Fate of Induced Pluripotent Stem Cells Following Tranplantation to Murine Seminiferous Tubules

vorgelegt von
Dipl.-Ing.
Jens Durruthy-Durruthy
aus Guantánamo, Kuba

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Promotionsausschuss:

Vorsitzender: Prof. Dr. Leif Garbe
Gutachter: Prof. Dr. Roland Lauster
Gutachter: Prof. Dr. Jens Kurreck


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"Seeds of great discoveries are constantly floating around us, but they only take root in minds well prepared to receive them."

Joseph Henry
The experimental part of this study was performed at

Stanford University School of Medicine
Institute for Stem Cell Biology & Regenerative Medicine
Lorry Lokey Stem Cell Research Building
265 Campus Drive, Rm 3015
Stanford, CA 94305, USA

under the direction of

Prof. Renee Reijo Pera
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Date: 
Studies of human germ cell development are limited in large part due to inaccessibility of germ cells during development. Moreover, although several studies have reported differentiation of mouse and human germ cells from pluripotent stem cells in vitro, differentiation of human germ cells from pluripotent stem cells in vivo has not been reported.

Here, we investigate in preliminary studies dynamic gene expression changes of recently discovered novel genes during nuclear cell reprogramming on a single cell level. We then test whether mRNA reprogramming in combination with xeno-transplantation may provide a viable system to probe the genetics of human germ cell development via use of induced pluripotent stem (iPS) cells. For this purpose, we derived integration-free iPS cells via mRNA-based reprogramming with OCT3/4, SOX2, KLF4 and cMYC alone (OSKM) or in combination with the germ cell specific mRNA, VASA (OSKMV). All iPS cell lines met classic criteria of pluripotency. Moreover, global gene expression profiling did not distinguish between undifferentiated OSKM and OSKMV iPS cells and only subtle differences were observed in expression of germ cell specific genes, epigenetic profiles and in vitro differentiation studies. In contrast, transplantation of undifferentiated iPS cells directly into the seminiferous tubules of germ cell-depleted immunodeficient mice revealed divergent fates of iPS cells produced with different factors. Transplantation resulted in morphologically and immunohistochemically recognizable germ cells in vivo, particularly in the case of OSKMV cells. Significantly, OSKMV cells also did not form tumors while OSKM cells that remained outside the seminiferous tubule proliferated extensively and formed tumors.

Results indicate that mRNA reprogramming in combination with transplantation is a viable strategy for genetic analysis of human germ cell development and may inform studies of fertility restoration in men.


Die Resultate zeigen, dass mRNA Reprogrammierung in Kombination mit Zelltransplantation eine brauchbare Strategie hinsichtlich genetischer Analysen der humanen Keimzellentwicklung ist. Zusätzlich können sie hilfreich für Studien sein, die die Wiederherstellung der Fruchtbarkeit von Männern untersuchen.
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Abbreviations

5mC  5-methylcytidine
AP   Alkaline phosphatase
ARCA Anti reverse cap analog
ATP  Adenosine-5’-triphosphate
BMP  Bone morphogenetic protein
CDS  Coding sequence
CNV  Copy number variant
CTP  Cytidine-5’-triphosphate
DAPI 4’,6-diamidino-2-phenylindole
DI   Deionized
DMEM Dulbecco’s modified Minimal Essential Medium
DNA Deoxyribonucleic acid
DTT  Dithiothreitol
EB   Embryoid body
EC   Embryonal carcinoma
EDTA Ethylenediaminetetraacetic acid
EGC  Embryonic germ cell
ESC  Embryonic stem cell
FBS  Fetal bovine serum
FDA  Food and drug administration
GMP  Good manufacturing practice
GTP  Guanosine-5’-triphosphate
HBA  Human alpha globin
HFF  Human fetal fibroblast
HPAT Human pluripotent associated transcript
<table>
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<td>HUF</td>
<td>Human fibroblast</td>
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<tr>
<td>ICM</td>
<td>Inner cell mass</td>
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<td>IVF</td>
<td><em>in vitro</em> fertilization</td>
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<td>ivT</td>
<td><em>in vitro</em> transcription</td>
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<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
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<tr>
<td>(m)RNA</td>
<td>(messenger) Ribonucleic acid</td>
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<td>MDS</td>
<td>Multi dimensional scaling</td>
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<tr>
<td>NEAA</td>
<td>Non-Essential amino acids</td>
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<td>ORF</td>
<td>Open reading frame</td>
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<td>OSKM(V)</td>
<td>OCT3/4, SOX2, KLF4, cMYC, (VASA)</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PB</td>
<td>piggyPac</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<td>PGC</td>
<td>Primordial germ cell</td>
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<tr>
<td>psi</td>
<td>Pseudouridine</td>
</tr>
<tr>
<td>(R)iPS</td>
<td>(RNA) induced pluripotent stem</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>RPF</td>
<td>Reprogramming factor</td>
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<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<tr>
<td>SCNT</td>
<td>Somatic cell nuclear transfer</td>
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<tr>
<td>SSB</td>
<td>Single-stranded binding</td>
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<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA</td>
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<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
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<tr>
<td>TSS</td>
<td>Transcription start site</td>
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<tr>
<td>UTP</td>
<td>Uridine-5'-triphosphate</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<td>VPA</td>
<td>Valproic acid</td>
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Chapter 1

Introduction

Mammalian development begins with a single cell resulted from the fertilization of a sperm and an oocyte followed by sheer complex developmental processes that have been under thorough investigation in biological research for decades. Modern developmental biology is elucidating those events by both studying cellular lineage specification, a decision making process wherein cells divide to make progeny that are functionally different from another, and by revealing key steps that are crucial during nuclear reprogramming, an event that describes the switch in gene expression of one cell type to that of another unrelated cell type. Understanding the molecular events that take place between fertilization and cell type specification will indeed have a tremendous impact on various related research areas including the induction of pluripotent stem cells, regenerative medicine and cell therapy [1].

1.1 Human embryonic and induced pluripotent stem cells

Stem cells possess two unique properties: the ability to self-renew through mitotic cell division to remain undifferentiated [2] and the ability to differentiate into any specific cell type to form mature tissues. The latter describes the potency of the cell, that is, depending on the developmental stage stem cells can be totipotent (ability to transform into all cell types), pluripotent (most cell types), multipotent (many cell types), oligopotent (few cell types) and unipotent (one cell type) [3]. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst, an early-stage embryo [4]. They give rise to each of the more than 220 cell types and tissues of the organism. Combined with the ability to replicate indefinitely they present a unique tool for biomedical research, developmental biology and regenerative medicine.
1.1.1 Nuclear reprogramming and induced pluripotency

Until the early 1950s [5], [6], [7] the validity of the dogma, that the process of a cell, to differentiate and eventually commit to its fate, is irreversible [8] has not been questioned [1]. It was until 1952, when early studies in frog cloning revealed the first evidence of nuclear reprogramming without altering the gene content postulating the invalidity of the tenet.

Many different strategies have been developed ever since, to induce nuclear reprogramming in differentiated somatic cells and to reset their mature, specialized state [9], a process that has been recognized with the Nobel Prize in 2012. Among these nuclear reprogramming techniques are: i) somatic cell fusion with pluripotent cells [10], [11], [12]; ii) somatic cell nuclear transfer (SCNT) [13], [14]; iii) pluripotent cell extract mediated reprogramming [15] and iv) transduction of exogenous genes into somatic cells [16].

1.1.2 iPS cell technology

The derivation of induced pluripotent stem (iPS) cells from fully differentiated somatic cells via ectopic expression of only a few transcription factors was a major breakthrough discovery in 2006 [17]. The striking resemblance of iPS cells to embryonic stem cells makes them a valuable tool for clinical and biomedical research, with potential applications in drug screening, disease modeling and/or novel cell-based therapeutic strategies [18], [19], [20]. Induction of pluripotency was originally achieved in mouse embryonic fibroblasts with four transcription factors, OCT3/4, SOX2, KLF4 and cMYC, also known as the Yamanaka factors, using retroviral vectors for ectopic gene expression [17]; however, other factors are also known to modulate iPS cell derivation including the addition of NANOG and LIN28 [21], [22]. The iPS cell technology has witnessed dramatic changes over the past seven years with respect to safety (integrative versus non-integrative) and efficiency [23]. Recent developments are summarized in Table 1.1.
Chapter 1. Introduction

1.1.2.1 Integrative methods

Retroviruses and lentiviruses were used to introduce foreign genes into the host genome of cells that were derived towards the very first iPS cell lines [16]. The integration of the Yamanaka factors into the genome leads to the overexpression of these exogenous genes. However, a major drawback of these viral strategies is possible mutations and silencing of indispensable genes and/or the induction of tumorogenicity [46]. It has also been shown that the integrated provirus can alter expression of neighboring host genes [47]. Despite the limitations, a number of developments have been suggested and successfully proven to be viable including drug inducible transgenic systems [26], [27], [36], [48] and the usage of reprogramming cassettes that can also be LoxP-flanked [34], [49] (Tab. 1.1).

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<th>Delivery/Expression method</th>
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<td></td>
<td>Adenovirus</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>Episomal vector</td>
<td>[37]</td>
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<td></td>
<td>Minicircle</td>
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<tr>
<td>Non-integrative, DNA-free</td>
<td>ES protein extract</td>
<td>[39]</td>
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<td></td>
<td>Recombinant proteins</td>
<td>[40], [41]</td>
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<tr>
<td></td>
<td>Sendai vectors</td>
<td>[42]</td>
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<tr>
<td></td>
<td>Chemicals</td>
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<tr>
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<td>[44]</td>
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<tr>
<td></td>
<td>miRNA transfection</td>
<td>[45]</td>
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</table>

Table 1.1. Summary of nuclear reprogramming methods by ectopically expressed factors since its discovery 2006. Adapted from [1].
Several additional gene delivery systems have been described, including the piggyback (PB) transposon/transposase and the Sendai virus, a non-viral and viral systems, respectively [32], [33], [42]. They are useful strategies to deliver large genetic elements and to increase reprogramming efficiencies in mammalian cells [50].

1.1.2.2 Non-integrative methods

With the potential to impact clinical applications, iPS cell derivation strategies shifted from genome-modifying to that of non-integrative methods, including the use of chemical compounds [43], proteins [41], [40] and plasmids [37], [51]. These results demonstrate that somatic cell reprogramming does not require genomic integration or the continued presence of exogenous reprogramming factors. A fourth, more recent method for integration-free iPS cell derivation was developed in 2010 [44] and then further optimized by the Reijo Pera laboratory [1]. Here, synthetic modified mRNA molecules, each encoding the Yamanaka factors, are applied to differentiated cells over an extended period of time. To date, this is the most powerful approach to derive iPSC lines efficiently with the luxury to control stoichiometry of the Yamanaka factors in a timely manner. In addition, it opens up novel directed differentiation and trans-differentiation procedures and addresses a critical safety concern for its use in regenerative medicine (see 1.4).

1.1.3 The black box of nuclear reprogramming

Nuclear reprogramming is complex, dynamic and likely to follow a specific set of sequential events involving epigenetic changes combined with the conversion of an entire transcriptional network [13], [52], [53], [54], [55].

With the accumulation of additional reprogramming factors (RPFs) and chemical compounds, the molecular identity of derived iPS cell lines is inherently altered regardless of their developmental potential. Indeed, we are just beginning to understand how iPS cell lines differ from each other and from hES cells [56]. The quality and phenotype of a derived iPS cell line is primarily determined by: i) identity and number of RPFs [57], ii) ratio of RPFs to each other [52], iii) reprogramming strategy [34], [44] and iv) duration of ectopic expression during reprogramming [58]. In fact, Carey et al. have shown that minor differences in expression ratios of reprogramming factors lead to different iPS cell line qualities and altered epigenetic states [52].

It is believed that the process of nuclear reprogramming may involve a sequence of stochastic epigenetic events and sequential activation of pluripotency markers [59]. In mice, alkaline phosphatase (AP) and SSEA1 positive cells are already detected 3 and 9 days,
respectively, after viral reprogramming factor transduction, whereas GFP expressed from the endogenous OCT3/4 or NANOG loci first appear only after 2 weeks. The virally transduced factors need to be expressed for about 2 weeks to initiate the reprogramming process [26].

Another model suggests that reprogramming may be described as a two-stage process [60]. In the first stage, exogenous OCT3/4 and SOX2 cause the downregulation of lineage-associated genes and upregulation of a subset of ES cell-specific genes. In addition, the first stage of reprogramming comprises widespread epigenetic remodelling: epigenetic enzymes that are most likely activated by reprogramming factors induce a global unfolding of chromatin and catalyze the removal of repressive chromatin modifications from key pluripotency genes. This allows these pluripotency genes to be targeted and activated by exogenous OCT3/4 and SOX2, resulting in the revival of the interconnected autoregulatory loop and reactivation of the ES cell transcriptional network. At the same time, transgene silencing that was initiated during the first reprogramming stage reaches completion, leading the fully re-established pluripotent state to be independent from continuous transgene expression.

Despite the fact that key pluripotency markers such as OCT3/4, SOX2, and NANOG have been found to be crucial for establishing and maintaining pluripotency during reprogramming our knowledge of what defines a fully reprogrammed cell is still in its infancy. Indeed, we have to understand what specifies a bona fide pluripotency network.

Pluripotency can be defined in both molecular and functional terms. Latter describes from the developmental biological perspective the ability to differentiate into all derivatives of the three primary germ layers: ectoderm, mesoderm, and endoderm; as mentioned earlier. In order to suggest a molecular definition one has to consider both, important molecules and pathways that come into play to maintain or induce pluripotency and support its functional properties. Various methods have been used for identifying pluripotency factors using both stem cell lines and embryos. Whereas in vivo studies in embryos have relied on candidate gene analysis by knocking out alleles followed by examination of the phenotype, studies in cell lines focused primarily on large-scale, whole genome approaches. Descriptive strategies include gene expression profiling, epigenetic profiling, and protein expression profiling [61], [62], [63], [64], [65]. On the other hand large-scale functional approaches, including RNAi screens and overexpression screens [66], [67] also promoted our understanding of the meaning of pluripotency [1].

As the list of pluripotency-associated genes continues to grow there has recently been added another layer of complexity to the pluripotency network by introducing long-noncoding RNAs (LincRNAs) [68] and miRNA [45] that may be essential to pluripotency
and lineage commitment possibly through their association with specific epigenetic modifiers. Particularly, Au et al. very recently discovered novel genes and isoforms of which some may be LincRNAs that are exclusively expressed in human pluripotent stem cells [69].

It remains a challenge to accurately define pluripotency but with the emergence of new key players we set the stage to unravel the complexity behind the pluripotent transcriptome.

1.2 Mammalian germline formation

Human reproduction is achieved through the formation of the germline. Cells of the mammalian germline are unique in that they are the only cell type to be incorporated into the next generation to eventually generate new genotypes. The fundamental process of the germ cell lineage involves the differentiation into two types of highly specialized cells, sperm and oocyte, and the subsequent fusion of both that results in the regeneration of germ cells. Despite its fundamental position in the human life cycle, only little is known about the mammalian germline.

