ser-iPS cells and MEF-iPS cells, as well as teratomas of ES cells (Figure 4.25B).

Taken together, the expression of MHC class I and MHC class II, costimulatory molecules (CD80 and CD86), Hormad1 and Zg16 genes do not seem to be responsible for the reduced immunogenicity of Ser-iPS cells. ICAM1, PGE2 and arginase 1 genes, which relate to the immune-privileged function of Sertoli cells, were found differently expressed in teratomas or EBs derived from Ser-iPS cells and MEF-iPS cells. Thus expression of ICAM1, PGE2 and arginase 1 genes might contribute to the reduced immunogenicity of Ser-iPS cells. However, Ser-iPS cells failed to retain Sertoli cell immune functions, which switch type 1 to type 2 immune response and favor Treg generation *in vivo*.

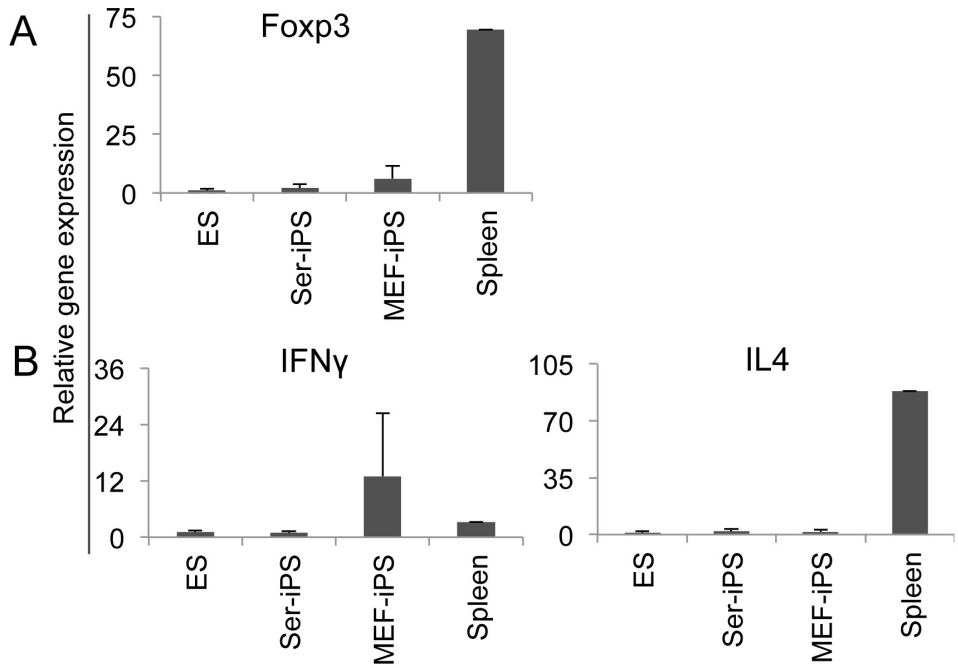

Figure 4.25. Expression of Foxp3, IFNγ and IL-4 genes in teratomas from Ser-iPS cells.

A-B: Foxp3, IFNγ and IL-4 gene expression in Ser-iPS cell teratomas was examined by qRT-PCR. Teratomas from MEF-iPS cells and ES cells were used as controls. Spleen was taken as a positive control. Average mRNA level in ES cell teratomas was arbitrarily set to 1. Average values

of Ser-iPS cell 1, 2 and 3 and MEF-iPS cell 1 and 2 in (A) and (B) are as in Figure 4.11. All Ser-iPS cells and MEF-iPS cells are passage 9-15 (early-passage). Bars represent mean ± standard deviation.

4.6 Transdifferentiation MEF to hematopoietic cells

With different culture conditions and appropriate transcriptional factors ectopic expression, somatic cells can be directly reprogrammed into another kind of adult stem cells or differentiated cells without establishing a pluripotent state (Szabo et al., 2010). Cell types with tissue-specific conditional mutants are very important for this process, since it is much easier for monitoring successful transdifferentiation.

4.6.1 Generation of Vav-iCre/Rosa26R-fGFP mice

For transdifferentiation of MEF into hematopoietic cells, Vav-iCre mouse line was selected, which expresses Cre recombinase in hematopoietic tissues under the control of Vav regulatory elements (de Boer et al., 2003; Kwon et al., 2008; a kind gift of Dimitris Kioussis, London, UK and Meinrad Busslinger, IMP, Vienna, Austria; Figure 4.26A, upper panel). Vav gene expresses in virtually all murine hematopoietic cell types and also hematopoietic stem cells (Adams et al., 1992; Katzav et al., 1989 and Bustelo et al., 1993). Furthermore, the Vav promoter elements can efficiently and exclusively initiate

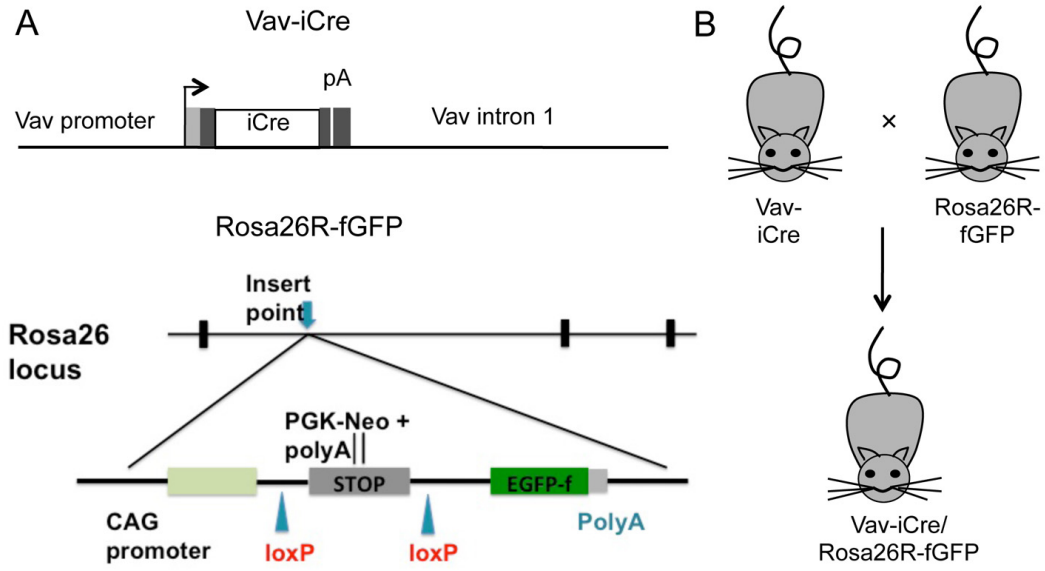

Figure 4.26. Schematic representation of Cre expression and Rosa26R-fGFP structure used to generate Vav-iCre/Rosa26R-fGFP mice.

A: Cre expression under the control of Vav promoter (upper panel). Rosa26R-fGFP structure contains loxP sites and EGFP under the control of CAG promoter (lower panel). pA represents poly A sequence. CAG promoter: chicken beta-actin promoter with cytomegalovirus enhancer. B: Scheme for generation of Vav-iCre/Rosa26R-fGFP mice.

transgene expression in almost all nucleated hematopoietic cells independent of integration position (Ogilvy et al., 1999a and Ogilvy et al., 1999b). Another mouse line used is Rosa26R-fGFP mouse, which contains loxP sites flanking a STOP cassette in front of farnesylated eGFP (Rawlins et al., 2009; a kind gift of Emma L Rawlins, Gurdon Institute, Cambridge, UK). Membrane-targeted eGFP is expressed after Cre-mediated excision of the STOP cassette (Figure 4.26A, lower panel). To generate Vav-iCre/Rosa26R-fGFP mice that express eGFP in hematopoietic cells under the control of Vav promoter, Vav-iCre mice were crossed with Rosa26R-fGFP mice (Figure 4.26B). Heterozygote mice (Vav-iCre/Rosa26R-fGFP$^{+/-}$) were obtained. To test GFP expression in hematopoietic cells, blood and BM samples from these heterozygote mice were isolated and analyzed for GFP expression in different types of hematopoietic cells by flow cytometry or microscopy.