1.2.1 Primordial germ cell formation

Germ cell specification requires precise orchestration of transcriptional regulation, changes in physiology and location to ensure timely restriction from the soma (Fig. 1.1). In mammals, germ cell establishment occurs via inductive signaling, in contrast to lower organisms where germ cell specification occurs through the inheritance of germ cell determinants already present in the egg [70], [71], [72]. The molecular mechanisms of inducing the germline early in mammalian development from pluripotent progenitors are far from understood, especially in humans. It is in the early mouse embryo post-implantation and gastrulation, while the epiblast and the three germ layers (endoderm, ectoderm, mesoderm) are established, when primordial germ cell (PGC) formation begins [73]. At this time a small group of cells is induced to segregate from the ICM and to be set physically apart from the extra-embryonic ectoderm (yolk sac) of the embryo to become PGCs by signals produced by neighboring cells [74], [75], [76]. Among those signaling molecules are Bone Morphogenetic Proteins (e.g. BMP4 and BMP8a), which ensure the survival, the proliferation and migration of PGCs through the embryo. Little is known about the specific molecular regulations and underlying pathways that guide PGC specification in humans mainly due to the limited access to early human embryonic samples. Despite that circumstance it has been shown that similar pathways may direct PGC formation in the human embryo [77], [78], [79], [80], [81].
In mice, BLIMP1 is a key transcriptional regulator of PGC specification which is both involved in a unique germ cell specific transcriptional network [72], [82], [83], [84] and partially represses the somatic program in PGCs [82], [85]. The expression of BLIMP1 (PRDM1 in human) is unique to a small number of cells in the posterior, proximal mouse epiblast at E6.25 followed by upregulation of STELLA (DPPA3 in human) around E7.25 [86]. Interestingly, many pluripotency-associated genes including OCT3/4, SOX2, and NANOG are reactivated in PGC specification [81], [87], which is unique as it is the only pathway during lineage specification from epiblast cells that involves reactivation of such pluripotency marker genes. Moreover, it underlines the striking resemblance to pluripotent stem cells.

Additional key markers that are specific for PGC specification include CXCR4 in mice and the proto-oncogene c-KIT and its ligand, KIT in both mice and humans [88], [89], [90] and DAZL, the DAZ gene homologue.

1.2.2 VASA, a key germ cell marker

Upon migration and arrival to the developing gonads, another key germ cell specific marker, VASA, is expressed in both mouse and human PGCs. The VASA gene DDX4
(MVH in mouse) encodes a highly conserved and germ cell specific RNA-binding protein whose role in germline development remains unclear but has been suggested to act as a chaperone enabling correct folding of different target RNAs in germ cells [91]. Notably it is exclusively expressed in both male and female pre-meiotic germ cells [92] making it an interesting candidate for examining early germ cell specification.

Between E10.5 and E11.5 (in mouse) sex-determination is controlled by chromosomal constitution of gonadal somatic cells. While female germ cells subsequently enter meiosis, male germ cells, gonocytes, continue to proliferate until they differentiate to pre-spermatogonia that enter mitotic arrest [93]. It is believed that the expression of the male specific gene SRY on the Y chromosome in precursors of Sertoli cells triggers the expression of SOX9 which is essential for both Sertoli cell differentiation and male gonadal development [94], [95]. Germ cells then associate with Sertoli cells to form testis cords which induces the expression of VASA in post-migratory PGCs. VASA expression is induced in both male and female PGCs and persists until these cells enter meiosis and after which its levels diminish [90], [96], [97].

In the absence of either SRY or SOX9, the genital ridge develops into an ovary and the supporting cells become follicle cells instead of Sertoli cells [98]. Removing the gonads before they start to develop into testes or ovaries results in the development of a female, independent of the carried sex chromosome [99].

1.3 From hES/iPS cells to male germ cells

A significant challenge in reproductive biology is to recapitulate bona fide human germ cell specification and differentiation both in vitro and in vivo.

Recent studies in the mouse suggest that complete reconstitution of mammalian germline development from pluripotent stem cells is possible [100], [101], [102], [103]. In these studies, a critical advance was made by the induction of a transient epiblast-like cell state from mouse ES (mES) cells and iPS cells by addition of key cytokines or via overexpression of PRDM14, STELLA and TFAP2C transcription factors alone. Resulting cells were developmentally competent to form sperm and oocytes that produced live, healthy offspring after fertilization.
1.3.1 *In vitro* differentiation of male germ cells from mouse embryonic and induced pluripotent stem cells

It was not until the mid 80s when Bradley *et al.* showed the first ever *in vivo* evidence for mouse embryonic stem cell-derived germ cells [104]. After injection of mES cells into blastocysts the group observed germline chimera formation which suggests that mES cells, if cultured under the right conditions, can be differentiated to PGCs. Now, almost three decades later, we have learned a lot more about germ cell development in the dish and how mouse embryonic and germ cell specification can help us understanding human gamete formation in more detail.

Tooyaka *et al.* reported in 2003 the first *in vitro* derived male germ cells [105]. In this study, a mES cell reporter that harbored the VASA homologous gene promoter MVH to drive GFP expression was used to visualize and examine early germ cell specification. *In vitro* germ cell differentiation depended on embryoid body (EB) formation and was further enhanced by induction of BMP4-producing trophectoderm cells. Mouse ES-derived MVH-positive cells could participate in spermatogenesis when transplanted into reconstituted testicular tubules, demonstrating that ES cells can produce fully functional and differentiated sperm cells *in vitro*.

A second study reported mouse spermatogenesis up to haploid cells *in vitro* [106]. Similar to Tooyoka *et al.* PGCs were isolated from EBs. In addition, the group continued to derive lines of embryonic germ cells (*in vitro* cultured PGCs) which showed erasure of the imprinting marks of the IGF2R and H19 genes, a property characteristic of the germ lineage. PGC-like cells stained positive for SSEA1, OCT3/4, PIWIL2, DAZL and in some cells SRY. The specification to a male germline was confirmed by upregulated acrosin and heparin, suggesting that the default phenotype of female germ cells was suppressed in cultured EBs. PGCs matured into haploid male gametes, which when injected into oocytes developed into blastocysts.

A third study reports virtually complete spermatogenesis *in vitro* which upon injection into mouse oocytes resulted in blastocyst formation and viable offspring that eventually died prematurely [107]. Collectively, these three studies have proved that gametes can be obtained in culture, and moreover that mES cells that share many developmental features and biological markers with PGCs can be used as a starting point. Despite these efforts it was not until five years later before *in vitro* derived germ cells were capable of producing healthy offspring that was fertile.

In 2011, Saitou’s group generated *in vitro* derived primordial germ cell-like cells (PGCLCs) from mouse embryonic stem cells and induced pluripotent stem cells through epiblast-like cells (EpiLCs), a cellular state highly similar to pregastrulating epiblasts but distinct from
epiblast stem cells (EpiSCs) [101]. PGCLCs, when transplanted into the seminiferous tubules of W/W\(^v\) mice, exhibited complete spermatogenesis. Sperm, collected for ICSI contributed to fertile offspring.

One year later, the same group convincingly reported that oocytes, derived from \textit{in vitro} derived PGCs contributed to fertile offspring after \textit{in vitro} maturation and fertilization [100]. In both studies, cytokines were used to induce a PGC-like phenotype from mES and iPS cells.

The most recent study to date demonstrates the activation of such a PGC-like phenotype through the simultaneous overexpression of three transcription factors without the use of cytokines [102]. This was a breakthrough since it, for the first time indicated that one could change the cell fate intrinsically towards that of a PGC-like phenotype without stimuli from outside the cell. BLIMP1 (PRDM1), PRMD14 and TFAP2C (AP2\(\gamma\)) over-expression directs EpiLCs, but not mES cells, into PGCs that contributed to spermatogenesis and fertile offspring. This transcription-factor based reconstitution and regulation of murine spermatogenesis is indeed a powerful tool that provides an invaluable resource to shed light on mammalian gametogenesis \textit{in vitro}.

1.3.2 \textit{In vitro} differentiation of male germ cells from human embryonic and induced pluripotent stem cells

As outlined earlier germ cells undergo highly sophisticated developmental programs including specification, migration, and sex determination during fetal life, and gametogenesis after sexual maturation. Recapitulating this complexity \textit{in vitro} is one of the biggest challenges in human stem cell research.

The first study of human germ cell differentiation was reported in 2004 and involved spontaneous differentiation of human ES cells through EB formation [77]. The group identified upregulation of pre-meiotic and meiotic marker genes including DAZL, STELLA, VASA, and c-KIT during two-week cultures. More importantly, a subset of germline-associated genes (DAZL, c-KIT) was also observed in undifferentiated hES cells, suggesting that hES cells are indeed not the equivalent to cells of the ICM.

Similar to what has been reported in mice, the addition of BMPs can promote germ cell specification in human ES/iPS cells [78]. In this follow-up study even higher expression of germ cell markers was observed during an optimized EB formation protocol. Additional reports surfaced in subsequent studies that highlight the importance of those inductive signaling molecules [80], [108], [109].
In 2009, Park et al. introduced a more sophisticated strategy to enrich germ cells based on the cell surface marker expression SSEA1 and c-KIT and co-culture of hES cells with stromal cells from the human fetal gonad [110]. In this approach cells from the human fetal gonad were used to more accurately mimic the in vivo environment in the dish. The authors reasoned that with the secretion of paracrine factors produced from fetal stromal cells, PGCs would differentiate more efficiently from primitive germ cells. Indeed, cells expressed numerous germ cell marker such as VASA, PRDM1 and DAZL and revealed evidence of partial epigenetic reprogramming. In line with the idea to create an environment in vitro that closely resembles the germ cell niche in vivo, additional small molecules have been introduced such as fibroblast growth factor 2 (FGF2) [111], retinoic acid [112], SCF [114], WNT3A [115] or feeder cells (MEF) [111] and porcine ovarian fibroblast [116].

Based on these previous reports another hypothesis surfaced and that is that a small population of cells within a hES cell population is pre-destined to become germ cells. Accordingly, germline differentiation could be promoted by simply optimizing culture conditions of hES cells. Bucay et al. tested this hypothesis and observed a subpopulation of cells expressing CXCR4, a membrane receptor implicated in PGC migration which upon isolation differentiated into putative PGCs and Sertoli cells [117].

Deriving haploid gametes in vitro from hES/iPS cells is a significant step forward and a phenotype that researchers have struggled with for years. In 2009, the Reijo Pera group derived human haploid gametes for the first time, though only in a small number of differentiated cells [79]. Three major genes of the Deleted-in-Azoospermia (DAZ) family, DAZ, DAZL, and BOULE were overexpressed in hES cells and then spontaneously differentiated in adherent culture conditions for seven days. Haploid (1n) cells expressed various germ cell-specific markers and progressed through meiosis as indicated by the presence of SCP3, a key member of the synaptonemal complex that forms during meiosis.

Collectively, these findings illustrate the difficulties associated with human germ cell differentiation in vitro and while this field of research has made a lot of progress in the past decade (Table 1.2) it is still at its infancy and alternative strategies to study human PGC specification are likely to be needed. Only then can we investigate the mechanisms of human germ cell development and advance the field for developmental engineering technologies in the future.
<table>
<thead>
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<th>Cell source (gender)</th>
<th>Culture condition</th>
<th>In vitro derived derivatives</th>
<th>Phenotypic features, marker expression</th>
<th>Reference</th>
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<td>Gonocytes</td>
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<td>PGCs</td>
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Table 1.2. *In vitro* germ cell differentiation in primates. *from Cynomolgus monkey, **from Common marmoset, *** in review, hESCs = hES cells, iPSCs = iPS cell. Adapted from [118].
1.4 Applications

There are two main distinct types of applications for pluripotent stem cells and in particular iPS cell research: (i) regenerative medicine and (ii) *in vitro* application. Latter comprises drug screening, disease modeling, toxicology and iPS cell assays and has already been shown in several studies to be successful [20]. In contrast translating the findings from iPS and germ cell research from the bench to bedside is still far from reality. The ultimate goal is to generate functionally competent *in vitro* differentiated germ cells to generate healthy and fertile offspring, which has been shown very recently in mice [100]. It will require time, effort, and innovative strategies until we successfully accomplish one of the greatest achievements in human stem cell research.

1.4.1 Clinical applications

Among stem cells, human ES cells are considered to hold the greatest potential for clinical and biomedical research. Since iPS cells do not raise ethical concerns in contrast to ES cells they may represent an ideal source for various applications (Fig. 1.2).

Hanna *et al.* demonstrated in a proof-of-concept study that iPS cell-based cell replacement therapy has indeed a promising potential. Using a humanized sickle cell anaemia mouse model in which iPS cells were first created following *in vitro* differentiation into hematopoietic progenitors, the group was able to successfully correct the defect [119].

A similar approach is desired for *in vitro* derived gametes. Ten to fifteen percent of couples are infertile with male infertility contributing to half of the cases. In 1-2% of men, there is a severe deficiency in germ cell development. In the US. alone there are 55,000 childhood cancer survivors annually, many of whom were unable to bank sperm before receiving sterilizing treatments.

Despite the desperate need of such applications, a number of hurdles have to be overcome. First, high quality patient-specific iPS cells have to be fully characterized with standardized methods and meet Good Manufacturing Practice (GMP) regulations (see 1.4.2). Such characterization involves among others the screening for mutations and chromosomal aberrations (reviewed in [121]). Secondly, protocols for *in vitro* derived germ cells currently in its infancy have to be in a streamlined and efficient manner to produce high quality *in vivo* counterparts. Thirdly, gene expression, epigenetic modifications, and genome ploidy of human ES/iPS cell-derived germ cells have to be evaluated prior use and their functional capabilities including fertilization and embryogenesis has to be strictly and inevitably regulated.
Figure 1.2. Reprogramming technology and iPS cells have the potential to be used to model and treat human disease. In this example, the patient has a neurodegenerative disorder. Patient-specific iPS cells - in this case derived by ectopic co-expression of transcription factors in cells isolated from a skin biopsy - can be used in one of two pathways. In cases in which the disease-causing mutation is known (for example, familial Parkinson’s disease), gene targeting could be used to repair the DNA sequence (right). The gene-corrected patient-specific iPS cells would then undergo directed differentiation into the affected neuronal subtype (for example, midbrain dopaminergic neurons) and be transplanted into the patient’s brain (to engraft the nigrostriatal axis). Alternatively, directed differentiation of the patient-specific iPS cells into the affected neuronal subtype (left) will allow the patient’s disease to be modelled in vitro, and potential drugs can be screened, aiding in the discovery of novel therapeutic compounds. Adapted from [120].

1.4.2 Use of GMP grade cells

Data suggest that clinical applications of human induced pluripotent stem (hiPS) cells will be realized. It is critical that derivation of high quality hiPS cells for clinical applications should be rapid, efficient and cost effective. This is especially important considering that, after reprogramming, clonal lines require characterization and may require genome correction (in the case of genetic diseases) and differentiation to a transplantable cell type. All of these steps require extensive maintenance in culture and are therefore associated with mutagenesis that can affect quality of the cells and ability of the cells to engraft and function properly [122], [123]. It is important that methods are compatible with
future uses in pathologies associated with tissue-degeneration where a fast and efficient cell therapy is crucial and cost-effective.

Clinically compliant cell products are required for potential cell based therapies. Previous regulatory oversight suggests that two methods may be acceptable for this purpose: 1) Derivation of cells and cell products under GMP requirements and 2) Conversion of cells or cell products derived under research-grade conditions to GMP quality standards\(^1\). Latter has recently been demonstrated with a lentiviral-derived hiPS cell line \[124\]. This requires that the cells be successfully transferred to a GMP facility and cultured, frozen and thawed, and expanded extensively in such an environment. Also, rigorous tests should be passed to exclude the presence of adventitious agents that could have been present in the research-grade reagents used during the cell derivation process. Thus, it is important to develop a standardized platform of iPS cell derivation that is integration-free, fast, efficient, scalable and easily transferred to GMP compliant conditions.

1.4.3 Transplantation of iPS, hES and germ cells

Transplantation of iPS cells in various organs has successfully been reported in different disease mouse models emphasizing their potential for cell therapy \[125, 126\] (see \[13\] for review). In these studies and similar transplantation-based approaches \[127, 128, 129\] transplanted cells involved the cell lineages that are more easily derived from pluripotent stem cells. The generation of germ cell lineage cells with *bona fide* differentiated characteristics has been more difficult as outlined earlier.

Despite these tremendous achievements many challenges are associated with iPS cell-based therapy: i) detection of high quality iPS cells, ii) efficiency of cell-lineage-specific differentiation, iii) proper preclinical animal models, iv) successful engraftment, v) risk of teratoma formation, vi) maturation level and functionality of cells, and vii) costs associated with patient-specific therapy.

The potential of teratoma formation upon donor cell transplantation represents one major concern. The frequency of teratoma formation following human ES cell transplantation into animal hosts is directly related to the degree of immunosuppression \[130\] prompting for the question whether or not autologous iPS cell derivatives which should elicit no immune reactivity against transplanted donor cells, result in an even greater rate of teratoma formation compared to animal studies. While it is likely that the use of pre-differentiated cells in transplantation assays will greatly reduce the teratoma forming

\(^1\)http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070273.pdf
potential; at present, it is unclear whether those differentiated cells and available strategies to separate them from residual pluripotent cells will eliminate the risk of teratoma formation [131].
Chapter 2

Objective

A significant challenge in reproductive biology is to recapitulate *bona fide* human germ cell specification and differentiation both *in vitro* and *in vivo*. Successful reconstitution of human germ cell development will enable studies of basic development and potentially contribute to clinical applications in assisted reproduction. Recent studies in the mouse suggest that complete reconstitution of mammalian germline development from pluripotent stem cells is possible. In these studies, a critical advance was made by the induction of a transient epiblast-like cell state from mouse ES and iPS cells by addition of key cytokines or via overexpression of PRDM14, STELLA and TFAP2C transcription factors alone. Resulting cells were developmentally competent to form sperm and oocytes that produced live, healthy offspring after fertilization.

In the first part of this thesis we examined recently discovered novel genes that are specifically expressed in human pluripotent stem cells. In particular, we reprogrammed somatic cells into induced pluripotent stem cells and interrogated their dynamic gene expression changes over time during cell fate transition on a single cell level, thus revealing their putative implications in establishing and/or maintaining pluripotency.

The second and major part of this thesis encompasses also cell fate transitions from somatic cells towards induced pluripotent stem cells but focuses then on human germ cell development. Fundamental insights gained from mouse studies described above, along with knowledge of genetic control of PGC fate in diverse organisms and previous studies of human germ cell differentiation *in vitro* prompted us to hypothesize that developmental ‘priming’ by induced expression of key germ cell specific genes in combination with xenotransplantation might overcome current roadblocks to efficient human germ cell development. Due to inherent differences between human and mouse pluripotent stem cell,
and based on previous studies, we predicted that induced expression of translational regulators such as VASA, DAZ, DAZL and BOULE might promote human germ cell formation. Thus we included VASA during mRNA reprogramming to iPS cells in order to overcome significant hurdles that we and others have encountered with human germline derivation in vitro. We then transplanted the induced germ cells directly into the seminiferous tubules of germ cell-depleted mice, in order to evaluate the contribution of VASA-primed and non VASA-primed cells to germline development in vivo.

Collectively, we established a novel approach to study germ cell forming potential of undifferentiated cells in vivo using a mouse xenotransplant model and examine differences between different iPS cell phenotypes.
Chapter 3

Materials and Methods

3.1 Molecular Biology Materials and Methods

3.1.1 Materials

3.1.1.1 Chemicals and enzymes

All chemicals, enzymes and tissue culture equipment used in this work are of molecular biology quality and were acquired from Life Technologies, Sigma Aldrich, Becton Dickinson and Company (BD), and Fisher Scientific. Additional materials and commercial kits used are described in the following sections.
3.1.1.2 Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2% Gelatin*</td>
<td>1 g Gelatin from porcine skin, type A dissolved</td>
</tr>
<tr>
<td>PBS (Invitrogen)</td>
<td></td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>0.1% TritonX-100, 4% goat serum in PBS</td>
</tr>
<tr>
<td>Fixation buffer</td>
<td>4% Paraformaldehyde (PFA) in PBS</td>
</tr>
<tr>
<td>Permeabilization buffer</td>
<td>0.2% TritonX-100 in PBS</td>
</tr>
<tr>
<td>TAE buffer (10x)</td>
<td>400 mM Tris-acetate, 10 mM EDTA</td>
</tr>
<tr>
<td>Fixation buffer**</td>
<td>1% PFA in DW (pH 9.2) + 0.15% Triton X-100 + 3 mM dithiothreitol</td>
</tr>
<tr>
<td>Wash buffer**</td>
<td>0.1% Tween-20 (PBST)</td>
</tr>
<tr>
<td>Xylene</td>
<td></td>
</tr>
<tr>
<td>0.01 M Sodium citrate buffer</td>
<td></td>
</tr>
<tr>
<td>(pH 6.0)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. Buffers and solutions. *solutions autoclaved after preparation, ** for meiotic spreads.

3.1.1.3 Bacterial hosts, media and culture plates

<table>
<thead>
<tr>
<th>Strain</th>
<th>One Shot®TOP10 Chemically Competent <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>F-mcrA ∆(mrr-hsdRMS-mcrBC) ϕ 80lacZΔM15 ∆lacX74 recA1 arAD139 ∆(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ−</td>
</tr>
<tr>
<td>LB broth*</td>
<td>250 ml DI water + 5 g LB Agar pulver (Sigma-Aldrich)</td>
</tr>
<tr>
<td>LB agar plates*</td>
<td>10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl, DI water up to 1 L, 15 g Bacto-agar (Gibco-BRL)</td>
</tr>
</tbody>
</table>

Table 3.2. Bacterial hosts, media and culture plates. *solutions autoclaved after preparation.

3.1.1.4 Vectors

- *Addgene*

**pcDNA3.3-KLF4**
Vector backbone: pcDNA3.3
Drug resistance: Ampicillin
Insert: human KLF4 (insert size: 1596 bp)
Chapter 3. *Materials and Methods*

**pcDNA3.3-SOX2**
Vector backbone: pcDNA3.3  
Drug resistance: Ampicillin  
Insert: human SOX2 (insert size: 1137 bp)

**pcDNA3.3-OCT4**
Vector backbone: pcDNA3.3  
Drug resistance: Ampicillin  
Insert: human OCT4 (insert size: 1266 bp)

**pcDNA3.3-c-MYC**
Vector backbone: pcDNA3.3  
Drug resistance: Ampicillin  
Insert: human cMYC (insert size: 1504 bp)

**pcDNA3.3-LIN28A**
Vector backbone: pcDNA3.3  
Drug resistance: Ampicillin  
Insert: human LIN28A (insert size: 813 bp)

**pcDNA3.3-eGFP**
Vector backbone: pcDNA3.3  
Drug resistance: Ampicillin  
Insert: human eGFP (insert size: 903 bp)

**pcDNA3.3-d2eGFP**
Vector backbone: pcDNA3.3  
Drug resistance: Ampicillin  
Insert: human d2eGFP (insert size: 846 bp)

- *DNA2.0*

**pJ201:61044**
Vector backbone: pJ201  
Drug resistance: Kanamycin
Chapter 3. Materials and Methods

Insert: custom design (insert size: 255 bp)

**pJ201:VASA**
Vector backbone: pJ201  
Drug resistance: Kanamycin  
Insert: custom design (insert size: 2174 bp)

**pJ201:KLF4**
Vector backbone: pJ201  
Drug resistance: Ampicillin  
Insert: human KLF4 (insert size: 1596 bp)

**pJ201:SOX2**
Vector backbone: pJ201  
Drug resistance: Ampicillin  
Insert: human SOX2 (insert size: 1137 bp)

**pJ201:OCT4**
Vector backbone: pJ201  
Drug resistance: Ampicillin  
Insert: human OCT4 (insert size: 1266 bp)

**pJ201:c-MYC**
Vector backbone: pJ201  
Drug resistance: Ampicillin  
Insert: human cMYC (insert size: 1504 bp)

### 3.1.1.5 Instruments and software

- NanoDrop Technologies, ND-1000
- Gel Logic 200 Imaging System
- AirClean 600 PCR work station (ISC BioExperts)
- Biomark Genetic Analysis (Fluidigm)
Chapter 3. Materials and Methods

- VWR water bath
- Centrifuge 5804 (Eppendorf)
- Centrifuge 5424 (Eppendorf)
- Fisher Vortex Genie 2 (Fisher Scientific)
- Leica DM IL microscope
- LEICA DMI 4000 B microscope (Leica Application Suite v3.6.0)
- MCO-36AC CytoGROW™GLP Series CO2 incubator (Sanyo Scientific)
- Steric GARD III Advance dissection hood (The Baker Company)
- Class II Type A2 Biological Safety Cabinets
- Beckman Coulter Allegra X-22R centrifuge
- Veriti 96 Well Thermal Cycler PCR machine (Applied Biosystems)
- 7300 Real Time PCR Systems (Applied Biosystems)
- Confocal Microscope Zeiss LSM 700
- Illumina Genome Analyzer IIx (llumina HiSeq 2000 platform)

3.1.2 Methods

3.1.2.1 Transformation of competent bacteria

Transformation was performed with the heat shock method. Chemically competent One Shot®TOP10 E. coli have been used for plasmid DNA amplification. Up to 100 ng of DNA was gently mixed with one vial of One Shot®cells and incubated on ice for 30 min. The cells were heat-shocked for 30 sec at 42°C without shaking and immediately placed back on ice for 2 min. 250 µl of pre-warmed S.O.C. Medium was added and each vial shaken at 37°C for 1 h at 225 rpm. 10 and 50 µl were spread on pre-warmed selective agar plates and incubated overnight at 37°C. Transformation efficiency was calculated according to the manufacturer’s instructions.
3.1.2.2 Plasmid DNA purification

Isolation of plasmid DNA was performed according to the manufacturer’s handbook using buffers and equipment contained in the kit (QIAprep Spin Miniprep Kit using a microcentrifuge). For preparative purposes, *E. coli* was plated and grown over night, then one colony was picked and transferred into a glass tube containing 5 ml LB broth + ampicillin (1:1000). *E. coli* incubated overnight at 37°C on a shaker and was expanded to a flask containing 250 ml LB broth + ampicillin (1:1000) the next day to incubate for another day.

3.1.2.3 Agarose gel electrophoresis

Analysis and purification of DNA fragments was performed by agarose gel purification (1 % agarose in 1X TAE buffer). Ethidium bromide (0.5 µg/µl, Bio-Rad) was used for DNA visualization under UV light with excitation at 312 nm. DNA samples were mixed 1:10 with 10x BlueJuice Gel Loading Buffer (Invitrogen) and separation was done at 100 V at room temperature. A 1 Kb Plus DNA ladder (Invitrogen) was used as a standard marker.

3.1.2.4 Construction of ivT templates

An outline for ivT template construction is schematized in Fig. C.4. A "backbone sequence" containing 5' and 3' UTR regions, T7 promoter and multiple cloning site was synthesized by DNA2.0 (Menlo Park, CA). ORF PCRs were templated from different plasmids as follows: human OCT3/4, SOX2, KLF4, cMYC, LIN28 + eGFP, d2eGFP from Addgene, VASA our group. PCR reactions were carried out with HiFi Hotstart (KAPA Biosystems, Woburn, MA). Specific primer pairs were synthesized by the Protein and Nucleic Acid Facility (PAN, Stanford University). Products on agarose gel were cut and purified using Qiaquick gel extraction kit (QIAGEN, Valencia, CA). ORF fragments and DNA2.0 vector containing the "backbone sequence" were digested with *AgeI* and *NheI* (New England Biolabs, Ipswich, MA) for 45 min at 37°C, followed by agarose gel verification and purification with Qiaquick gel extraction kit (QIAGEN). Enzyme treated ORFs and DNA2.0 vector were ligated for 2 h with a PCR cycle program (30 sec: 10°C, 30 sec: 30°C) using a *T4 ligase* (New England Biolabs). Transformation was carried out with OneShot®TOP10 Chemically Competent *E. coli* (Invitrogen) according to the manufacturer’s instructions and positive clone selection was verified with a Qiaquick Mini-Prep purification kit (QIAGEN) followed by a test digestion with *SpeI* (New England Biolabs). Plasmid inserts were excised by restriction digest and purified using Qiaquick PCR purification kit (QIAGEN) before being subject to template tail PCR. Vectors from Addgene
containing the Yamanaka factors were used in addition to our own designed mRNA. A polyA tail was added with a T_{120}-heeled reverse primer and carried out with HiFi Hot-start (KAPA Biosystems) PCR reaction. Purification of amplicons was carried out with Qiaquick PCR purification kit (QIAGEN) and amplicons were used for ivT reactions.

3.1.2.5 **In vitro transcription of modified RNA**

Synthesis for mRNA was carried out with the MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer’s instructions with slight modifications. A custom ribonucleoside blend was used comprising 6 mM 3’-0-Me-m⁷G(5’)ppp(5’)G ARCA cap analog (New England Biolabs), 7.5 mM of adenosine triphosphate and 1.5 mM of guanosine triphosphate (Ambion), 7.5 mM of 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies, San Diego, CA). Reactions were incubated for 4 h at 37°C, followed by DNase treatment for 15 min at 37°C. DNase treated RNA was purified using the MEGAclean kit (Ambion). Correct RNA synthesis and RNA purification was verified and quantitated by Nanodrop (A230/A260 between 1.7-2.0) and concentration was adjusted to 100 ng/ml. RNA reprogramming cocktails were prepared by pooling individual 100 ng/µl RNA stocks to produce a 100 ng/µl total blend. Different ratios of individual mRNAs were tested to prior reprogramming to optimize feeder-free-reprogramming with regard to reprogramming efficiency and cell viability. We found that molar ratios of 3:0.5:1:0.5:(1) (OSKM[V]) were optimal. Stocks were stored at -80°C. Working space and instruments were cleaned with RNaseZap (Ambion) to prevent RNA degradation by RNases.

3.1.2.6 **Denaturing formaldehyde-agarose gel electrophoresis**

mRNA transcripts were analyzed for specificity of ivT reaction and correct size. A 1.5% denaturing formaldehyde-agarose gel was prepared dissolving 0.75 g agarose in 36 ml DI water, 5 ml 10X MOPS running buffer (Ambion) and 9 ml 37% formaldehyde (12.3 M, Sigma-Aldrich). mRNA samples (1 µg) were mixed with 3x the volume of Formaldehyde Loading Dye (Ambion) and 0.25 µl ethidium bromide (10 mg/ml, Bio-Rad) followed by heat denaturation for 15 min at 70°C. RNA ladder (RNA Millenium marker, Ambion) was treated like mRNA samples and used for size comparison.

3.1.2.7 **mRNA transfection**

mRNA transfection was carried out with RNAiMAX (Invitrogen) according to the manufacturer’s instruction. Briefly, RNA and reagent were first diluted in Opti-MEM basal
medium (Invitrogen). 100 ng/µl RNA was diluted 5x and 5 µl of RNAiMAX (Invitrogen) per µg RNA was diluted 10x. Both dilutions were combined, briefly vortexed and incubated for 15 min at room temperature. After complex formation, mix was drop wise dispensed to culture medium. RNA transfection was performed in hES cell medium or Pluriton mRNA reprogramming medium (Stemgent) supplemented B18R interferon inhibitor (eBioscience, San Diego, CA) at 200 ng/ml.

3.1.2.8 Cells

BJ human fibroblast cells (passage 6) were established from normal human fetal foreskin and purchased from Stemgent (Cambridge, MA). Human adult dermal fibroblasts were derived from a 28 year old healthy control male (HUF1), 30 year old healthy control female (HUF3), a 60 year old male with a chromosome 2 pericentric inversion (HUF58) and a 31 year old female with premature ovarian failure (HUF9). GM13325 fibroblasts were purchased from Coriell. H9 and H1 human embryonic stem cells were received form our lab.

3.1.2.9 Tissue acquisition

Written approval for all somatic derivations and subsequent iPS cell generation performed in this study was obtained from the Stanford University Institutional Review Board (IRB protocol 10368) and the Stanford University Stem Cell Research Oversight Committee (SCRO protocol 40), and written informed consent was obtained from each individual participant.