In blood samples from heterozygote mice, about 95% CD45^{+} cells expressed GFP, while still 5% CD45^{+} cells were GFP^{-}. For T cell (CD4 and CD8) and DC (CD11b), almost all the cells expressed GFP (100%); for B cell (CD19) and macrophage (F4/80), about 99% of these cells were GFP^{+}, only 1% of the cells were GFP^{-} (Figure 4.27A). The expression of GFP fluorescence in BM cells from heterozygote mice was detected with microscopy. Most of the BM cells expressed GFP fluorescence, in contrast to BM cells from wt mice, which were absolutely GFP^{-} (Figure 4.27B). These data indicate that Vav promoter and Cre-mediated excision of the loxP sites are effective in hematopoietic cells from Vav-iCre/Rosa26R-fGFP$^{+/-}$ mice. Thus, Vav-iCre/Rosa26R-fGFP mice can be used as cell source for detection of successfully transdifferentiate somatic cells into hematopoietic cells.

4.6.2 Vav-MEF preparation by MACS

The purity of initiating cells is extremely important for successful transdifferentiation. Here, terminally differentiated MEF were used for transdifferentiation. MEF were isolated from day 13.5 pregnant Vav-iCre/Rosa26R-fGFP$^{+/+}$ mice; in the following referred to as Vav-MEF. Vav-MEF were prepared as described before (Methods 3.2) and purified with MACS to avoid potential contamination of hematopoietic cells. MACS selected Vav-MEF were infected with retroviral vectors expressing Oct4 or Oct4 plus Bmi1 to generate hematopoietic cells. Cells were cultured under HSC condition in the presence of cytokines (SCF, hyper-IL-6, Flt3 ligand and human IGF1; hereafter referred to as

complete HSC medium), which is a commonly used medium for HSC in our lab. When non-adherent cells emerged, cells were collected and analyzed by flow cytometry (Figure 4.28).

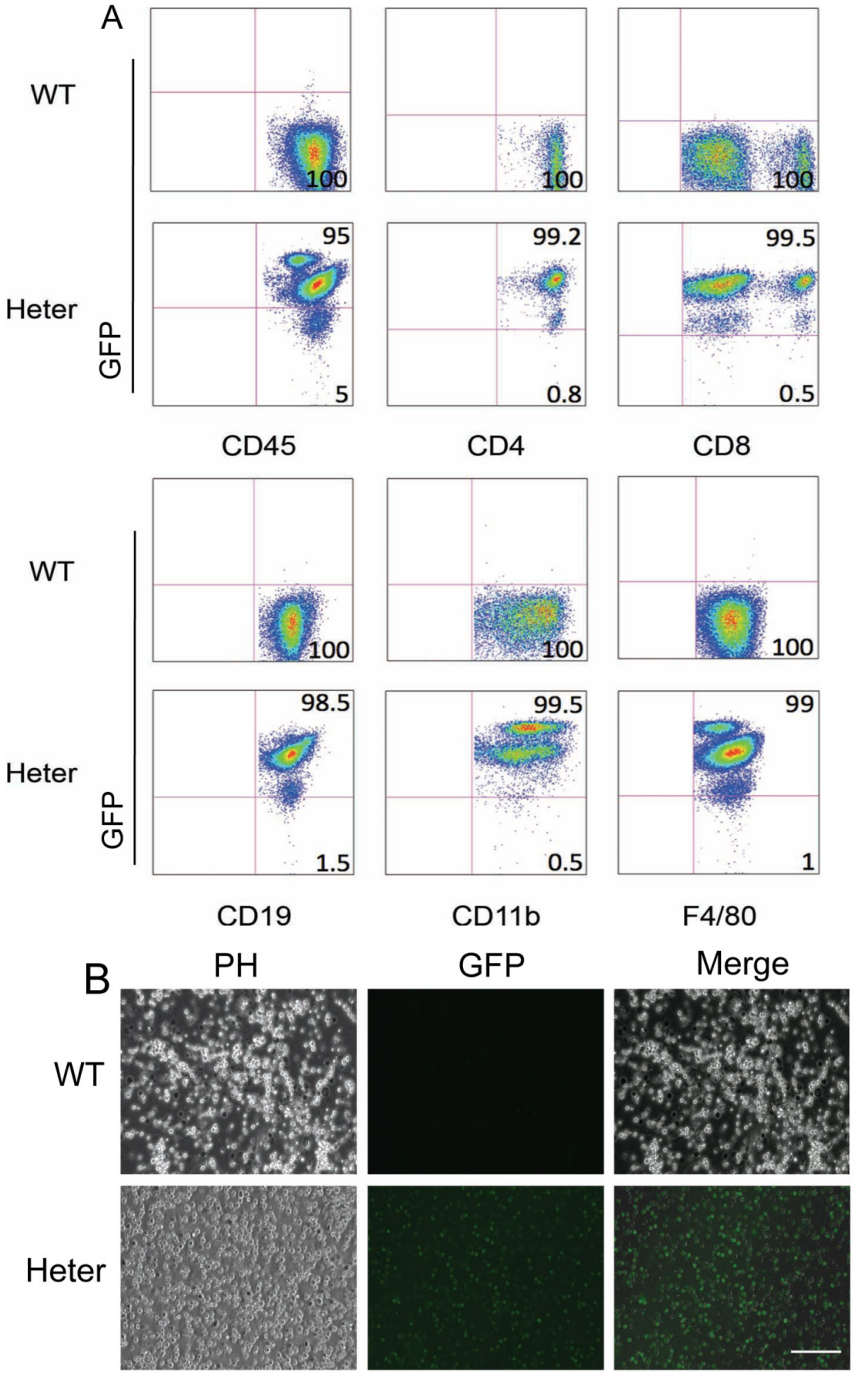

Figure 4.27. GFP expression in hematopoietic cells from Vav-iCre/Rosa26R-fGFP+/- mice.

A: Flow cytometry analysis of blood cells from heterozygote Vav-iCre/Rosa26R-fGFP mice. Blood cells from wt mice were used as control. Cells were stained with antibodies at 4°C for 30 min. GFP expression in different types of hematopoietic cells was examined. Numbers depict the percentages of corresponding populations. WT: wild type; Heter: heterozygote.

B: Microscopy of BM cells from heterozygote Vav-iCre/Rosa26R-fGFP mice. Representative images of GFP expression in hematopoietic cells from wt mice (upper panel) and heterozygote mice (lower panel) are shown. Images were taken immediately after the isolation of BM cells. PH: phase contrast. Scale bar represents 800 µm.

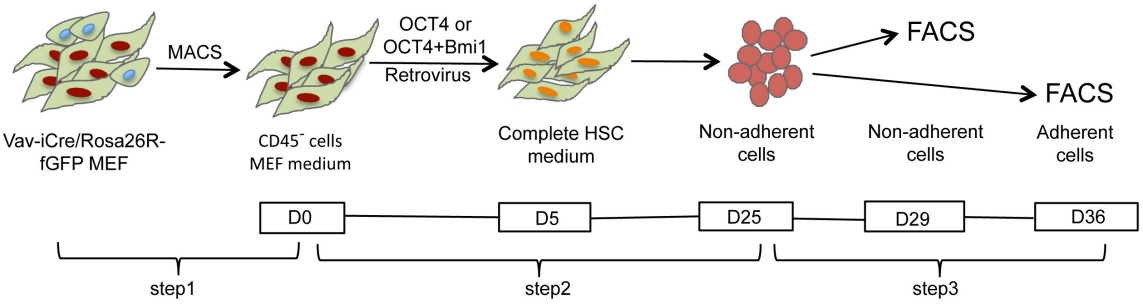

Figure 4.28. Schematic representation of transdifferentiation Vav-MEF into hematopoietic cells.