3.1.2.10 Cell culture

Human fibroblast cell lines were cultured in Dulbecco’s modified Minimal Essential Medium + GlutaMAX (DMEM, Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) on 0.2% gelatin (Sigma, St. Louis, MO) coated wells. Cells were passaged using TrypLE Express (Invitrogen) and replated at a dilution of 1:3.

Human embryonic stem cells (H9 and H1) and induced pluripotent stem cells were cultured in DMEM/F12 (Invitrogen) supplemented with 20% Knockout Serum Replacer (Invitrogen), 2 mM L-glutamine, 0.1 mM Non-Essential Amino Acids (NEAA, Invitrogen), 0.1 mM 2-Mercaptoethanol (Millipore, Billerica, MA) and 10 ng/ml b-FGF (Invitrogen). For feeder-independent maintenance of human ES cells and iPS cells basal mTeSR 1
medium (STEMCELL Technologies, Vancouver, BC, Canada) supplemented with 5X mTeSR1 supplement (STEMCELL Technologies) was used. Culture plates were pre-coated with matrigel (BD Biosciences, Franklin Lakes, NJ). Cells were passaged mechanically or enzymatically using 200 units/ml of collagenase IV (Invitrogen), washed and replated at a dilution of 1:2 to 1:5. Differentiated cells were removed and/or cleaned under the dissection hood.

Cultures were maintained at 37°C and 5% CO₂ and medium changed every day. For mRNA reprogramming medium was changed every day. Cells were frozen down in Bambanker (Wako) (for pluripotent cells) and FBS (Invitrogen) and 10% dimethyl sulfoxide (DMSO, Sigma) for fibroblasts.

For GMP-compliant lines reagents listed in Table D.3 were used.

### 3.1.2.11 Derivation of mRNA induced pluripotent stem cells

For feeder-free reprogramming target fibroblasts were seeded at 1x10⁴-4x10⁴ cells per well of a 6 well plate on 0.2% gelatin coated wells and cultured in MEF media. 24 h later MEF media was replaced with NuFF- (NuFFs, GlobalStem) conditioned Pluriton media (Stemgent) supplemented with Pluriton supplement (Stemgent) and B18R (200 ng/ml, eBioscience). Cells were transferred to a low oxygen environment (5%) for higher reprogramming efficiencies before the first transfection. After 2 h of equilibration in low oxygen conditions mRNA cocktail containing OSKM(V)g (OCT3/4, SOX2, KLF4, cMYC, (VASA), d2eGFP) was transfected and repeated every 24 h until colony formation was observed. Incubation of mRNA and transfection mix with cells was carried out for 4 h. Primary colonies were picked onto fresh culture dishes coated with Matrigel (BD Bioscience) and media was replaced with mTeSR1 (STEMCELL Technologies) supplemented with 5X mTeSR1 supplement (STEMCELL Technologies). Established iPS cell lines were cultured under 20% oxygen conditions.

### 3.1.2.12 Conversion, culture and characterization under GMP conditions

Cells were gradually converted from Pluriton to mTeSR1/Nutristem as follows: 1:0, 0.8:0.2, 0.5:0.5, 0.2:0.8, 0:1 with x:x being the ratio of Pluriton:mTeSR1/Nutristem. The ratio was altered with each daily media change. The same conversion strategy was applied for mTeSR1/Nutristem to TeSR2/Nutristem conversion. Cells were manually passaged with glass tools during the entire conversion on Synthemax (Corning), an extra cellular matrix with cell adhesion promoting peptides. Synthemax is a synthetic, non-biological, xeno-free, gamma sterilized (SAL 10-6) substrate and quality tested. Cells that were
converted to xeno-free conditions were then transferred to the UCLA GMP compatible facility, expanded under xeno-free conditions and cryobanked for future applications. Fully converted cells were subject to characterization including immunocytochemistry, sterility tests (Gram positive/negative bacteria, fungi, mycoplasma), karyotyping and STR analysis (Cell Line Genetics).

Our GMP-compliant facility is built with ISO 7/ Class 10,000 clean room and ISO 5/ Class 100 biosafety cabinet (BSC). The equipments in the facility are monitored on a daily basis and SOPs are followed for cleaning and operation. External certified vendors calibrate the biosafety cabinets and monitor air as per ISO standards and perform viable particle count in clean rooms and BSCs. Temperature and gases are also continuously monitored in the equipments through a wireless network system operated by contract vendors. Periodic calibrations are done to ensure functioning of the equipments as stipulated. External vendors also perform calibration and qualification of pipettes. Adventitious agents testing is done by clinically certified laboratories. Our in-house qualification method involves cleaning and decontamination for water baths, incubators, refrigerators, freezers, and centrifuges.

3.1.2.13 Immunostaining of live cells

Stemgent StainAlive DyLight 488 anti-Human TRA-1-60/TRA-1-81 antibody was diluted in fresh cell culture medium to a final concentration of 5\(\mu\)g/ml. Old medium was aspirated and replaced with diluted primary antibody. Cells were incubated for 30 min at 37°C and 5% CO\(_2\). The primary antibody was aspirated and cells were washed gently 2x with cell culture medium. Fresh cell culture medium was added and cells were examined under a fluorescent microscope using the appropriate filters. Cells were kept in culture after examination.

3.1.2.14 Alkaline phosphatase staining

Alkaline phosphate (AP) staining was performed using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) following the manufacturer’s instructions.

3.1.2.15 Gene expression analysis

Gene expression assays were performed using Fluidigm (San Francisco, CA) analysis and following the protocol “Single-Cell Gene Expression Using EvaGreen DNA Binding Dye”.
Chapter 3. *Materials and Methods*

Briefly, purchased forward and reverse primer pairs were mixed together to a final concentration of 20 µM. All primer pairs were pooled together at a final concentration of 200 nM each for pre-amplification. CellsDirect 2x Reaction Mix, SuperScript III RT (Reverse Transcriptase) Platinum Taq Mix, 4X Primer Mix (200 nM) and TE buffer were prepared at total volume of 9 µl. Cells (1-50) were added in 1 µl to each reaction and the following thermal cycling protocol was set: Reverse Transcription - 50°C for 15 min, Inactivate RT/Activate Taq - 95°C for 2 min, 18 Cycles - 95°C for 15 sec and 60°C for 4 min, 4°C for infinite. ExoSAP-IT treatment removed unused material and was performed at 37°C for 15 min (digest) and 80°C for 15 min (inactivation). Reaction was diluted 1:5 in TE buffer and stored at -20°C or immediately used for Sample Pre-Mix. Sample Pre-Mix, Samples and Assay Mix was prepared according to the protocol. The Fluidigm chip was primed and loaded with the assay and sample mix. Data were collected and analyzed using the Fluidigm Data Collection Software v.3.0.2.

For single-cell gene expression analysis single cells were collected and harvested in 2x Reaction Mix for storage at -80°C or for immediate processing. For cell lysis, reverse transcription, and pre-amplification of cDNA the C1 Single Cell Auto Prep System from Fluidigm was used and the protocol (PN 100-4904 D1) according to manufacturer’s instructions. For pluripotency validation of HPATs a multiple tissue panel of cDNA was used from Clontech (Mountain View).

### 3.1.2.16 EB formation and *in vitro* differentiation

iPS cells from one confluent 10 cm dish were harvested, washed and seeded into 2 wells of an ultra low attachment plates (Corning) containing DMEM + 20% FBS. 7 days after cells grew in suspension, embryoid bodies were transferred to gelatin-coated wells of a 24 well plate containing the same medium to allow the cells to attach. Medium was changed every 2-3 days for up to 2 weeks. Cells were fixed in 4% PFA for staining of representative germ layer markers.

### 3.1.2.17 Teratoma formation

Kidney capsule injection was performed as follows: Cells were prepared (manually picked in 100-200 cell clumps in 30 µl culture media) and the recipient mouse was anesthetized. The mouse was placed on its abdomen on a sterile paper towel. A vertical incision was made through the skin along the animal’s spine, about 2 cm from the base of the tail to the top curve of the spine. With forceps the edge of the incision on one side was gripped and skin was carefully separated from the peritoneum by placing closed scissors under the skin and gently opening the scissors. A small incision into the peritoneum was made and
the kidney was localized. With a little pressure on the abdomen, the kidney was exposed and popped out of the body cavity. A small tear with a sharp glass pipet was made in the kidney capsule and cells were inserted beneath the kidney capsule using a mouth pipet. The kidney was placed back into the body cavity, the skin was pulled upward and skin was clamped together using sterile surgical clamps. 3-4 weeks after the surgery tumors were dissected and fixed overnight in 4% PFA diluted in PBS.

Fixed samples were sent to AML Laboratories (Baltimore, MD) for paraffin embedding, sectioning and staining with hematoxylin and eosin. Sections were then examined for the presence of tissue representatives of all three germ layers.

3.1.2.18 Differentiation towards primordial germ cells

The developmental potential for \textit{in vitro} PGC formation was assessed by treating cell cultures with BMP4 (R&D Systems, 50 ng/ml) or vehicle (4 mM HCl + 0.1% BSA) in culture media. Samples were collected at day 0, 2, and 4 and gene expression analysis was performed using Fluidigm.

3.1.2.19 Bisulfite sequencing

Genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen) and processed using the Epitect Bisulfite Kit (Qiagen) according to manufacturer’s instructions. Converted DNA was amplified by polymerase chain reaction (PCR) using primers published previously [132], [133]. PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C (Fig. B.2). PCR products were purified with QIAquick PCR purification kit (Qiagen), cloned using TOPO TA cloning kit (Invitrogen) and the resulting plasmids transformed into OneShot® Top10 chemically competent \textit{E. coli} (Invitrogen). 10 bacterial clones per genomic region (for pluripotency genes) and 20 bacterial clones per genomic region (for imprinted genes) were either sent to Sequetech for sequencing or picked and DNA was extracted using QIAprep Spin Miniprep kit (Qiagen) followed by reverse-sequencing using the M13 reverse primer (PAN facility Stanford University). Sequenced clones were aligned by Geneious software (Biomatters, Auckland, New Zealand, www.biomatters.com) and CpG methylation analysis was performed by BioQ Analyzer software (Max Planck Institut Informatik, Saarbruecken, Germany, www.mpi-inf.mpg.de). Significant differences of methylation patterns of imprinted genes were calculated as follows: For every clones/sample the methylated/nonmethylated fraction per CpG island was determined. The mean and standard deviations of fractions across all CpG islands was calculated per sample and Fisher’s exact test was applied.
3.1.2.20 Meiotic spreads

Differentiated cells were harvested in PBS and put on a glass slide dipped in a solution of 1% PFA in DW (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol (Sigma-Aldrich) [134]. The slide was then incubated overnight in 4°C. After fixation, the slide was washed in 0.4% Photoflo (Kodak) in DW and dried for 30 min at room temperature. Subsequent immunostaining was performed as follows. The samples were washed with PBS containing 0.1% Tween-20 (PBST) and treated with 0.5% Triton for 15 min. After washing with PBST, they were incubated in 1% BSA in PBS overnight at 4°C. The samples were incubated with a primary antibody diluted in 1% BSA/PBS, anti-SCP3 (1/400, Novus Biologicals) and anti-CENPA (1/400, Abcam) for 3 h at room temperature. After washing with 1% BSA/PBS, the cells were incubated with a 1:1000 dilution of A488-conjugated anti-mouse IgG (Invitrogen) and A594-conjugated anti-rabbit IgG (Invitrogen) for 1 hr. After brief washing in 1% BSA/PBS the samples were then mounted on a glass slide in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA) containing 3 µg/mL 4’,6-diamidino-2-phenylindole (DAPI) followed by fluorescence microscopy.

3.1.2.21 RNA seq

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) per manufacturer’s instructions and subjected to cDNA synthesis. 100 ng of total RNA was subjected to first and second cDNA synthesis using the Ovation RNA-Seq System V2 (NuGEN Technologies, Inc.; San Carlos, CA) following the fragmentation with an average size of 200-300 bases using the Covars S-Series System. Briefly, 1-5 µg of each cDNA sample was diluted in 120 µl 1X TE buffer. The Covaris S-Series System settings were as follows: duty cycle -10%, intensity -5, cycles/burst -100, time - 5 min. Illumina library construction was then performed using the NEBNext DNA Sample Prep Master Mix Set 1 and Agencourt AMPure XB beads for clean up. Briefly, 10-25 ng of fragmented DNA was end repaired following the manufacturer’s instructions of the NEBNext DNA Sample Prep Master Mix Set 1 kit followed by cleanup with the Agencourt RNAClean XB beads. End repaired DNA was subject to dA-tailing followed by a second clean up. dA-tailed DNA and adaptor were ligated followed by a PCR for enrichment of adaptor ligated DNA (98°C for 30 sec; 17x 98°C for 10 sec, 65°C for 30 sec, 72°C for 30 sec; 72°C for 5 min, 4°C on hold). Samples were cleaned once again and the built library was analyzed on a HS Agilent DNA chip using a Bioanalyzer. Samples were sequenced in 2 lanes of Illumina HiSeq 2000 platform (Illumina, Inc) as 100 bp paired end reads. Quality check of raw data was processed through the web-based Galaxy platform using the FASTQC tool. Reads with
a median score lower than 20 were trimmed using FASTQ Trimmer. Reads were then mapped using TopHat v.2.0.5 with default settings. The mean insert sizes as determined by the Bioanalyzer were employed in the TopHat mapping. Transcript assembly and expression level quantification of transfrags was performed using Cufflinks v.2.0.2 to filter out background and artifactual transfrags [135]. Each sample was assembled individually and all assemblies were merged together using Cuffmerge. Bowtie indexes and annotation files were downloaded from http://cufflinks.cbcb.umd.edu/igenomes.html (UCSC, h19). Transcripts with a $p \leq 0.05$ were considered to be differentially expressed. Visualization of differential gene expression analysis was performed with CummeRbund v.1.2.0 in R. Scatter plot and volcano plot (type of scatter plot) analysis helped in identifying changes in gene expression in RNAseq derived datasets between all four lines. Specifically the volcano plot displays significance versus fold-change on the y- and x-axes, respectively. The plots are constructed by plotting unstandardized signal (fold-change) against noise-adjusted/standardized signal (negative log(10) of the p-value) on the x- and y-axes, respectively. Genes with low p-values (highly significant) appear towards the top of the plot. The Jensen-Shannon (JS) divergence was calculated to measure similarities between two lines with regard to their probability distributions of global gene expression. The more similar two samples are the more close is their JS distance. Principal component analysis (PCA) and multi dimensional scaling (MDS) were used to visualize the level of similarity between all 4 samples in a 2 dimensional plot. The plots contain the information that are measured in the Jensen-Shannon distance matrix.

3.1.2.22 Xenotransplantation assay

Human cell lines were transplanted into the testes of busulfan-treated, immune-deficient nude mice (NCr nu/nu; Taconic) as previously described for primate [136] and human [137] spermatogonia. Briefly, immunodeficient nude mice were treated with a single dose of busulfan (40 mg/kg, Sigma) at 6 weeks of age to eliminate endogenous spermatogenesis. Xenotransplantation was then performed 5 weeks after busulfan treatment by injecting cell suspensions containing 10% trypan blue (Invitrogen) into the seminiferous tubules of recipient testes via cannulation of the efferent ducts. Approximately 7 µl of cell suspension was injected per testis.

Eight weeks after transplantation, recipient mouse seminiferous tubules were dispersed gently with Collagenase IV (1 mg/mL) and DNase I (1 mg/mL) in D-PBS and fixed in 4% paraformaldehyde, as previously described [136] [137]. Clusters of human cells were observed on the basement membranes of recipient mouse seminiferous tubules by staining with a rabbit anti-primate testis cell primary antibody [136] and a goat anti-rabbit IgG
secondary antibody conjugated with AlexaFluor488 (Invitrogen). All dehydration, rehy-
dration, and staining steps were carried out in 12-mm Transwell baskets (Corning Life
Sciences) to prevent loss of seminiferous tubules.