Step 1: Vav-MEF were isolated from day 13.5 pregnant homozygote Vav-iCre/Rosa26R-fGFP mice. After one passage, Vav-MEF were purified by negative selection with CD45 MicroBeads to remove hematopoietic cells.
Step 2: MACS purified Vav-MEF were infected with retroviral vectors expressing transcriptional factor Oct4 or the combination of Oct4 and Bmi1. Infected cells were cultured in MEF medium. Five days after infection, MEF medium was refreshed with complete HSC medium and changed every two days until non-adherent cells emerged.
Step 3: At day 29 after infection, non-adherent cells were collected and analyzed by flow cytometry. At day 36 after infection, remaining adherent cells were trypsinized into single cells and harvested for flow cytometry.

Purity of Vav-MEF was determined by flow cytometry immediately after MACS selection. Unstained CD1 MEF were used as control. Actually, freshly isolated Vav-MEF were not absolutely homogeneous population. The purity of Vav-MEF increased from 97.5% (before MACS) to 99% (after MACS; Figure 4.29). This purification procedure is necessary for hereafter transdifferentiation to avoid the possible contamination of hematopoietic cells.

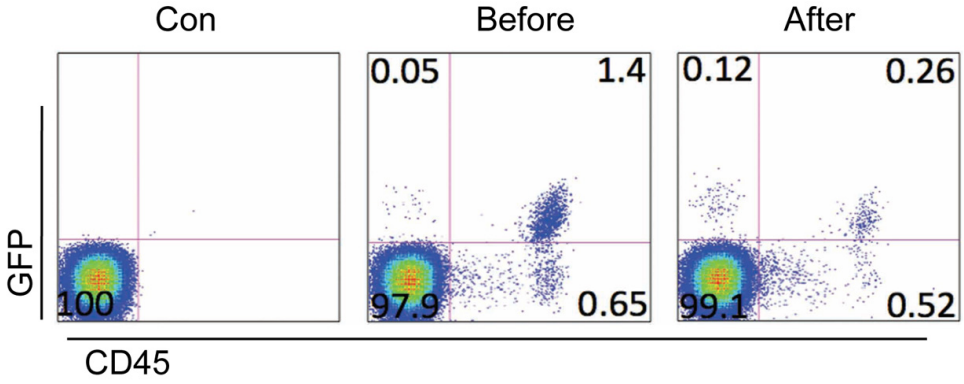

Figure 4.29. Purity of Vav-MEF after MACS selection.

Vav-MEF were purified by negative selection with CD45 MicroBeads. Unstained CD1 MEF were used as control. Purified Vav-MEF were analyzed by flow cytometry as $CD45^{-}GFP^{-}$ cells.

Numbers depict the percentage of corresponding populations. Con: control. Before: purity of Vav-MEF before MACS selection. After: purity of Vav-MEF after MACS selection.

4.6.3 Transdifferentiation MACS selected Vav-MEF to hematopoietic cells

To generate hematopoietic cells, MACS purified Vav-MEF were infected with retrovirus expressing Oct4 or Oct4 plus Bmi1 in the presence of PB (8 μg/ml). Infected Vav-MEF were cultured in complete HSC medium. At about day 23 after infection, non-adherent cells emerged from both Oct4 and Oct4 plus Bmi1 infected Vav-MEF (Figure 4.30).

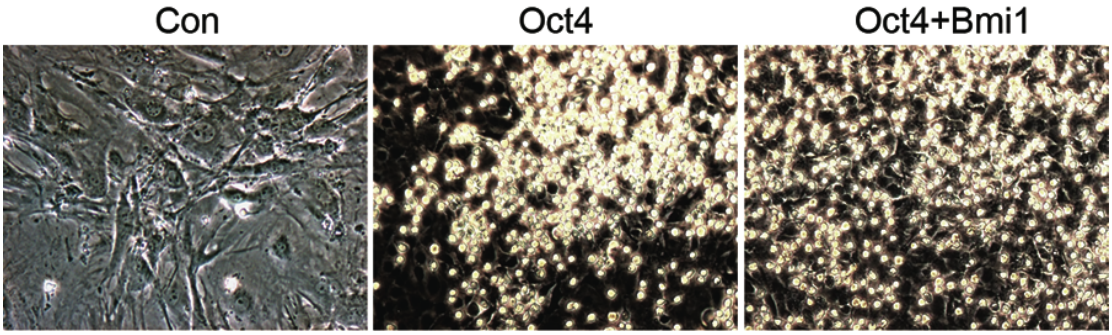

Figure 4.30. Emergence of non-adherent cells from retrovirus infected Vav-MEF.

Non-adherent cells emerged at about day 23 after infection. Images were taken at day 25 after infection. Con: Vav-MEF cultured in complete HSC medium without retrovirus infection.

The non-adherent cells emerging from Oct4 plus Bmi1 infected Vav-MEF were 3 days ahead in time compared to Oct4 infected Vav-MEF. The non-adherent cells emerged as clones and kept proliferating for several days. At about day 27 after infection, non-adherent cells seemed to stop proliferating and the quantity of non-adherent cells decreased. Then at day 29 after infection, these non-adherent cells were collected by gently washing with PBS for several times and stained the cells with APC-Cy7-conjugated CD45 and Pacific Blue-conjugated F4/80 antibodies. The remaining adherent cells were kept in culture with complete HSC medium.

Compared to control MEF, there were obvious two populations of non-adherent cells from Oct4 and Oct4 plus Bmi1 infected Vav-MEF. The two populations were gated separately and analyzed for the expression of CD45, F4/80 (macrophage marker) and GFP. In population I, most of the non-adherent cells were $CD45^+$ cells (Oct4: 98.5% vs Oct4+Bmi1: 99.1%), while only part of the cells expressed GFP (Oct4: 36.7% vs Oct4+Bmi1: 19.6%). These data do not fit the tested blood samples from heterozygote mice, in which about 95% $CD45^+$ cells were GFP^+ (Figure 4.27A). This is probably because microscopy was used for selection of homozygote embryos for Vav-MEF

preparation. Due to autofluorescence by microscopy, it might be difficult to guarantee only homozygote embryos were selected and this might cause that only some $CD45^+$cells were also GFP^+. There was almost no expression of F4/80 gene in population I (Figure 4.31).

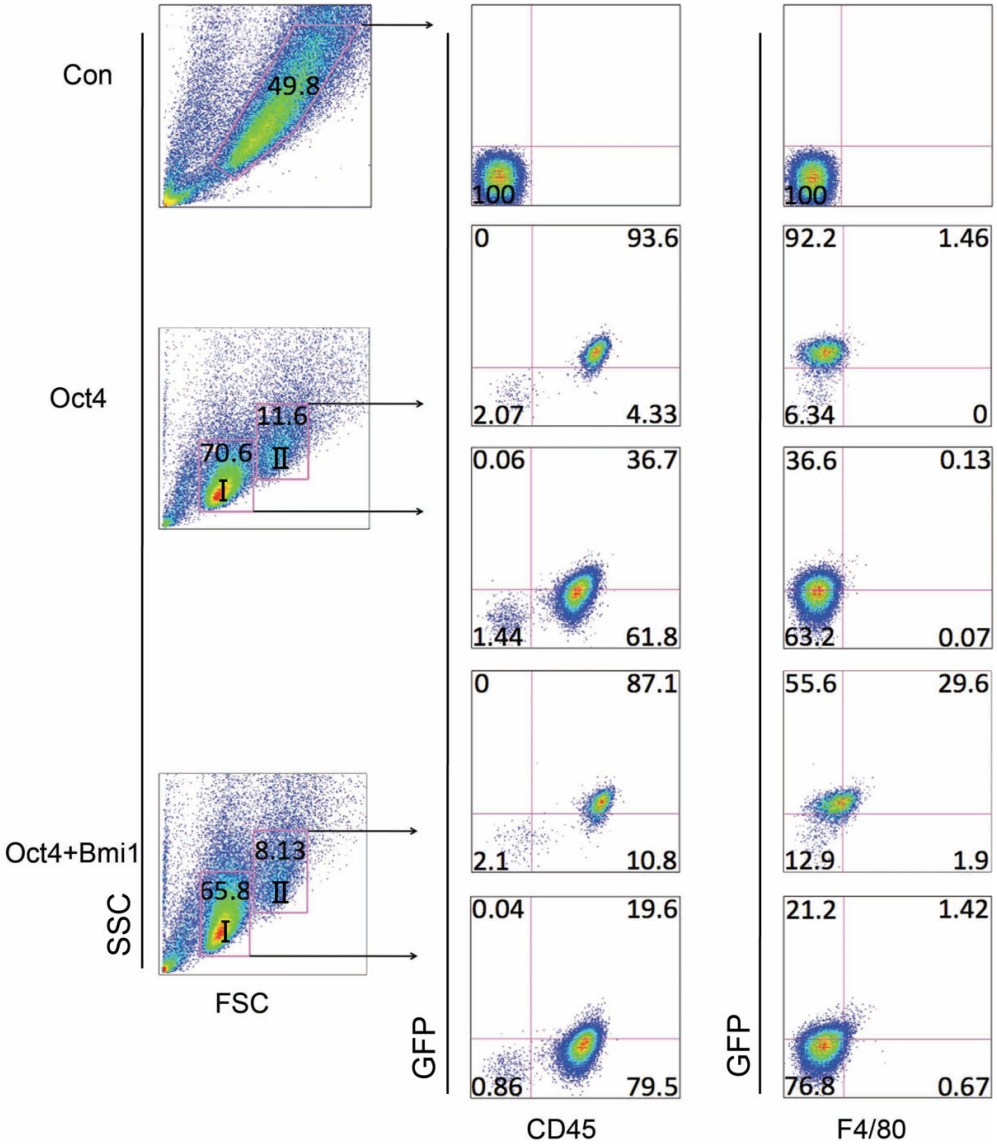

Figure 4.31. Generation of hematopoietic cells from Vav-MEF.

Non-adherent cells were collected at day 29 after infection and stained with APC-conjugated-CD45 and Pacific Blue-conjugated-F4/80 antibodies. Expression of CD45, F4/80 and GFP was determined by flow cytometry. Numbers depict the percentage of corresponding populations. I, II represent two different populations in non-adherent cells. Con: control. FSC: Forward Scatter. SSC: Side Scatter.

In population II, most of the cells expressed both CD45 (Oct4: 98% vs Oct4+Bmi1: 98%) and GFP (Oct4: 93.6% vs Oct4+Bmi1: 87%), only really small part of the cells were $CD45^-GFP^-$ (about 2%). There was still almost no expression of F4/80 gene in Oct4

infected Vav-MEF (1.5%). However, for Oct4 plus Bmi1 infected Vav-MEF, much more macrophages were found (31%; Figure 4.31).

However, after several days culturing of remaining adherent cells with complete HSC medium, no non-adherent cells emerged anymore. Then after 36 days of infection, these adherent cells were trypsinized, stained with CD45 and F4/80 antibodies and analyzed by flow cytometry. These remaining adherent cells, even cultured in complete HSC medium, failed to be converted into hematopoietic cells. They did not express CD45 and F4/80, and also no GFP^+ cells emerged (Figure 4.32). Thus, MACS purified Vav-MEF can be transdifferentiated into hematopoietic cells by infection with retrovirus expressing Oct4 alone or Oct4 plus Bmi1.

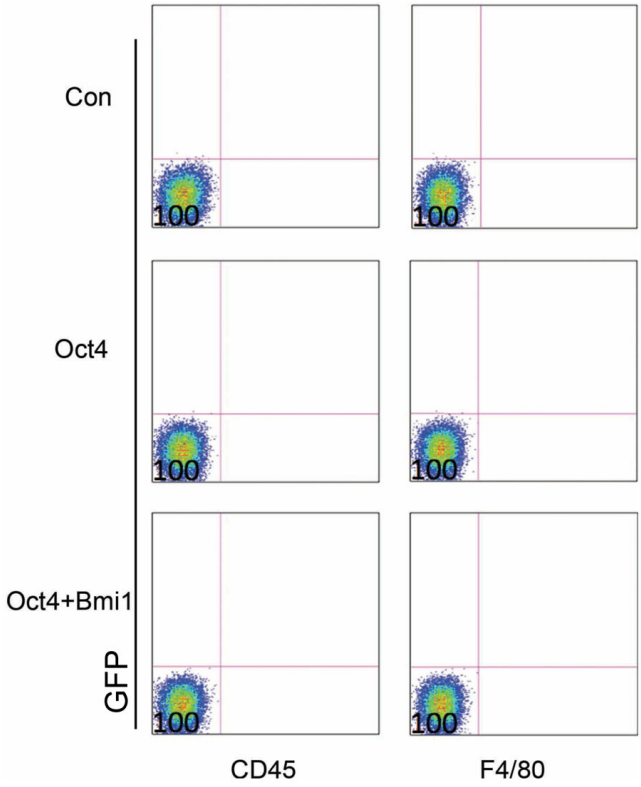

Figure 4.32. Expression of hematopoietic cell markers in remaining adherent cells.

Remaining adherent cells were trypsinized to single cells 36 days after infection. These cells were stained with APC-conjugated-CD45 and Pacific Blue-conjugated-F4/80 and analyzed by flow cytometry with the expression of CD45, F4/80 and GFP. Numbers depict the percentage of corresponding populations.

4.6.4 Vav-MEF preparation by FACS sorting

For MACS purified Vav-MEF, there were still about 1% of $CD45^+$ and/or GFP^+ cells left before infection with retrovirus expressing Oct4 and Oct4 plus Bmi1. This small part of

potential hematopoietic cells might serve as a source of hematopoietic cells and keep proliferating during *in vitro* culture in complete HSC medium. To further purify Vav-MEF and avoid possible contamination from remaining hematopoietic cells, $CD45^{-}GFP^{-}$ Vav-MEF were selected by FACS. To ensure Vav-MEF were obtained from Vav-iCre/Rosa26R-$fGFP^{+/+}$ embryos, Vav-MEF were isolated separately from each embryos and homozygote Vav-MEF were selected according to genotyping results. Vav-MEF were isolated from day 13.5 pregnant homozygote mice as aforementioned (Methods 3.2) and sorted for $CD45^{-}GFP^{-}$ cells. Desired Vav-MEF ($CD45^{-}GFP^{-}$) were gated strictly to avoid potential contamination of hematopoietic cells. Primary Vav-MEF were heterogeneous and did include $CD45^{+}$ and/or GFP^{+} cells (about 4.4%; Figure 4.33A). After FACS sorting, Vav-MEF were kept in culture to let them recover from the sorting stress. Purity of Vav-MEF was immediately tested by flow cytometry. Before FACS sorting, the purity of Vav-MEF ($CD45^{-}GFP^{-}$) was about 98%, and the purity of Vav-MEF increased to 99.8% after FACS sorting (Figure 4.33B).