3.1.2.23 Immunohistochemistry (IHC)

Formalin-fixed mouse testes from xenotransplants were sent to AML Laboratories (Bal-
timore, MD) for paraffin embedding and sectioned into serial cross-sections of 10 mm
thickness each. For immunostaining, testes sections were deparaffinized in xylene (thrice,
10 min each), rehydrated through a graded series of treatment with ethanol (100%, 90%, 80%,
and 70%, 5 min each) and rinsed in tap water. For all samples, antigen retrieval
was performed by boiling the sections in 0.01 M sodium citrate buffer (pH 6.0) for 20 min,
followed by incubation at room temperature for 30 min. For DNA methylation analy-
sis, sections were incubated in 4 N HCl for 20 min prior to blocking. A 10% solution of
normal donkey serum (Jackson Immunoresearch) in PBS was used as a blocking buffer.
Sections were incubated with the following primary antibodies diluted in blocking solution
(0.25% BSA, 0.3% Triton X-100, sterile PBS) overnight at 4°C: VASA (R&D Biosystems),
NUMA, STELLA, DAZ, DAZL (Abcam), PLZF (Calbiochem), UTF1 (Millipore), 5-MC
(1:1500; Eurogentec), 5-hMC (1:1500; Active Motif) and GATA4 (Santa Cruz Biotech).
The sections were washed and immunofluorescence staining was performed by incubating
sections with fluorescently labelled secondary antibodies raised in donkey. Stained sec-
tions were mounted in ProLong Gold Anti-fade mounting media containing DAPI (Life
Technologies). Negative controls included incubation with Rabbit IgG antibodies and
omission of the primary antibody for all samples.

3.1.2.24 Statistical Analysis

Analysis of variance and statistical comparisons were performed using R, GraphPad Prism
(La Jolla, CA) and SPSS (IBM, Armonk, NY) with statistical significance set at 0.05.
Student’s two-tailed t test was used to determine statistical significance for data generated
from Fluidigm gene expression assays and quantitative analysis of cross-sections. Fisher’s
exact test was performed for bisulfite sequencing analysis. For single-cell analysis and
visualization R packages were used.
3.2 Biophysical Materials and Methods

3.2.1 Materials

3.2.1.1 Microscope and software

- Images were acquired with a LEICA DMI 4000 B fluorescence microscope and a LSM 200 confocal microscope.

- Leica Application Suite (v3.6.0) for database and image handling.

- ImageJ and Adobe Photoshop for image acquisition.

3.2.2 Methods

3.2.2.1 Image Acquisition

Image acquisition for quantitative time course experiments was performed with a LEICA DMI 4000 B microscope. Images were acquired with same microscope settings (gain, brightness, saturation) to standard conditions for software analysis. Each acquired images was representative for each well. Background correction was done with the plugin Subtract Background and BG Subtraction from ROI under ImageJ followed by a manual adjustment. A script was written to ensure high throughput analysis of fluorescence intensities.

Quantification of stained sections for NUMA/VASA double staining was determined manually from 3-5 independent 10x fields taken from 3-5 different depths in testis tissue and from at least 3 separate biological replicates (confocal microscope). Data for statistical analysis followed normal distribution. N for each samples was as follows: iPSC.OSKM - 8, iPSC.OSKMV - 8, Human Testis - 3, H1 - 4, H9 - 4.

3.2.2.2 Data handling

Images were collected and saved as LAN files and converted into TIFF images.
3.3 Technical notes

The protocols for mRNA reprogramming used in this study are multisteped and complex. In addition, RNA work requires extra precaution and an RNase free work environment. Therefore, it is advised that in order to apply this methodology, all steps described are followed rigorously and are quality controlled. In detail, synthesized mRNA needs to be verified by denaturing formaldehyde agarose gel for correct size and specificity. An aliquot of each batch of synthesized mRNA needs to be transfected and expression of proteins must be confirmed 24 h later by immunostaining. The inclusion of GFP during any long term reprogramming experiment monitors the transfection ability and is therefore suggested.
Chapter 4

Results

4.1 Novel isoforms in human pluripotent stem cells and their role during nuclear reprogramming

Au et al. recently discovered novel transcribed isoforms in a human embryonic stem cell line (H1) by combining second and third generation RNA sequencing and applying a custom method annotation [69]. Among those novel transcripts were 273 that are transcribed from previously unidentified gene loci. We selected the top 23 highest expressed novel genes (referred as HPAT for Human Pluripotent Associated Transcript) and conducted a single cell gene expression analysis to elucidate their putative role in pluripotency maintenance and/or establishment.

4.1.1 Novel genes are specifically expressed in a pluripotent context

In the study by Au et al., a male human embryonic stem cell line (H1) was subject to RNAseq analysis. We tested whether the discovered novel genes were also expressed in other pluripotent samples and whether or not they can be detected in different tissues. Using qPCR we verified their pluripotence specificity as we could only detect expression in two independent hES cell lines (H1 and H9) and one induced pluripotent stem cell line (RiPSC.HUF1). Novel genes were undetectable or expressed at very low levels in a collection of cDNAs obtained from different adult human tissues (Fig. 4.1, A). Controls included genes whose expression is highly enriched in pluripotent stem cells and tissue-specific markers (Appendix C.1, Supplementary Fig. S1 and Appendix C.2, Supplementary Fig. S2).
Figure 4.1. **Gene expression validation of HPAT.** (A) Gene expression analysis by qPCR was performed on two hES cell samples (H1 and H9), one iPS cell line (RiPSC.HUF1), and a collection of cDNAs from fetal and adult tissues. (B) Gene expression profiling of HPAT genes in blastocysts. (C) Gene expression profiling of one HPAT gene and two control genes in single blastomeres of eight-cell embryos. E1 and E2 denote the embryos from which blastomeres were isolated.
We also examined expression during human development, in single blastomeres of eight-cell embryos and in human blastocysts. We observed three different categories of expression: genes (HPAT1, HPAT15, and HPAT19) detected in the blastocysts but not in the eight-cell-stage blastomeres (Fig. 4.1, B), genes abundantly expressed in both blastocysts and eight-cell embryos (HPAT21) (Fig. 4.1, C), and genes that were not expressed in either stage of preimplantation development.

4.1.2 Single cell gene expression during nuclear reprogramming

We then used single cell gene expression analysis during nuclear reprogramming to further study the novel genes and elucidate their implications, if any, during iPS cell derivation. We hypothesized that in contrast to bulk sample gene expression, single cell gene expression has the power to unravel putative connections to known pluripotency markers or other epigenetic markers that play crucial roles during nuclear reprogramming. We used the Fluidigm C1 technology to measure the expression of up to 96 different genes in a single cell and tracked gene expression changes over the course of reprogramming in 96 single cells collected on day 0 (fibroblast), 2, 5, 7, 10, 12 and established iPS cell line. 578 single cells passed our quality checks and were subsequently used for this analysis.

Based on gene expression kinetics over time we were able to group novel gene expression into three categories: (1) gradual, (2) late, and (3) expressed only in fully established iPS cell lines (Fig. 4.2).

Among the top 23 expressed novel genes we also included a panel of other markers that were specific for fibroblast cells (CD13, COL1A1, PDGF3B, CD90, VIM), pluripotent cells (LIN28A, DNMT3B, SOX2, NANOG, TDGF1, POU5F1, UTF1, SALL4), epigenetic modifiers (BPTF, DNMT1, EED, GLP, G9A, P300, EZH2, JARID2, KDM3B, MBD3, MCRS1, MLL2, BRG1, SNF2H, HP1, TAF1, TET1, THAP11, WDR5, NPM1), ES cell cycle regulators (BUB1, CDC20, CDKN1A, LATS2, MAD2L1, RBL1), and members of specific pathways (CDH1, CDKN2A, GRB2, LEFTY2, LMNB1, MAPK1, MAPK3). This allowed us to calculate positively and negatively correlated pairs of genes during reprogramming identifying possible regulatory linkages (Fig. 4.3, A). Among the highest positively correlated gene pairs were POU5F1/SOX2, LIN28A/SOX2 and DNMT3B/SALL4, all well established interactions that have been reported in the literature [138], [139], [140]. Interestingly, one of the novel genes (HPAT3) revealed strong positive correlation with DNMT3B, a DNA methyltransferase, implicating its possible (in)direct interaction in epigenetic modifications (Fig. 4.3, B).

To establish cell-type specific patterns of gene expression that may aid our understanding of network activity and cell state transitions, we performed hierarchical clustering
and PCA using the expression data for all genes including novel transcripts in all 578 cells. PCA can visually separate different populations (time points at which cells were collected) based on single cell gene expression (Fig. 4.3, C). It is also clear that established pluripotent cells separate the most from the rest of the populations indicating their mature phenotype. We then only considered pluripotency markers for PCA and revealed a clear separation between fibroblast, early reprogrammed cells (day 2-5), and late reprogrammed cells (day 7-12) (Fig. 4.3, D). Established pluripotent cells clustered within the late (day 12) cell population and were indistinguishable from late reprogrammed cells. This is in contrast when only considering novel genes for PCA. Our analysis revealed a clear separation between late (Day 12) and iPS cells from the rest of the populations (Fig. 4.3, E). Moreover, gene expression of novel factors has the potential to separate fully established iPS cells from cells that are about to become an iPS cell, even the late reprogrammed cells, suggesting that these novel markers may be bona fide pluripotency markers that are more reliable to screen for fully reprogrammed cells.
Figure 4.3. **Multivariate data analysis to study novel genes** (A) Correlation analysis of all genes on all 578 single cells during reprogramming. Positively correlated gene pairs in blue and negatively correlated gene pairs in red. (B) Top 11 positively correlated gene pairs and their correlation coefficient R. (C) Principal component analysis (PCA) on all genes across all 578 single cells. Each dot represents a single cell. (D) PCA on all pluripotency associated genes including novel genes. (E) PCA on exclusively all novel genes. Novel genes are denoted based on their location on the human genome.

Correspondence analysis reveals that some of the novel genes (in red) play an important role late during reprogramming since they cluster closer together with single cells from day 10-12 (Fig. 4.4, A). Key pluripotency markers (SOX2, POU5F1) cluster with single cells that were collected at day 7 suggesting an earlier role during reprogramming. Classic fibroblast markers (CD13) cluster with control cells (GFP transfected only) or fibroblast cells.

Unsupervised clustering in a heatmap plot underlines the finding that each population that was collected at a different time point represents a unique transcriptome despite the heterogeneous character of single cells within each single population (see PCA plots) (Fig. 4.4, B).

To examine whether reprogramming involves random or sequential activation of marker
Figure 4.4. Elucidating putative functions of novel genes. (A) Correspondence analysis plot. Genes in red and samples (single cells) in green. The proximity of a given gene to a sample determines its importance for the separation of that sample from the rest of the samples. (B) Heatmap of gene expression of all 578 single cells. (C) Bayesian network with pluripotency associated genes including novel genes. Novel genes are denoted based on their location on the human genome.
genes, we derived a Bayesian network with pluripotency associated genes at all stages during reprogramming. A Bayesian network is a probabilistic model that represents a set of random variables and their conditional dependencies. We applied this model to our multivariate data set (Fig. 4.4, C). The network predicted that the activation of endogenous SALL4 initiates a series of consecutive steps leading to the activation of many pluripotency genes. It also predicted that three key pluripotency markers - OCT3/4, SOX2 and NANOG - form a network that has been shown to be crucial for establishing the pluripotency network and for maintaining pluripotency. Moreover, the network suggested that three of the novel transcripts may have putative roles in this cascade of gene activation as they are in close proximity to well established pluripotency markers.

4.2 Derivation of induced pluripotent stem cells with two factor combinations

4.2.1 Addition of VASA to OSKM reprogramming mix does not affect reprogramming efficiencies or kinetics

Synthesized modified mRNAs, including VASA mRNA, were produced and validated prior to use as shown (Appendix C.3, Supplementary Fig. S3 and Appendix C.4, Supplementary Fig. S4A-C); protein expression and localization was confirmed by immunocytochemistry (Fig. 4.5). We added synthesized VASA mRNA to OSKM factors in molar ratios of 3:0.5:1:0.5:1 (OSKMOV) and examined reprogramming efficiency and kinetics of different somatic lines [BJ (XY), HUF1 (XY), HUF3 (XX), and HUF9 (XX)] with either the OSKM or OSKMOV cocktail in parallel. We adapted a previously reported protocol [141] in which feeder-free iPS cell derivation was accomplished with 6 mRNA factors (OSKM + NANOG + LIN28A) and modified OCT3/4. We successfully derived feeder- and xeno-free iPS cells with OSKM alone and with OSKMOV (Appendix C.4, Supplementary Fig. S4D-F). We observed colony formation with both OSKM and OSKMOV reprogramming with no significant differences in colony number (around 0.5 % efficiency) or timing of colony appearance (6-12 days post first-transfection). We then further analyzed the XY lines, HUF1 and BJ, and compared OSKM- and OSKMOV-derived lines across a series of functional and molecular assays beginning with a comparison of morphology, alkaline phosphatase (AP) staining and embryoid body (EB) formation. We did not observe notable differences, regardless of reprogramming cocktail used (Appendix C.5, Supplementary Fig. S5A). Colonies reprogrammed with OSKMOV were characterized by a high nucleus/cytoplasm ratio, prominent
nucleoli, well defined borders and a distinguishing chromatin structure and nuclear architecture (speckles and heterochromatin domains), all features that are very similar to OSKM reprogrammed colonies and human embryonic stem cells [142].

![Immunostaining of VASA in mRNA transfected fibroblasts 24 h after transfection. VASA protein localized correctly in the cytoplasm. Mock transfected, secondary antibody stained only and non-transfected samples served as negative controls. Scale bar, 10 µm.](image)

**Figure 4.5. Functional validation of mRNA expression encoding for VASA.** Immunostaining of VASA in mRNA transfected fibroblasts 24 h after transfection. VASA protein localized correctly in the cytoplasm. Mock transfected, secondary antibody stained only and non-transfected samples served as negative controls. Scale bar, 10 µm.

### 4.2.2 Transient ectopic VASA expression alters gene expression signatures of derived iPS cell lines

As part of the assessment of pluripotency, we examined endogenous gene and protein expression of various markers associated with pluripotency including POU5F1, NANOG, SALL4, and DNMT3B in both OSKM and OSKMV colonies (Appendix C.5, Supplementary Fig. S5C). Notably, expression of a subset of markers associated with pluripotency was lower \((p \leq 0.05)\) in lines reprogrammed with OSKMV relative to their OSKM counterpart, with PRMT5, SALL4, and DPPA4 being the most significantly different \((p \leq 0.001)\).

We also confirmed a similar reduction in expression of a subset of genes in lines that were derived with OSKM or OSKMV via a lentiviral reprogramming strategy to exclude reprogramming strategy related events (Appendix C.5, Supplementary Fig. S5C). We then examined effects of transient ectopic expression of VASA during reprogramming on expression of genes associated with early germ cell development. We observed that the majority of markers showed gene expression levels similar to the lines reprogrammed with OSKM alone and/or the parental fibroblast line, indicating no gene activation (exemplified by PRDM1). However, a subset (PRDM14, DPPA3 [STELLA], and VASA) was expressed at significantly higher levels \((p \leq 0.001)\) in iPS cell lines reprogrammed with OSKMV relative...
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to OSKM derived colonies, indicated for iPSC.HUF1 cells (Fig. 4.6A). Results were partially mirrored by the lentiviral-derived HUF1 iPS cell line and mRNA derived iPSC.BJ cells. We detected a smaller subset of germ cell markers at a higher expression level in OSKMV derived lines of which only PRDM14 (for iPSC.BJ) was significantly upregulated compared to the OSKM counterpart (Appendix C.5, Supplementary Fig. S5D). We note that gene expression was measured at two different passages (passage 4 and 14) to eliminate the possibility of expression from exogenous mRNA and to demonstrate stability of the distinct endogenous gene expression profile. We further confirmed endogenous VASA gene expression in OSKMV cells with immunocytochemistry (Appendix C.5, Supplementary Fig. S5B).