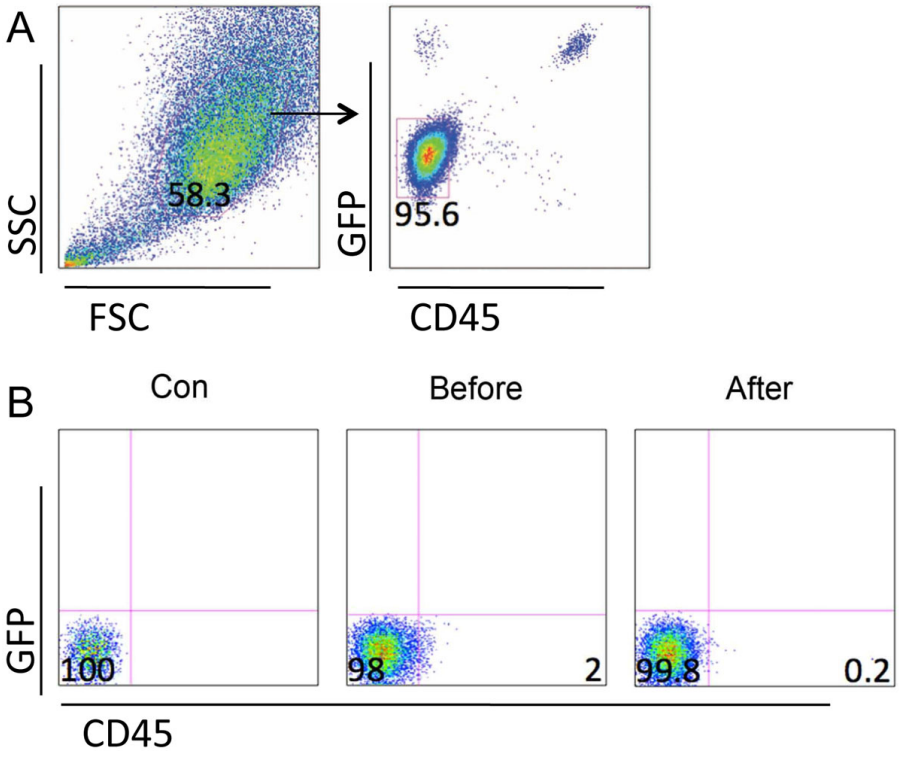

Figure 4.33. Purity of FACS purified Vav-MEF.

A: FACS selection of purified Vav-MEF as $CD45^{-}GFP^{-}$ cells. Numbers depict the percentage of corresponding populations. FSC: Forward Scatter. SSC: Side Scatter.
B: Purity of Vav-MEF was analyzed by flow cytometry. Purified Vav-MEF were referred to as $CD45^{-}GFP^{-}$ cells. Unstained CD1 MEF were used as control. Con: Control. Before: Purity of Vav-

MEF before sorting. After: Purity of Vav-MEF after sorting. Numbers depict the percentage of corresponding populations.

FACS sorting purified Vav-MEF were infected with retroviral vectors expressing Oct4 and Oct4 plus Bmi1. Infected cells were cultured in the same condition as MACS purified Vav-MEF (see 4.6.3). However, after 30 days of culture with complete HSC medium, there were still no non-adherent cells emerged as MACS purified Vav-MEF and FACS sorting purified Vav-MEF became apoptotic. Therefore, FACS sorting purified Vav-MEF failed to be transdifferentiated into hematopoietic cells after infection with retrovirus expressing Oct4 and Oct4 plus Bmi1 in complete HSC medium.

In summary, MACS purified Vav-MEF were successfully transdifferentiated into hematopoietic cells by retrovirus expressing Oct4 and Oct4 plus Bmi1. However, FACS sorting purified Vav-MEF failed to be transdifferentiated into hematopoietic cells in the same conditions. It is possible that transdifferentiated hematopoietic cells from MACS purified Vav-MEF might come from the proliferation of residual hematopoietic cells (1% $CD45^+$ and/or GFP^+ cells). While residual hematopoietic cells from FACS sorted Vav-MEF were only 0.2% ($CD45^+$ and/or GFP^+ cells), which might explain the different results from the same infection. Therefore, Oct4 or Oct4 plus Bmi1 over-expression in Vav-MEF dose not seem to possess the potential to transdifferentiate Vav-MEF into hematopoietic cells.

5 DISCUSSION

iPS technology represents a new reprogramming method that directly induce somatic cells into a pluripotent state by resetting the somatic cell epigenetic state. During this reprogramming process, somatic gene expression is switched off and at the same time, the pluripotency program is activated, which further progresses into a stable pluripotent state. However, somatic gene expression is not completely inactivated in the early stage of pluripotency. Incomplete inactivation of somatic gene expression results in somatic memory, which will in turn affect the characteristics and functions of resulting iPS cells. In view of somatic memory, choosing the appropriate starting cells for cellular reprogramming is critical for further applications of iPS cells.

The first part of this thesis focuses on immunogenicity of iPS cells derived from immune-privileged Sertoli cells. This study demonstrates that Ser-iPS cells exhibit low immunogenicity both *in vivo* and *in vitro*. Ser-iPS cells formed teratomas at a higher frequency compared to MEF-iPS cells upon syngeneic transplantation into B6 mice. Moreover, Ser-iPS cell teratomas exhibited less T cell, B cell and DC infiltration, and tissue damage and necrosis compared to those of MEF-iPS cells. Furthermore, EBs formed by Ser-iPS cells showed lower T cell stimulation potential in *in vitro* co-culture experiment. Thus, Ser-iPS cells show reduced immunogenicity and this may be due to some residual somatic memory, originating from immune-privileged Sertoli cells used for reprogramming.

Transdifferentiation is another promising method to reprogram one cell type directly into another. It avoids potential tumorigenesis risk of iPS cells, because transdifferentiation bypasses the pluripotent stage during reprogramming. The second part of this thesis focuses on transdifferentiation Vav-MEF into hematopoietic cells. In this study, Vav-MEF were used that allow monitoring of hematopoietic cells, which emerge after transdifferentiation. Both MACS and FACS purified Vav-MEF were used for transdifferentiation into hematopoietic cells. MACS selected Vav-MEF yielded hematopoietic cells by infection with retrovirus expressing Oct4 and Oct4 plus Bmi1, which might be due to some residual hematopoietic cells in the starting cell population. Hematopoietic cells failed to emerge after transfection of FACS purified Vav-MEF with the same transcription factors. Thus, Oct4 and Oct4 plus Bmi1 transcriptional factors do not seem to have the potential to transdifferentiate Vav-MEF into hematopoietic cells.

5.1 Reduced immunogenicity of Ser-iPS cells

Autologous iPS cells are an attractive cell source for personalized regenerative medicine, yet their immunogenicity is still a great challenge for their future applications. Previous studies showed the immunogenicity of iPS cells by transplantation them into syngeneic hosts (Zhao et al., 2011). However, the immunogenicity of iPS cells and iPS cell-derived cells has remained highly controversial (Araki et al., 2013; Guha et al., 2013; de Almeida et al., 2014). As cells of origin impact on the properties of iPS cells, using less immunogenic cells for iPS cell generation might translate into a low immunogenicity of obtained iPS cells (Boyd et al., 2012; Liu et al., 2013). Here immune-privileged Sertoli cells were used to generate iPS cells and Ser-iPS cells did possess reduced immunogenicity compared to the corresponding MEF-iPS cells.