4.2.3 OSKMV derived lines, similar to OSKM derived lines, are fully pluripotent

To further assess whether mRNA-reprogrammed OSKM and OSKMV lines are both fully pluripotent, we performed a variety of molecular and functional assays. Spontaneous differentiation revealed that all lines form all three germ layers in vitro (Appendix C.6, Supplementary Fig. S6A). Epigenetic analysis revealed similar methylation status of OCT3/4 and NANOG promoters of both OSKM and OKSMV derived HUF1 lines comparable to hES cells, which were substantially different relative to the parental fibroblast line (Appendix C.6, Supplementary Fig. S6B). Moreover, we observed that all clones were karyotypically normal and differentiated to all three germ layers in vivo in teratoma assays, following kidney capsule injection into SCID mice (Appendix C.6, Supplementary Fig. S6C and D). Taken together, these results indicate that mRNA derived iPS cell clones from multiple independent fibroblast lines, with and without addition of VASA are fully reprogrammed by the most stringent assays available to date for human pluripotent cells [143].

4.2.4 OSKMV and OSKM derived clones respond differently to BMP4 treatment

VASA overexpression from lentiviral vectors has been reported to drive differentiation into primordial germ cells (PGCs) and more mature germ cells when overexpressed in both human ES and iPS cells [81]. In mice, PGC specification is characterized by localized BLIMP1 (PRDM1) expression in a small number of cells in the posterior, proximal mouse epiblast at E6.25 followed by upregulation of STELLA around E7.25 [144]. Several groups have demonstrated in vitro differentiation of PGCs from both mES and hES cells via media supplementation with bone morphogenetic proteins (BMPs) [78], [79], [80],
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Thus, we hypothesized that lines derived with OSKMV might reveal a different potential in their ability to respond to BMP4 relative to OSKM cells. We supplemented culture media of both OSKM and OSKMV derived lines with BMP4 over the course of four days and assessed activation of gene expression of early germline markers. We included two controls - an mRNA derived (OSKM) BJ fibroblast line and a hES cell line (H9). Although a subset of genes, including NANOS2, RET, and Y chromosome DAZ2 among others, did not show significant changes in gene expression in either the OSKM or OSKMV derived lines, expression of NANOS3, VASA, and DPPA3 was significantly upregulated (p ≤ 0.01) post-BMP4 treatment in the line derived with OSKMV after four days of BMP4 treatment (Fig. 4.6B). iPS cells derived with OSKM as well as controls did not demonstrate differential expression of these germ cell markers in response to BMP4 treatment, indicating that observed differences are confined to different reprogramming factor cocktails used during iPS cell derivation. We also examined gene expression changes of pluripotency-associated markers during BMP4 supplementation (Appendix C.7, Supplementary Fig. S7A and B). DNMT3B, POUF51, and SALL4, all genes that are strongly expressed in hES and iPS cells, showed a rapid decrease in gene expression after four days of BMP4 treatment in both OSKM and OSKMV derived lines as well as both control lines, as expected (Appendix C.7, Supplementary Fig. S7B).

4.2.5 OSKMV and OSKM derived lines display distinct SCP3 staining patterns

Synaptonemal Complex Protein 3 (SCP3) encodes a meiosis-specific protein that is essential for formation of meiotic synaptonemal complexes of the maternal and paternal homologous chromosomes [145]. Its localization and distribution along the chromosomes indicates meiotic progression in pluripotent stem cell differentiation to germ cells [79], [80], [146]. Ectopic overexpression of VASA in pluripotent human iPS cells has recently been shown to promote meiotic progression as judged by positive SCP3 staining patterns [81]. Thus, we compared expression and localization patterns of SCP3 protein in OSKM and OSKMV differentiated cells. Our analysis revealed that the majority of cells regardless of VASA mRNA addition during reprogramming stained negative for SCP3 protein indicating no meiotic activity (Fig. 4.6C and Appendix C.7, Supplementary Fig. S7C). Nonetheless, clusters of differentiated cells showed a punctuated staining pattern, indicative of early pre-meiotic events (leptotene stage); moreover, cells reprogrammed with OSKMV displayed slightly higher percentages (p ≤ 0.05) of cells positive for SCP3 staining. In addition, we noticed a distinct staining pattern that was most prominent in H1 hES cells and in iPS cells reprogrammed with OSKMV consisting of elevated SCP3
clusters with elongated structures indicative of late, albeit disorganized, assembly of meiotic chromosomes in zygotene, pachytene or diplotene meiotic prophase I stages [79], [80]. This staining pattern was observed in approximately 16% of cells, a percentage that is significantly higher (p ≤ 0.05) than in the OSKM reprogrammed cells or the female hES cell control (8% and 10%, respectively) (Fig. 4.6C). Few cells in the undifferentiated negative female control (H9 hES cell) displayed SCP3 staining patterns as expected. Interestingly, the second differentiated male control (H1 hES cell) line displayed the greatest number of cells with elongated SCP3 staining pattern comparable to differentiated OSKMV cells.

4.2.6 Analysis of epigenetic status and global transcription profiles

We next examined the epigenetic status and global transcriptional profiles of derived lines (Appendix C.7, Supplementary Fig. S7D and E). We analyzed four imprinted genes via bisulfite sequencing: KCNQ1OT1- and PEG1/MEST-linked differentially methylated regions (DMRs), both maternally imprinted [147], [148], as well as the H19 DMR and H19 promoter loci, both paternally imprinted [149]. Our analysis revealed slight but significant differences (p ≤ 0.0001) in methylation status of both maternally- and paternally-imprinted loci, in particular, the maternally-imprinted KCQ1OT1 and the paternally-imprinted H19 promoter region (Appendix C.7, Supplementary Fig. S7E). For both loci, the OSKMV line showed a significantly lower percentage of methylated sequences compared to the OSKM counterpart and the parental HUF1 fibroblast line. To further test our findings, we also included an additional control of three independent pooled male sperm samples. We observed little if any methylation at the KCNQ1OT1 and PEG1/MEST loci as expected for maternal-specific germline methylated loci. Also, as expected, the paternally imprinted H19 DMR region demonstrated near-complete methylation. In contrast, to the H19 DMR, which acquires methylation during gametogenesis, the H19 promoter is further methylated following implantation [150] and was characterized by few methylated sequences (18%). Equal proportions of fully methylated and fully unmethylated DNA was detected for PEG1/MEST in both, the OSKM and OSKMV line (p ≥ 0.05, X^2 test). We note that methylation patterns of imprinted genes are diagnostic of germ cells and that cells were analyzed in their undifferentiated state.

To examine global gene expression, we performed RNAseq followed by differential gene expression analysis on the OSKM and OSKMV derived HUF1 line together with controls (parental HUF1 and H9). As expected, gene expression profiles between HUF1 fibroblasts and the three pluripotent populations (OSKM, OSKMV, H9 hES cells) were significantly different (p ≤ 0.05) (Appendix C.8, Supplementary Fig. S8). Interestingly, HUF1 fibroblasts and H9 hES cells expressed more genes at significantly different levels (p ≤ 0.05) than
Figure 4.6. Functional and molecular studies of iPSC.HUF1 derived with OSKM and OSKMV. (A) Gene expression analysis of markers associated with the germline lineage in an undifferentiated state. Three genes (PRDM14, VASA, DPPA3) showed significantly higher expression in OSKMV derived clones (purple bar) compared to OSKM. (student t-test, mean ± s.d.; n ≥ 16 for each gene and sample *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). (B) Gene expression analysis of clones derived with OSKM and OSKMV, iPSC.BJ line, and H9 during in vitro differentiation into primordial germ cells with BMP4. Samples were isolated at day 0, 2, and 4 post BMP4/vehicle treatment. Three key markers (NANOS3, VASA, DPPA3) were upregulated in OSKMV derived clones (student t-test, mean ± s.d.; n ≥ 16 for each gene and sample *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). (C) Quantification of meiotic spreads. Approximately 200-300 cells were counted for each line. Staining patterns were classified as negative, punctuated or elongated, p = 0.0299 for elongated and p = 0.0077 for punctuated staining between OSKM and OSKMV cells. (student t-test, mean ± s.d.; n ≥ 200 for each sample).
HUF1 fibroblasts compared to their iPS cell derivatives (Appendix C.8, Supplementary Fig. S8C). Pairwise volcano and scatter plots emphasize the gene expression differences between each sample (Appendix C.8, Supplementary Fig. S8A and B). We further explored the relationship between all four lines and visualized the Jensen-Shannon (JS) distances in a heatmap and a significant genes (p≤0.05) overview matrix across all genes (Appendix C.8, Supplementary Fig. S8C and D). Principal component analysis (PCA) and multi-dimensional scaling (MDS) revealed a very similar global gene expression in the OSKM and OSKMV derived lines that were significantly different (p≤0.05) from the parental fibroblasts (Appendix C.8, Supplementary Fig. S8E and F). Derived iPS cell lines also were distinct from the H9 hES cell control but revealed a similar global gene expression phenotype (all three pluripotent lines with a positive second PC in PCA analysis). Hierarchical clustering analysis (Appendix C.8, Supplementary Fig. S8G) and a heatmap of differentially expressed genes (Appendix C.8, Supplementary Fig. S8H) highlighted the relationship between all pluripotent stem cell lines. When we compared both derived iPS cell lines with hES cells, we observed only a few genes, isoforms and transcription start sites (TSSs) with statistically significant differential expression, consistent with previous reports [151], [152].

4.3 In vivo differentiation of iPS cells into the germ cell lineage

4.3.1 Xenotransplantation directs germ cell formation in OSKM and OSKMV reprogrammed cells

There are no previous reports of transplantation of human pluripotent stem cells to the seminiferous environment in vivo. The use of testicular transplantation is common for teratoma analysis; however, as commonly used, teratoma formation assays rely on transplantation under the testis capsule. In contrast, here we made use of a xenotransplant assay in which cells from reprogrammed lines were directly injected into the seminiferous tubules of busulfan-treated immune deficient nude mice as previously described [136], [153]. We used this assay to investigate OSKMV and OSKM donor cell differentiation into germ cells in vivo with injection directly into mouse seminiferous tubules. Specifically, we hypothesized that the inclusion of VASA during reprogramming would confer a unique germ cell competent state. As previously reported, transplanted human spermatogonial stem cells migrate to the seminiferous tubule basement membrane and proliferate to form chains and patches of spermatogonia that persist long-term [153]. Note that complete spermatogenesis is a function of evolutionary distance such that rat transplantation into
mouse tubules or human transplantation into nonhuman primates is expected to yield complete spermatogenesis whereas human transplantation of germ cells into mouse or rat tubules will be limited to formation of prospermatogonia, spermatogonia or and/or possibly early meiotic derivatives [153]. As a positive control, we transplanted human fetal testicular cells (22 week old tissue) into busulfan-depleted spermatogenic tubules of immunodeficient mice and observed clusters and chains of spermatogonia two months post-transplantation (Fig. 4.7A, left panel). Transplantation of H1 hES cells (XY karyotype) and H9 hES cells (XX karyotype) served as controls; we hypothesized that H9 hES cells would result in formation of few, if any, germ cells in seminiferous tubules relative to the XY hES cells. Two standard methods to examine the potential to form germ cells in transplants were pursued: Immunohistochemistry of serial sections of fixed tissue and whole mount staining of testis to assess potential differentiation to chains of spermatogonia, in vivo. Whole mounting is only possible when engraftment occurs in the absence of large masses post-transplantation. For immunohistochemical (IHC) analysis of serial cross-sections of transplanted tissues, we used a panel of very well-characterized germ cell markers [79], [96], [100], [154], [155], [156].

We injected undifferentiated OSKM and OSKVM iPS cells into seminiferous tubules of 8 testes each to evaluate their potential to form PGCs, gonocytes or spermatogonia in vivo in a transplant assay. Depending on which factors were used during reprogramming, we obtained strikingly different results. Testes injected with OSKVM cells maintained their naïve tissue structure two months post injection and 1 out of 8 testes was positive for a cluster of human cells that persisted long term (Fig. 4.7A, right panel and Appendix D.1, Supplementary Tab. S1). In contrast, testes transplanted with OSKM cells developed large internal proliferating cell masses with no signs of teratoma formation in all 8 out of 8 transplanted testes - transforming the testis into an enlarged tissue (Fig. 4.8A and B and Appendix C.9, Supplementary Fig. S9A). To examine outcomes more extensively, we then repeated the transplantation and used the same method of direct comparison of germ cell activity across all transplantations. Our replicate transplantations consisted of OSKVM cell injections into the seminiferous tubules of an additional 6 testes with subsequent IHC analysis of serial cross sections regardless of tissue structure. We confirmed previous observations that indicated that OSKVM-derived iPS cells do not form large masses of cells but instead leave the mouse testis structure intact. In direct contrast, testes transplanted with undifferentiated H9 (Fig. 4.8A, v) and H1 (Fig. 4.8A, iii) hES cells, developed enlarged tissues indicative of tumors similar to OSKM cells (Fig. 4.8A, ii) and in addition formed teratoma-like structures (in H9 cells only, Fig. 4.8A, vi) indicative of multiple germ layer tissue formation (see also Appendix D.1, Supplementary Tab. S1). We observed that H1 cells that were not localized to the basement membrane did not
Figure 4.7. Transplantation of OSKM and OSKMV cells into busulfan-treated mouse testes. (A) Whole mount analysis on transplanted human fetal testis cells and OSKMV cells into mouse testes. Chain (white dashed rectangle) and cluster formation (white arrows) visible in human fetal testis control cells. Transplanted OSKMV cells gave rise to cluster formation indicated by white dashed rectangle. Scale bar, 50 µm. (B) Histology cross-sections of tubules inside mouse testes stained with co-localizing human-specific NUMA (red) and VASA (green). White arrows indicate transplanted cells positive for VASA germ cell marker; red dashed arrows indicate VASA negative transplanted donor cells. Scale bar, 50 µm. (C) Histology cross-sections of tubules of mouse testes stained with additional germ cell specific marker DAZ demonstrating NUMA co-localization. Scale bar, 50 µm. (D) H1 and H9 hES cell controls. H1 cells are positive for VASA/NUMA co-staining; H9 cells are negative for germ cell marker selection. White arrows indicate transplanted cells positive for VASA germ cell marker; red dashed arrows indicate VASA negative donor cells. Scale bar, 50 µm.
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demonstrate clear differentiation to either germ cells or somatic cells. Instead, based on
the histology of multiple xenografts, H1 and OSKM cells outside the tubules resembled the
histology of embryonal carcinoma (EC) cells and yolk sac tumors (Fig. 4.8A, vii-viii) [157].

Next, we focused on directly comparing germ cell activity and extensively screened se-
rial cross sections for a panel of well-established germ cell markers in all transplanted
mouse testis (OSKM, OSKMV, H1, H9). We compared our results to cross sections
from a 22 week-old human fetal testis sample which contain abundant prospermatogonia
and spermatogonia. Our analysis depended on the identification of human donor cells
and the ability to discriminate them from mouse cells. For this purpose, we used a hu-
man specific antibody, NUMA, that stains the cell nucleus and validated its specificity
by staining various tissues of human and mouse origin with latter staining negative for
NUMA (Appendix C.9, Supplementary Fig. S9B and C). Our analysis revealed germ
cells inside tubules of OSKM and OSKMV transplanted testes that are of human origin
(nuclear NUMA) and co-localize with several germ cell specific markers including VASA
and DAZ (Fig. 4.7B and C). In contrast, transplanted H9 (XX female) cells that were
detected inside tubules did not stain positive for any germ cell markers (Fig. 4.7D). Note,
busulfan-depleted seminiferous tubules bear the risk of incomplete germ cell depletion re-
sulting in infrequent mouse germ cells present, thus we carefully discriminated positively
stained germ cells that are of human or mouse origin (Fig. 4.9A) in all of our analysis and
subsequent quantification efforts.