5.1.1 Teratoma formation of Ser-iPS cells

Many factors might influence the immunogenicity of *in vitro* differentiated cells in transplantation, such as *in vitro* culture conditions (Scheiner et al., 2014), cell types for transplantation (Cao et al., 2014) and transplantation sites (Scheiner et al., 2014). *In vivo* immunogenicity of Ser-iPS cells was examined by teratoma assay. Teratoma assay is frequently used to evaluate the immunogenicity of pluripotent stem cells and their derivatives *in vivo* (Koch et al., 2008; Zhao et al., 2011). Teratoma harbors a variety of differentiated cell types similar to the normal derivatives of all three germ layers (Tapper and Lack 1983). Thus, teratoma provides an useful model for simultaneously assessing the immunogenicity of various differentiated cell types from iPS cells (Boyd et al., 2012; Zhao et al., 2011). In this assay, tumor formation indicates that the recipient's immune system fails to reject tumor-forming cells (Dressel 2011).

Here, we found that Ser-iPS cells have a much stronger capacity to regulate the recipient's immune response compare to MEF-iPS cells. This immune regulatory ability allows Ser-iPS cells to form teratomas at high frequency. Thus, Ser-iPS cells possess low immunogenicity in teratoma formation assays in *in vivo*. Matrigel, which contains numerous active growth factors, can increase teratoma formation efficiency of both mouse and human ES cells (Lawrenz et al., 2004; Prokhorova et al., 2009). Therefore, matrigel and DMEM mixture (1:4) was used to suspend iPS cells and ES cells for teratoma assay.

5.1.2 Immune responses induced by Ser-iPS cells *in vivo*

Ser-iPS cells formed teratomas at a high efficiency upon syngeneic transplantation, which is first evidence of their reduced immunogenicity. Furthermore, Ser-iPS cells appear to prevent efficient infiltration of host derived immune cells into teratomas, such as T cell, B cell and DC. Low T cell infiltration in Ser-iPS cell teratomas was confirmed by both immunohistochemical staining and qRT-PCR. Such low T cell infiltration is consistent with less tissue damage and necrosis in Ser-iPS cell teratomas, since tissue destruction goes along with infiltration of activated T cells (Boyd et al., 2012; Zhao et al., 2011). DC, as robust antigen-presenting cells, presents exogenous antigens to recipient T cells either by direct or indirect pathway (Kadereit and Trounson 2011). Such antigen-presenting pathways correspond to respective T cell rejection patterns (direct and indirect T cell rejection, Kadereit and Trounson 2011; Liu et al., 2013). iPS cells did not directly differentiate into DC (Robertson et al., 2007), and thus antigen recognition in teratoma assay is most likely through indirect pathways, where donor antigens are recognized by recipient DC, and further presented to recipient T cells, which results in immune activation (Kadereit and Trounson 2011).

5.1.3 Immune responses induced by Ser-iPS cells *in vitro*

In vivo immune responses are complex processes, which involve multiple interactions between different immune cell types, including T cell, B cell and DC. The important role of CD4 T cells during immune responses has been reported (Drukker et al., 2006; Swijnenburg et al., 2008). *In vitro* co-culture is commonly used to investigate possible immune responses induced by stimulator cells. Thus, further insights of Ser-iPS cell immunogenicity were obtained by *in vitro* co-culture of CD4 T cells with undifferentiated and EBs of Ser-iPS cells. EB assay represents the *in vitro* equivalent of teratoma formation *in vivo*. EBs of Ser-iPS cells exhibited less potential in stimulating T cell proliferation compared to that of MEF-iPS cells. However, both Ser-iPS cells and MEF-iPS cells in their undifferentiated state showed T cell response similar to ES cells. Thus, the immunogenicity of iPS cells was found to be related to the somatic cells used for reprogramming and to the differentiated state.

Previous studies indicated that the immune-privileged function of sertoli cells includes the generation of Tregs (Dal Secco et al., 2008; Doyle et al., 2012). Therefore, whether Ser-iPS cell immunogenicity was related to the generation of Tregs was investigated here. Ser-iPS cells showed a Treg profile similar to MEF-iPS cells and ES cells in co-culture experiments. Results obtained here also confirmed Treg generation function of Sertoli cells (Dal Secco et al., 2008). Since there is a difference in *in vivo* and *in vitro*

environments, it is also worth investigating the frequency of Tregs in teratomas. Foxp3 expression in teratomas from Ser-iPS cells and MEF-iPS cells was found to be comparable. Thus, the reduced immunogenicity of Ser-iPS cells *in vitro* and *in vivo* appears to be unrelated to the Treg profile.

5.1.4 Possible molecules involve in immunogenicity of pluripotent stem cells

Considering the immunogenicity of iPS cells, syngeneic transplantation was used and several genes were identified during immune rejection (Zhao et al., 2011). Hormad1 and Zg16 genes, which were expressed in regressing iPS cell teratomas but not in ES cell teratomas, were reported to contribute to iPS cell immunogenicity (Zhao et al., 2011). However, this observation is still highly controversial and was not confirmed in follow up studies (Araki et al., 2013; Guha et al., 2013). This observation is also in contrast to the result reported here. A correlation between Hormad1 and Zg16 expression and iPS cell immunogenicity was not observed here. Hormad1, also called cancer/testis 46, involves in chromatin binding and highly expresses in testis. It has been identified as a tumor antigen (Chen et al., 2005). Zg16 is highly expressed in pancreas and down-regulated upon injury (Neuschwander-Tetri et al., 2004). In line with Arakia et al. and Guha et al. results, no obviously different expression of Hormad1 gene was observed between teratomas from Ser-iPS cells and MEF-iPS cells. Unexpectedly, much higher Zg16 expression in Ser-iPS cell teratomas was found, which correlate inversely with the less immunogenicity of Ser-iPS cells, according to the results from Zhao et al.. Due to the elusive roles of Zg16 during teratoma formation, the exact roles of Zg16 during this immune response can not be explained at present. Thus, the level of Hormad1 and Zg16 expression appears not to be related to the regression of teratomas and seems not to be important for the immunogenicity of iPS cells.

Previous studies showed that ES cells possessed low immunogenicity compare to other somatic cells. Low expression of MHC class I, MHC class II and costimulatory molecules (CD80 and CD86) on ES cells partly contribute to their low immunogenicity and result in immune tolerance (Magliocca et al., 2006). Here the expression of MHC class I, MHC class II and costimulatory molecules (CD80 and CD86) was similar between Ser-iPS cells and MEF-iPS cells both in undifferentiated cells or day 14 differentiated EBs, as well as in corresponding ES cells. Therefore, the expression of MHC class I, MHC class II and costimulatory molecules seems to be unrelated to the reduced immunogenicity of Ser-iPS cells.

Further to the aforementioned genes Hormad1 and Zg16, other genes might be implicate in the immune reponses and were investigated. The anti-inflammatory cytokines IL-10 and cell membrane protein ICAM1 were expressed at lower levels in teratomas from Ser-iPS cells. IL-10 is an important immune-modulatory molecule and can suppress the production of pro-inflammatory cytokines, costimulatory molecules and antigen presenting. The primary function of IL-10 appears to limit and terminate inflammatory responses (Moore et al., 2001). Thus, IL-10 seems to serve as an immune suppressive cytokine. Unexpectedly, IL-10 gene was expressed at lower levels in Ser-iPS cell teratomas as compared to MEF-iPS cells, which correlates inversely to reduced immunogenicity of Ser-iPS cells. However, the expression pattern of IL-10 gene is consistent with a previous study (Robertson et al., 2007), where IL-10 seems more related to tissue destruction. On the other hand, IL-10 is a responsive cytokine, and responses to pro-inflammatory stimuli (Li et al., 2012). Thus, it is possible that due to the higher immune responses during MEF-iPS cell transplantation, IL-10 might accelerate its production to blunt pro-inflammatory stimuli.