4.3.2 OSKMV in contrast to OSKM reveals greater germ cell forming
potential

In order to quantify our immunohistochemical analysis, we counted tubules that stained
positive for all VASA/NUMA cells to determine the average fraction of tubules that had
residing human germ cells present (Fig. 4.8C). We observed 7 and 18 % of tubules po-
itive for both OSKM and OSKMV transplanted cells, respectively (Fig. 4.8D and E).
In contrast, we observed over 80 % of tubules filled with NUMA/VASA double positive
cells in the human fetal testis cross sections (notably, these are non-transplanted cells
and naturally-existing germ cells). We then considered only those tubules positive for
NUMA/VASA activity and counted individual cells across all sections; results indicate a
more than 4-fold difference between OSKM and OSKMV with the latter being superior
(Fig. 4.8F). To compare germ cell production between OSKM and OSKMV derivations
and our human fetal testis control, we calculated relative germ cell numbers by multiply-
ing the percentage of occupied tubules by the number of cells per tubule and compared
them to our positive control. This indicated a 5-fold difference between OSKM compared
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Figure 4.8. Transplantation of OSKM and OSKMV followed by quantification analysis. (A) Hematoxylin and eosin staining of histology cross-sections of tubules inside mouse testes. i-v: Black arrows indicate individual tubules. Yellow arrows indicate cartilage formation in H9 transplanted cells as example for teratoma formation. Scale bar, 100 µm. vi: H9 transplanted cells formed teratomas indicated by three germ layers. vii-viii: H1 and OSKM cells formed fibrotic structures that resembled embryonal carcinoma (EC) cells and yolk sac tumors. (B) Weight of testes two months after transplantation of cells (OSKM and OSKMV cells with three controls: human fetal testis, H1 hES cell and H9 hES cell line). (C) Representative low magnification image of histology cross-section of multiple tubules stained with NUMA and VASA indicating that only a fraction of tubules formed VASA positive cells. Red dashed rectangle indicate tubules with NUMA only cells along the basement membrane. White rectangle indicate NUMA/VASA positive tubules. Scale bar, 50 µm. (D) Representative image of histology cross-section of one single tubule (for OSKMV). Single NUMA/VASA positive cells were counted for quantification. Scale bar, 50 µm. (E-G) Quantification of immunohistochemistry results of cross-sections from OSKM and OSKMV cells compared to both controls. All serial sections were subject to counting (see Materials and Methods) Scale bar, 50 µm. (E): Percentage of tubules with positive VASA/NUMA co-staining calculated (against total number of counted tubules). iPSC.OSKMV is also significantly different from H9 control \( p = 0.0432 \) (F): For each positive tubule, VASA positive cells that co-stained with NUMA were counted and calculated against positively stained tubules. iPSC.OSKMV is also significantly different from H9 control \( p = 0.0397 \) (G): Relative germ cell forming potential calculated by multiplying fraction of positively stained tubules with number of VASA/NUMA co-stained cells for each sample. iPSC.OSKMV is also significantly different from H9 control \( p = 0.0417 \).
to OSKMV and the human fetal testis, which was significant (Fig. 4.8G). Analysis of H1 donor-derived cells revealed results that were similar to OSKM transplanted cells. As highlighted from the IHC analysis, mouse testes transplanted with H9 cells had lower germ cell activity in our quantitative analysis, which, however, was not significant (Fig. 4.8E-G).

In order to further verify that germ cells were induced from iPS cells in vivo, we extended our analysis of previously characterized germ cell markers [79], [96], [100], [154], [155], [156] and demonstrated co-localization of three sets of PGC and pre-meiotic germ cell markers (VASA/DAZL, STELLA/UTF1, and VASA/UTF1) with NUMA (Fig. 4.9C, Panel 1 and 2) for OSKM and OSKMV transplanted cells in conjunction with the positive control (Fig. 4.9C). In addition we observed, in rare instances, cells that stained positive for GFRα1 cells (Fig. 4.9C, Panel 3), suggesting that OSKMV iPS cells are not only superior in germ cell formation to OSKM iPS cells in vitro but also in vivo.

To evaluate whether transplanted cells differentiated to other lineages (such as Sertoli cells) or remained in their undifferentiated state, we co-stained for NUMA and germ cell markers with GATA4, a Sertoli cell marker. We observed GATA4 positive cells (Fig. 4.9B) at the basement membrane of tubules that did not co-stain with NUMA, indicating their murine origin; we note that the GATA4 cells resided in the vicinity of NUMA/DAZ double positive cells highlighting their supportive phenotype in germ cell differentiation. Tubules that were entirely filled with large numbers of cells both intra-tubular and extra-tubular appeared to be unorganized, of human origin and OCT3/4 positive, suggesting an excess of transplanted cells that retained their undifferentiated state (Appendix C.9, Supplementary Fig. S9D). This is in contrast to OCT3/4 positive cells that did not co-localize with NUMA but instead revealed an organized staining pattern along the basement membrane indicating endogenous cell of mouse origin. We observed that a small number of the transplanted iPS cells may escape during injection particularly in H1 and H9 hES cell-containing testes. Specifically, H1 and H9 donor cells appeared to exit the seminiferous tubules and entered the extra-tubular space within the mouse testis as illustrated by positive NUMA staining outside mouse tubules (Appendix C.9, Supplementary Fig. S9D, middle panel). Since the cells are undifferentiated iPS cells, however, they may subsequently proliferate.
Figure 4.9. Germ cell formation of OSKM and OSKMOV transplanted cells in vivo. (A) Observed endogenous germ cell activity inside the basement membrane of transplanted mouse testes. Cells stained positive for VASA, PLZF and DAZ but do not co-localize with NUMA indicating residual germ cells after busulfan treatment. Scale bar, 50 µm. (B) Detection of Sertoli cells indicated by GATA4 staining that is of mouse origin and near OSKMOV transplanted cells at the basement membrane of mouse testes. Scale bar, 50 µm. (C) Additional germ cell markers stain positive with NUMA in OSKM and OSKMOV transplanted cells in mouse testes and co-localize with each other. GFRα1 was only detected in OSKMOV transplanted cells and human fetal testis control sections. Yellow dashed rectangles indicate magnified snapshots in each panel and for each sample. Each panel shows single individual protein and DAPI (left) and a zoomed out merged image (right). Scale bar, 80 µm
4.3.3 Epigenetic analysis of donor-derived germ cells from H1 hES cells, OSKM and OSKMV iPS cells

In order to evaluate if germ cells derived from donor iPS cells were specifically undergoing epigenetic remodeling events such as demethylation and conversion of 5-methylcytosine (5-MC) to 5-hydroxymethylcytosine (5-hMC) [158], [159], we performed immunohistochemistry for 5-MC and 5-hMC in human fetal testes and in recipient mouse testes xenografts as previously demonstrated for embryo analysis [160] (Fig. 4.10). We observed that in fetal testes, the levels of 5-MC appeared reduced in a large number of germ cells compared to 5-hMC levels. This was particularly evident in that a subset of VASA-positive germ cells had little to no 5-MC signal and a robust 5-hMC signal in fetal germ cells (Fig. 4.10A). We stained for NUMA/VASA in adjacent tissue sections and observed that OSKM, OSKMV
and H1 hES cell donor-derived cells exhibited a similar pattern of 5-MC and 5-hMC signals to fetal germ cells (Fig. 4.10, arrows). 5-MC levels were low to absent in NUMA+VASA+ donor cells indicating that global DNA demethylation was initiated but only partially complete owing to the maintenance of 5-hMC levels, which is an intermediate step in germ cell DNA demethylation. We also noted that NUMA-positive donor cells of OSKM and OSKMV xenografts that were VASA-negative and not considered germ cells expressed very high levels of both 5-MC and 5-hMC (Fig. 4.10C and D, right column). These observations collectively suggest an epigenetic transition from 5-MC to 5-hMC expression in fetal germ cells which is mirrored in germ cells derived \textit{in vivo} from undifferentiated OSKM and OSKMV donor cells.

Figure 4.11. Model of transplantation of undifferentiated iPS cells into mouse testes. A schematic summarizing the major findings of this study. iPS cells reprogrammed with OSKM and OSKMV displayed differences in their phenotype after derivation. When transplanted, iPSC.OSKMV cells specifically differentiate to PGCs inside the spermatogonial tubule niche where they contact Sertoli cells and were positive for a set of different stage-specific germ cell markers including GFRα1. iPSC.OSKM cells also localized inside the mouse testis niche and expressed key germ cell markers but to a lower extend and frequency. In addition, iPSC.OSKM cells developed enlarged cell masses inside the mouse testes that resembled embryonal carcinoma-like tissue. This is in contrast to iPSC.OSKMV where all transplanted mouse testes kept their naïve structure. The transplantation strategies proposed here offer potential avenue for fertility restoration for infertile men.
4.4 Moving one step forward towards clinical applications

4.4.1 Conversion of research grade iPS cells to GMP grade iPS cells

In order to make our lines GMP-compliant we next converted them to a xeno-free substrate and a fully defined media environment while maintaining a pluripotent phenotype. We focused in a proof-of-principal fashion on four OSKM derived lines including BJ, HUF1, HUF58, and GM13325 (Appendix D.2, Supplementary Tab. S2). Two strategies to fully convert our lines towards GMP-compatible conditions were applied: (1) Cells were first gradually converted to a 1:1 blend of TeSR2/Nutristem (both xeno-free) and then passaged onto a new substrate (Synthemax). (2) Cells were first passaged onto Synthemax before the media was gradually switched from mTeSR1/Nutristem to a 1:1 blend of TeSR2/Nutristem. The latter proved to be more feasible and was performed over a period of 14 days (Fig. 4.12). Noteworthy, the switch from mTeSR1/Nutristem to TeSR2/Nutristem blend resulted in a drastic change in cell morphology indicating widespread spontaneous differentiation. Colonies that retained morphology typical of undifferentiated cells were selected and manually passaged multiple times until a uniform and stable culture of undifferentiated cells was achieved. Following this procedure we successfully converted multiple lines including BJ and HUF1 derived with OSKM that have been used in this study to the new media and new substrate within the GMP facility. These cells passed stringent tests for sterility including tests for the absence of Gram positive and negative bacteria, fungi, mycoplasma and endotoxins (Appendix D.4, Supplementary Tab. S4).

Expression of key pluripotency markers remained positive (Fig. 4.13A) by immunofluorescence, emphasizing their undifferentiated character after full conversion. Karyotype...
analysis (Fig. 4.13B) was performed to exclude any chromosomal abnormalities due to the colony selection. Short tandem repeat analysis confirmed the clonal character of our lines and no match of the DNA fingerprint pattern of the cell lines with any other cell published in the ATCC, NIH or DSMZ website (Appendix D.4, Supplementary Tab. S4). Taken together, we demonstrate that our protocol of derivation of iPS cells can be used to derive pluripotent lines that could be successfully transferred into a GMP facility and that could also pass the GMP compliance tests for their identity, purity, safety and stability (Appendix D.2, Supplementary Tab. S2, Appendix D.3, Supplementary Tab. S3, Appendix D.4, Supplementary Tab. S4).

Figure 4.13. Molecular characterization of iPS cell lines in GMP conditions. (A) Immunocytochemistry showing expression of a panel of pluripotency markers in converted iPS cell clones (iPSC.HUF58). Scale bar, 150 µm. (B) Normal karyotype (46, XY) of iPSC.HUF58 after successful GMP conversion.

4.4.2 Derivation of iPS cells under fully defined culture conditions

Our initial protocol of derivation made use of substrates (porcine gelatin and Matrigel) and media (mTeSR1) which are not free of animal contaminants and which could not be traced for their quality. In an effort to make our protocol even more straightforward and easily reproducible in any GMP environment we attempted the derivation of iPS cell clones in a GMP compliant manner by using GMP compatible matrices for the initial seeding of the fibroblasts and by using only xeno-free culture media (Pluriton). We tested two different matrices: CELLstart and Synthemax. On both matrices, we derived multiple AP positive iPS cell colonies (around 100-200 on Synthemax and 50-100 on
CELLstart, as early as day seven. Colonies were immediately converted to a blend of TeSR2/Nutristem on the corresponding matrices, expanded and characterized. Notably the direct conversion to TesR2/Nutristem resulted in stable cultures of iPS cells that did not show any major sign of differentiation, resulting in xeno, integration and feeder free iPS cell lines. Derived lines proved to be fully pluripotent as they can differentiate in vitro into clinically relevant mesodermal, ectodermal and endodermal lineages (Fig. 4.14 and Appendix C.10, Supplementary Fig. S10).

![Supplementary Figure](image_url)

**Supplementary Figure.** Functional characterization of iPS cell lines in GMP conditions. FACS analysis and immunocytochemistry showing expression of a panel of differentiation markers of all three germ layers (endoderm, ectoderm, mesoderm) in converted iPS cell clones after directed in vitro differentiation. Scale bar, 150 µm.
Chapter 5

Discussion

Among the progeny derived from pluripotent stem cells, germ cells are possibly the most fascinating since they alone are able to create new individuals and to transmit genetic information across generations.

5.1 Defining pluripotency

Understanding the meaning of pluripotency is crucial for stem cell biologists as it is a fundamental prerequisite to discriminate between undifferentiated embryonic stem cells, induced pluripotent stem cells and cells that commit to a specific lineage. We have benefited tremendously from the breakthrough that occurred 15 years ago, when researchers, led by James Thomson, isolated human embryonic stem cells to grow them indefinitely in cell culture [4]. Despite thorough examination of human embryonic stem cells ever since, we still fail to grasp the scope of what it in fact means to be pluripotent. Recent studies suggest that it is possible to derive and culture novel pluripotent stem cells that share molecular characteristics and functional properties that are highly similar to mouse naïve ES cells, and distinct from conventional primed human pluripotent stem cells [161], [162]. Though, we still lack key information that define this specific cell state. In relation to this uncertainty, "hybrid sequencing” RNA-Seq analysis of a male hES cell line (H1), one of the most well-characterized pluripotent cell line, led to the discovery of thousands of novel isoforms of known expressed genes and hundreds of novel genes/LincRNAs from previously non-annotated gene loci. This exemplifies that our current understanding of pluripotent stem cells is far from complete and so is our annotation of its transcriptome. We examined a subset of new key players of pluripotency and their association with well-established pluripotency markers including OCT3/4, SOX2 and NANOG and find that
they may have putative roles in both establishing and maintaining pluripotency. Gene identification, even in well-characterized stem cell lines, is far from complete. It is essential to unravel the function of recently discovered genes that specifically are found in pluripotent stem cells and to add those findings to our existing knowledge of stem cell biology. Only then will we be able to accurately draw comparisons to induced pluripotent stem cells and to use both hES and iPS cells for efficient \textit{in vitro} and \textit{in vivo} differentiation studies.