ICAM1 is responsible for regulating migration and adherence of lymphocytes towards inflammatory sites. Increased expression of ICAM1 has been reported to associate with the activation of lymphocytes and maturation of macrophage-like cells (Dustin et al., 1986). Thus, the significantly higher expression of ICAM1 in MEF-iPS cell teratomas indicates a more severe immune responses upon syngeneic transplantation compared to Ser-iPS cells. Further to IL-10 and ICAM1 genes, other immune-related genes might be involved in this immune response, which warrants further investigation.

5.1.5 Possible somatic memory retained in Ser-iPS cells

The existence of somatic memory in iPS cells has been reported previously (Marchetto et al., 2009; Ghosh et al., 2010). Somatic memory refers to some remnants of the epigenetic profile from the cell type used for reprogramming (Kim et al., 2010; Ohi et al., 2011; Polo et al., 2010). This somatic memory influences the characteristics of iPS cells, such as iPS cells preferentially differentiate into cell lineage related to the cells used for reprogramming over unrelated lineages (Polo et al., 2010; Bar-Nur et al., 2011; Pfaff et al., 2012). However, the preferential re-differentiation of Ser-iPS cells into Sertoli cells was not observed here, based on the expresson of ABP, AMH and Sox9 in both Ser-iPS cell EBs and teratomas. Sox9 is continuously expressed in Sertoli cells (including pre-Sertoli cells), which is upregulated by Sry activity, and in turn upregulates other genes related to proper differentiation of Sertoli cells (Kashimada and Koopman 2010). AMH is a member of the transforming growth factor-β family and a target of Sox9. AMH is

expressed in early Sertoli cells and in charge of regression of Müllerian duct (Arango et al., 1999). ABP is produced by Sertoli cells and is a biological marker of Sertoli cell function (Hagenas et al., 1975). Thus, Ser-iPS cells do not have the propensity to differentiate into the cell lineage of their origin.

Immune protective molecules produced by Sertoli cells are the mainly contribution for the immune-privileged function of Sertoli cells (Doyle et al., 2012). These immune protective factors probably still preserved in Ser-iPS cells as somatic memory (Bar-Nur et al., 2011; Kim et al., 2010; Ohi et al., 2011; Polo et al., 2010). Arginase 1, which has an effect on urea cycle, hydrolyzes L-arginine into L-ornithine and urea (Guo et al., 2007). Arginase has been reported to be higly expressed in Sertoli cells (Guo et al., 2007). Indeed, higher arginase 1 expression was observed in Sertoli cells in my study. Interestingly, much higher arginase 1 expression in EBs of Ser-iPS cell was found compared to that of MEF-iPS cells, but not in teratomas of Ser-iPS cells and MEF-iPS cells. Consistently, the expression of CD3-ζ chain, the only known gene regulated by arginase 1, was no difference in iPS cell and ES cell teratomas. The differential expression pattern of arginase 1 between iPS cell EBs and teratomas might be due to the complex cell types in teratomas. Such teratomas contained infiltrated host macrophages that also express arginase (Chen and Broome 1980). Thus, it is of interest to examine the expression of CD3-ζ chain in T cells after co-culture with EBs of Ser-iPS cell, due to the higher expression of arginase 1 in Ser-iPS cell EBs.

PGE2, a pro-inflammatory factor with immune-suppressive activity, has been reported to suppress both innate and antigen-specific immune responses (Harris et al., 2002). PGE2 expression inhibited T cell proliferation, enhanced T cell apoptosis and improved Th 2 (protective) response while controlling the activation of CTL and NK cell mediated Th 1 (destructive) immunity (Kalinski 2012). Thus, high expression of PGE2 in Ser-iPS cell teratomas, which might originate from Sertoli cells, could possibly contribute to the reduced immunogenicity of Ser-iPS cells.

Sertoli cells provide an immune-privileged environment for developing germ cells partialy due to their ability to convert Th 1 (destructive) immune response to Th 2 immune response (Doyle et al., 2012), which more relates to immune modulation and tolerogenic (Meinhardt and Hedger 2011). However, the expression of IFNγ and IL-4, two genes representative for Th 1 and Th 2 immune response respectively, was similar in teratomas from Ser-iPS cells and MEF-iPS cells. Thus, the reduced immunogenicity of Ser-iPS

cells *in vivo* appears to be unrelated to Sertoli cell immune function that favors Th 2 immune response.

The mechanisms involve in Sertoli cell immune function are complicated and comprise further factors, such as multiple cytokines, chemokines, anti-inflammatory modulators, complement activation inhibitors and adhesion molecules (Doyle et al., 2012). Sertoli cells' complex immune mechanism coupled with the potentionally preserved somatic memory in Ser-iPS cells make the mechanisms of Ser-iPS cell reduced immunogenicity even more complicated. The reduced immunogenicity of Ser-iPS cells is probably due to the interaction of multiple factors. Further investigation is warranted to elucidate the mechanisms involved in Sertoli cell immune function and how they contribute to the reduced immunogenicity of Ser-iPS cells.

The reduced immunogenicity of Ser-iPS cells was observed in early-passage (p9-15) iPS cells. However, late-passage (p35-38) Ser-iPS cells exhibit an immunogenicity similar to the respective MEF-iPS cells in teratoma assay in syngeneic hosts. The low immunogenicity in early-passage Ser-iPS cells and its loss in late-passage Ser-iPS cells appears to be consistent with the presence of somatic memory in iPS cells. Somatic memory impacts on iPS cell properties at early stages (Kim et al., 2010; Ohi et al., 2011; Polo et al., 2010) and is erased upon continuous *in vitro* culture (Chin et al., 2009; Polo et al., 2010), similar to Ser-iPS cell immunogenicity reported here. However, the molecular events occurring during extended periods of culture have remained elusive: whether pluripotent cells get selected and occur in a high frequency or whether the exogenous reprogramming factors get silenced and/or the epigenetic reprogramming continues (Papp and Plath 2011). The exact underlying mechanisms for the loss of Ser-iPS cell reduced immunogenicity during extended passaging have to await further studies.

In summary, the immunogenicity of iPS cells derived from two different somatic cell types, immune-privileged Sertoli cells and MEF, was compared. Both Ser-iPS cells and MEF-iPS cells, and their derivatives showed immunogenicity. Additionally, Ser-iPS cells exhibited reduced immunogenicity compared to MEF-iPS cells, which however got lost upon continuous *in vitro* culture. These findings indicate that somatic cell types have an impact on the immunogenicity of respective iPS cells. Our results reinforce the concept of using immune-privileged somatic cells for iPS cell generation and derivation of differentiated progeny for transplantation.

5.2 Transdifferentiation Vav-MEF to hematopoietic cells

Transdifferentiation, an approach by which differentiated cell can be directly converted into other cell types, opens the perspective for to novel therapeutic strategies. With the increasing understanding of developmental biology, direct transdifferentiation by overexpression of critical transcriptional factors *in vitro* or by engineered cell transplantation *in vivo* has already been achieved (Islas et al., 2012; Yang et al., 2013; Rouaux and Arlotta 2013; Torper et al., 2013). Hematopoietic cells, which are normally difficult to differentiate from pluripotent stem cells *in vitro*, have also been obtained by infecting fibroblasts with different transcriptional factors (Szabo et al., 2010; Pereira et al., 2013). Several important molecules are involved in hematopoiesis. Bmi1, which belongs to polycomb repression complex 1, plays a vital role in HSC self-renewal and hematopoietic cell differentiation from ES cells *in vitro* (Iwama et al., 2004; Rizo et al., 2008; Ding et al., 2012).

Unexpectedly, only MACS purified Vav-MEF ($CD45^-GFP^-$, purity: 99.1%) were transdifferentiated into hematopoietic cells, while FACS sorting selected Vav-MEF ($CD45^-GFP^-$, purity: 99.8%) failed to be transdifferentiated. Considering the entirely different results from the same transfection method, starting cells might be one of the reasons for this result. First, the purity of Vav-MEF selected by MACS and FACS sorting was different. The purity of Vav-MEF ($CD45^-GFP^-$) after MACS selection was 99.1%, which means an about 1% possible contamination with hematopoietic cells ($CD45^+$ and/or GFP^+); for Vav-MEF after FACS sorting, the possible residual hematopoietic cells were only about 0.2%. Thus, the hematopoietic cells obtained from MACS selected Vav-MEF are probably the result of proliferation of residual hematopoietic cells in the starting cell population. Second, the purification methods are different. Vav-MEF with high purity were obtained by FACS sorting, however FACS sorting exerts more stress on cells compared to MACS, such as lower growth rate and higher mortality of cells after FACS sorting. This might also lead to the failure of transdifferentiation FACS sorting purified Vav-MEF into hematopoietic cells.

In summary, successful transdifferentiation Vav-MEF into hematopoietic cells was not observed here as reported (Szabo et al., 2010). There are differences in preparation methods and purity of the starting cells, and also in the species used. Szabo and colleagues used human fibroblasts, while Vav-MEF were chosen as starting cells here. It is very well possible that the functions of same genes potentially vary between different species (human versus mouse).

5.3 Future perspectives

With the development of iPS technique, significant discoveries have been achieved in this field. iPS cells, which resemble ES cells, provide an attractive and renewable cell source for degenerative disease treatment. However, many questions about iPS cells still remain and should be addressed before their clinical application. For example, what are the exact mechanisms for iPS generation; which transfection method is appropriate to generate reliable iPS cells, such as viral vectors, RNA and protein; how to enhance the efficiency and purity for generating differentiated cells from iPS cells. Although many questions should to be addressed, iPS cells, which are a potential replacement of ES cells, remain a promising tool for future application. Recently, iPS cells could be obtained by reprogramming *in vivo* and resulting iPS cells showed totipotency characteristics (Abad et al., 2013). Thus, *in vivo* production of iPS cells might represent a new method to generate iPS cell-derived cells for replacement therapy.

iPS cells were generated from various somatic cell types, which in turn influence the properties of respective iPS cells through epigenetic mechanisms (Polo et al., 2010). Somatic memory in iPS cells leads to (i) DNA methylation according to parental somatic cells, (ii) preferential re-differentiation into cell lineages used for reprogramming and (iii) loss of somatic memory upon extended periods of *in vitro* culture (Bar-Nur et al., 2011; Kim et al., 2010; Ohi et al., 2011; Polo et al., 2010). As the origin of cells impact on the resulting iPS cells, choosing appropriate starting cells plays a critical role for subsequent application of iPS cells. As reported, neural progenitors from less immunogenic umbilical cord mesenchymal cell derived iPS cells exhibited lower immunogenicity compared to those from skin fibroblasts derived iPS cells (Liu et al., 2013). Reduced immunogenicity of iPS cells derived from immune-privileged Sertoli cells has been observed here. Thus, for generating desired differentiated cells from iPS cells for transplantation, somatic cells derived from immune-privileged sites, such as Sertoli cells, or somatic cells with low immunogenicity, such as cord blood, provide better cell sources. Furthermore, it is of interest to investigate the immunogenicity of specific differentiated cells from immune-privileged cell derived iPS cells (like Ser-iPS cells). Thus, the low immunogenicity of iPS cell derived cells exploiting somatic memory holds new promise for regenerative medicine.

Moreover, most of the studies focus on mouse iPS cells, the immunological activities of human iPS cells have not been systematically tested. Considering the time and cost that required for iPS cells generation from individual patient, several groups are trying to

establish iPS cell line banks that stock clinical-grade and human leukocyte antigen (HLA) matching iPS cell lines (Taylor et al., 2012; Kaneko and Yamanaka 2013). These HLA matched iPS cells will be used for future treatment of diseases. However, to what extent HLA matched iPS cells will avoid immune rejection and to what extent other factors (like culture conditions or miHA) will influence the immunogenicity of iPS cells remain elusive. So, it will be extremely important to select less immunogenic iPS clones or their differentiated derivaties as universal donors and monitor the immune responses after transplantation.

Transdifferentiation provides a rapid way to generate novel cell types and functional tissues. It enables the generation of specific cell types and avoids potential tumorigenesis of iPS technology. Several groups focus on transdifferentiation into neural cells, including neural stem cells or differentiated neural cells, with different combination of transcriptional factors (Ambasudhan et al., 2011; Han et al., 2012; Pfisterer et al., 2011; Thier et al., 2012; Vierbuchen et al., 2010). However, transdifferentiation into specific cell types, like hematopoietic cells, remains a big challenge.

Given the advantages of transdifferentiation technology, many questions still need to be addressed. For example, first, whether transdifferentiated cells, similar to iPS cells, inherit somatic memory from their original cells needs to be investigated. Second, more work should be done with human cells, as successful transdifferentiation in mouse cells might be not work equally for human cells. Third, transfection methods play also an important role for subsequent application of the cells obtained, as observed in iPS cells. The last but not the least is that of immunogenicity of obtained transdifferentiated cells. Analogous to immunogenicity of syngeneic iPS cells, the question is on the immunogenicity of syngeneic transplanted transdifferentiated cells, which warrants further investigations.

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7 APPENDIX

7.1 Conditions for genotyping of Vav-iCre/Rosa26R-fGFP mice

Conditions for genotyping of Vav-iCre mice

Cre for: 5'-GCCTGCCCTCCCTGTGGATGCCACCT-3'

Cre rev: 5'-GTGGCAGAAGGGGCAGCCACACCATT-3'

PCR reaction mix

10x Taq Pol buffer $(NH_4)_2SO_4$ with $MgCl_2$	5 µl
dNTP (10 mM each)	1 µl
Recombinant Taq DNA Polymerase	2 µl
for primer	1 µl
rev primer	1 µl
DNA sample	2 µl
Total volume	50 µl

Reaction scheme

Initiation	95°C	4 min	
Denaturation	95°C	30 sec	36×
Annealing	65°C	45 sec	
Extension	72°C	30 sec	
Final extension	72°C	10 min	
Termination	10°C	∞	

Conditions for genotyping of Rosa26R-fGFP mice

Rosa26 for: CACTTGCTCTCCCAAAGTCG

Rosa26 rev: TAGTCTAACTCGCGACACTG

CAG: GTTATGTAACGCGGAACTCC

PCR reaction mix

10x Taq Pol buffer $(NH_4)_2SO_4$	2 µl
25 mM $MgCl_2$	2 µl
dNTP (10 mM each)	0.4 µl
Recombinant Taq DNA Polymerase	0.2 µl
Rosa for primer	1.28 µl
Rosa rev primer	0.64 µl
CAG primer	0.64 µl
DNA sample	1 µl
H_2O	11.84 µl
Total volume	20 µl

Reaction scheme

Initiation	96°C	3 min	
Denaturation	96°C	30 sec	30×
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
Termination	4°C	∞	

7.2 Table of figures

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DECLARATION OF AUTHOR

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged and has not been submitted, either in part or whole, for a degree at this or any other university.

Aachen, October 22, 2014

Xiaoying Wang

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig und unter ausschließlicher Verwendung der angegebenen Literatur und Hilfsmittel erstellt habe. Die Arbeit wurde in gleicher oder ähnlicher Form bisher weder einer anderen Prüfungsbehörde vorgelegt noch veröffentlicht.

Aachen, Oktober 22, 2014

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