Patient-specific iPS cells will provide a platform for genetic analysis of naturally occurring deletions, insertions and mutations in human germ cell development. However, although there has been great progress in differentiation of mouse PGCs from pluripotent stem cells via a combination of \textit{in vitro} priming and \textit{in vivo} use of the niche to complete spermatogenesis and oogenesis \cite{100}, \cite{101}, \cite{102}, \cite{163}, \cite{164}, \cite{165}, several roadblocks must be overcome in order to achieve efficient, reproducible human germ cell differentiation amenable to developmental genetic studies and/or potential clinical applications. In this study, we tested the hypothesis that somatic cells could be reprogrammed to a pluripotent state with an additional germline factor, VASA, in concert with Yamanaka factors. We further hypothesized that the translational regulator, VASA would endow these cells with an ability to form human germ cells more easily than cells reprogrammed with Yamanaka factors alone. Findings presented here provide the most viable strategy to date to direct human PGC differentiation for several reasons: 1) iPS cells were produced via mRNA reprogramming, thus insuring they are integration and xeno-free and do not require extensive culture, which introduces genetic and epigenetic mutations to remove DNA-based reprogramming factors, 2) undifferentiated iPS cells are modified via inclusion of VASA in the reprogramming cocktail in order to enhance germ cell development and minimize tumorigenesis post-transplantation, and 3) our strategy makes use of the seminiferous tubule niche \textit{in vivo} in order to efficiently direct human germ cell formation from iPS cells, thereby potentially minimizing errors in erasure and establishment in sex-specific imprinted genes as have previously been observed in all human studies to date \cite{79}, \cite{80}, \cite{81}, \cite{146}, \cite{166}. Finally, OSKMV cells demonstrate a remarkable tendency to form germ cells \textit{in vivo} without excessive proliferation, tumor or teratoma formation, unlike transplanted OSKM, male and female hES cell lines.
5.2 VASA reprogrammed iPS cells are distinct from wild-type iPS cells

It is notable that efforts to simply translate results, in whole, from mouse to humans in diverse systems from hematopoietic, neural, cardiac, liver and others have all demonstrated a need to develop a specific protocol for differentiation of human tissues relative to the mouse. We have found this to be the case to date with differentiation of germ cells from mouse and human pluripotent stem cells. Although some methods and molecules are transferrable (see 1.2), others are not and thus, we focused here on alterations to accommodate human germ cell differentiation. Our data reveals that reprogramming with inclusion of VASA can endow a ground state of pluripotency that is unique from OSKM and hES cell lines. This is reflected at the undifferentiated state in OSKMV cells by notably lowered expression of OCT3/4 (POU5F1), TERT and SALL4. We suggest that the lowered level of these core pluripotency regulators is sufficient to maintain several phenotypic features in OSKMV cells consistent with conventionally derived OSKM iPS cells. Interestingly, ectopic VASA expression does not affect the global transcriptional programs of OSKMV and OSKM derived iPS cells. It appears that ectopic VASA expression during reprogramming to iPS cells provides a signal that down-regulates genes linked to pluripotency but does not induce in vitro differentiation under proliferative cell culture conditions. Our data also reveals that the OSKMV reprogramming process facilitates germ cell formation from these cells. Evidence for this is provided in part by significant upregulation of pre-meiotic germ cell markers NANOS3, VASA and DPPA3 upon BMP4 differentiation and a greater potential to enter meiosis compared to the OSKM counterparts. It is worth noting that PGC culture in vitro under proliferative conditions can result in derivation of pluripotent embryonic germ cell (EGC) lines capable of differentiating to somatic and germline progeny cells; moreover, several studies have provided evidence of extensive similarity between ES cells and early germ cells [77], [167]. In contrast, the ontogeny of mouse iPS cells versus mouse PGCs is a more distant one than in humans [101]. Mouse ES and iPS cells are considered to have a more naive ground state than hES cells and therefore, an epiblast-like state is induced prior to PGC formation [100], [101], [102].
5.3 Transplanted donor iPS cells form early germ cells in vivo and do not form teratomas when reprogrammed with VASA

Given the close relationship between human iPS cells and PGCs and the more distant relationship to mouse ES cells, we tested whether differentiation of RNA-reprogrammed iPS cells might simply be directed to the germline via injection directly into seminiferous tubules. Our observations reveal differences in OSKMV versus OSKM cells in favor of a germ cell fate. This interpretation is based on several observations: Firstly, NUMA+ OSKM and OSKMV cells in testicular cross-sections integrated into or proximal to the basal membrane of the mouse tubules where spermatogonia and gonocytes normally reside in early fetal stages. Secondly, OSKM and OSKMV donor cells expressed key pre-meiotic germline markers with specific expression of the early spermatogonial marker, GFRα1 limited to OSKMV cells only. Thirdly, the efficiency of derivation of germ cells was greater with OSKMV donor cells relative to OSKM cells. Fourthly, we detected partial and in some cases complete DNA demethylation of H1, OSKM and OSKMV donor-derived cells in a germ cell-specific manner, consistent with their progression through the germ cell lineage. The DNA demethylation pattern in donor-derived germ cells closely mirrored that observed in 2nd trimester fetal gonocytes and prospermatogonia and offered us a hint that donor cells may be undergoing a germ cell-specific event. However, in order to extend our results in future studies, locus-specific imprinting and chromatin modifications will be determined and a detailed analysis of donor-derived cells and their equivalent fetal germ cells will be performed in order to pinpoint the precise stage of germ cell differentiation that donor cells are progressing through. Finally, and of particular importance if we are to probe genetics of human germ cell development in the absence of complicating factors, cells reprogrammed with OSKMV formed no teratomas, tumors or undifferentiated cell masses in all injected sites (14 testes) in contrast to OSKM cells (similar to H1 [XY] control cells).

5.4 VASA and its involvement during nuclear reprogramming

Our studies indicate that the pluripotent stem cell state is under control of a transcriptional circuitry that includes the Yamanaka reprogramming factors [59]. Previous studies indicate that this transcriptional program is implemented in the context of an 'open' chromatin state, and it has been proposed that this state allows transcriptional programs
to switch rapidly upon induction of differentiation or reprogramming [142], [168]. We suggest that chromatin remodelers that maintain the ES/iPS cell state, may re-open somatic chromatin during reprogramming thus allowing VASA (and the Yamanaka factors) to activate the transcriptional network for pluripotency and/or PGC specification. Furthermore, we suggest that the activation of a germline differentiation pathway in OSKMV cells is mediated by a molecular ‘switch’, potentially a chromatin remodeler, that detects changes in the cell culture environment or when cells are exposed to the seminiferous tubule niche. In the case of OSKMV cells, this ‘switch’ guarantees a germline fate while in OSKM cells, the switch may require the function of additional molecular mechanisms to favor the same fate. In support of this notion, chicken ES cells primed with ectopic VASA (CVH) maintained pluripotency and exhibited increased germline competence when differentiated [169]. Collectively, we compare differential potential of OSKMV cells over OSKM cells to form PGCs in the environment of the seminiferous tubule without the risk of tumor formation (Fig. 4.11). We therefore highlight the inclusion of VASA during iPS cell reprogramming as a defined method to induce germ cell fate in humans. We further speculate that other factors with conserved functions in germline specification in mice and humans such as the transcription factor PRDM14 [101], [102] or the translational regulators, NANOS3, DAZL and PUM1/2 [79], [81], [170] may also be employed in mRNA-based reprogramming to confer higher germline potential on target cells.

5.5 Transplantation of iPS cells and derivatives

Transplantation of iPS cells in various organs has successfully been reported in different disease mouse models emphasizing their potential for cell therapy (see 1.4.3). We stress here that there are no previous reports of direct injection of human pluripotent stem cells into seminiferous tubules. Inclusion of VASA in mRNA-based derivation of iPS cells and techniques for transplantation provide a defined functional assay to characterize germ cell activity in derived iPS cell populations. However, it is important to note limitations associated with cell loss and failure of donor cell foci to exhibit spermatogonial features as previously described [136]. We suggest that the titration of number of cells transplanted may be critical to avoid overloading of the somatic niche and/or creation of an artificial niche that promotes proliferation of iPS cells rather than differentiation.
5.6 The use of GMP compliant iPS cells for future clinical studies

The clinical application of human iPS (hiPS) cells requires that production of hiPS cells has to meet GMP-compatible standards but also that the derivation of specified cell derivatives (used for transplantation) from pluripotent stem cells is performed under GMP conditions. Ideally the entire process, from the derivation of hiPS cells lines from patients’ somatic cells to the differentiation of such lines into transplantable fully differentiated cells, should be performed in a GMP-compliant environment. Nonetheless previous federal oversight has indicated (and accepted in the case of the Geron Inc. clinical trial, Geron 2009) that the conversion of pluripotent stem cell lines from a research grade environment to a GMP-grade environment is an accepted practice as long as rigorous tests are run on the converted lines to make sure that no detectable contamination of pathogens of animal origin can be found in the cells. Furthermore given the multitude of technologies for hiPS cell derivation developed in the last few years it is of great importance that a simple, fast, efficient and reproducible protocol of derivation is developed that will ensure the derivation of bona fide hiPS cell clones with no integration of foreign DNA and limited accumulation of mutations due to extensive culture.

In this study we have addressed both of the questions mentioned above. We have for the first time converted hiPS cells, derived using modified synthetic mRNAs under research-grade conditions, into GMP-grade conditions. Specifically, we slowly transitioned the cells from a xeno-containing substrate and media to xeno-free conditions that maintained the pluripotent character of the hiPS cells, within a GMP-compliant facility and cultured the cells using qualified defined reagents and a standardized protocol [171]. The converted lines maintained a normal karyotype and were free of measurable contaminants of non-human origin. We achieved a 100% rate of success in the conversion. We reason that such results are due to the optimization of our protocol of derivation. In light of the limited availability of GMP cellular manufacturing facilities, these results highlight an attractive potential mechanism for converting research-grade cell lines into putatively GMP/clinical-compliant biologics for future personalized cellular therapeutics. We anticipate that the availability of these GMP-compliant and fully characterized iPS cell lines will broadly benefit the scientific community because they represent a suitable platform for drug screening and toxicology tests. Furthermore the cells could be potentially used to produce cell derivatives within the GMP environment and following the strict release criteria that the U.S. Food and Drug Administration (FDA) requires for the clinical therapies and therefore represent a useful resource for the scientific community.
In an effort to develop a more standardized protocol of derivation of integration free iPS cells that could potentially be adopted by any GMP facility for the derivation of hiPS cells, we have also successfully derived iPS cells clones by making use only of chemically defined matrices and animal-free reagents that are already in use in GMP facilities. This newly established protocol greatly surpassed our original protocol and previously published protocols of derivation of iPS cells being shorter (and therefore less expensive and with a reduced handling of the cells), reproducible (shown to successfully reprogram a cohort of fibroblasts of different ages and genomic make-ups), up scalable, and fully defined. Therefore our fully defined protocol is easily adoptable by any GMP facility for derivation of iPS cells and represents a valuable resource in clinical applications of hiPS cells.
Conclusions

In summary, coupling of mRNA reprogramming and transplantation holds great promise for fertility restoration and preservation in men. We note that the mRNA based reprogramming method is likely to be preferable for potential clinical applications with germ cells as risk of integration is absolutely minimized. Given that infertility is remarkably common, affecting 10-15% of couples with half of all cases linked to a male factor [146], we suggest that it may be appropriate to further investigate our findings in nonhuman primate models towards the goal of complete recapitulation of spermatogenesis and potential clinical applications, under strict regulatory control [172], in hopes of ultimately alleviating the devastating consequences to quality of life that are encountered by many infertile men.

Moreover, we emphasize that defined practices need to be put in place for manufacturing and using hiPS cells in regenerative medicine. Our data demonstrate in proof of principle fashion the realization of hiPS cell under GMP-compliant conditions and represents a basis for the future use of hiPS cells in clinical trials.
Chapter 7

Perspectives

1998 and 2006 mark two breakthrough events in stem cell biology over the past 15 years as they are associated with the first ever isolation and culture of human embryonic stem cells as well as the induction of pluripotent stem cells from somatic cells, respectively. Developments within pluripotency induction, improvements in cell culture and innovative advances in whole genome and transcriptome sequencing technologies are moving in a breathtaking pace. Despite the milestones that the stem cell community has seen in the past, a tremendous amount of information remains to be revealed in order for us to truly understand human pluripotent stem cells. It is both hard and exciting to predict the next 15 years of stem cell research, yet with each milestone that we encounter we move one step forward to successfully apply our findings in regenerative medicine and to tackle to date incurable conditions and diseases including Parkinson’s [20] and Alzheimer’s disease [173] and infertility [102].

Ten to fifteen percent of couples are infertile with male infertility contributing to half of the cases. In one to two percent of men, there is a severe deficiency in germ cell development. To those who suffer male infertility, there are few options. It is hoped that studies such as this will form the foundation for better understanding of male infertility, specifically on a human genome background and ultimately provide a framework for technology that can be optimized for use in clinical applications in in vitro fertilization (IVF).

With respect to germ cell formation in vitro recent studies successfully demonstrated the reprogramming of one cell fate directly to another without passing through a pluripotent state [174] and while this has not yet been achieved for germ cells, these findings open up possible alternative strategies. Despite the tremendous accomplishments in human germ cell differentiation in vitro and in vivo it remains to be seen how capable they are functionally (potential of fertilization and embryogenesis). To elude ethical and legal aspects that are inherently associated one must move towards non-human primates to
expand studies of \textit{in vivo} germ cell derivation. Previous studies have already reported the \textit{in vitro} germ cell derivation from the common marmoset [175] and cynomolgus monkey (an OldWorld monkey) [176], [177], [178] (see [118] for review). Thus, studying \textit{in vivo} germ cell formation of human iPS cells in non-human primates is a promising model.

We think we can begin primate studies in the future based on these results; we anticipate that it will be difficult to overcome regulatory barriers but will seek to move forward with safety and efficacy as a foremost concern.
Appendix A

Construction of ivT templates

A.1 PCR of ORF of interest

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid with ORF</td>
<td>25 ng</td>
<td>As needed</td>
</tr>
<tr>
<td>Forward Primer (10 µM)</td>
<td>0.3 µM</td>
<td>0.75</td>
</tr>
<tr>
<td>Reverse Primer (10 µM)</td>
<td>0.3 µM</td>
<td>0.75</td>
</tr>
<tr>
<td>2x KAPA buffer ready mix</td>
<td>1x</td>
<td>12.5</td>
</tr>
<tr>
<td>DI water</td>
<td>-</td>
<td>As needed</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

Table A.1. PCR of ORF of interest.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp. [°C]</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>20 sec</td>
<td>27</td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>15 sec</td>
<td>27</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
<td>27</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A.2. PCR of ORF of interest. PCR program
A.2 Digest of DNA2.0 vector and ORF of interest

<table>
<thead>
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<th>Final Concetr.</th>
<th>Volume [µl]</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEBuffer I</td>
<td>1x</td>
<td>3</td>
<td>1: 37°C - 1 h</td>
</tr>
<tr>
<td>100x BSA</td>
<td>1x</td>
<td>0.3</td>
<td>2: 65°C - 20 min</td>
</tr>
<tr>
<td>ORF or plasmid</td>
<td>1 µg</td>
<td>As needed</td>
<td></td>
</tr>
<tr>
<td>NheI</td>
<td>2.5 U</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AgeI</td>
<td>2.5 U</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DI water</td>
<td>-</td>
<td>As needed</td>
<td></td>
</tr>
</tbody>
</table>

Total 30

Table A.3. Digest of DNA2.0 vector and ORF of interest.

A.3 Tail PCR

<table>
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<th>Final Concentration</th>
<th>Volume [µl]</th>
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<tbody>
<tr>
<td>Plasmid with ORF</td>
<td>25 ng</td>
<td>As needed</td>
</tr>
<tr>
<td>Forward Primer (10 µM)</td>
<td>0.3 µM</td>
<td>0.75</td>
</tr>
<tr>
<td>Reverse Primer (10 µM)</td>
<td>0.3 µM</td>
<td>0.75</td>
</tr>
<tr>
<td>2x KAPA buffer ready mix</td>
<td>1x</td>
<td>12.5</td>
</tr>
<tr>
<td>DI water</td>
<td>-</td>
<td>As needed</td>
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</table>

Total 25

Table A.4. Tail PCR.

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<th>Temp. [°C]</th>
<th>Time</th>
<th>Cycles</th>
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</thead>
<tbody>
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<tr>
<td>Denaturation</td>
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<td>20 sec</td>
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<td>Annealing</td>
<td>65</td>
<td>15 sec</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 sec</td>
<td>25</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>

Table A.5. Tail PCR. PCR program
Appendix B

Primer pairs

B.1 Primer pair for ORF amplification

Forward: AAAAAGCTAGCCACCATG-ORF
Reverse: AAAAAACCGGT-ORF

B.2 Primer pair for Tail PCR

Forward: TTGGACCCTCGTACAGAAGCTAATACG
Reverse: T_{120}CTTCTACTCAGGCTTTATTCAAAGACCA

B.3 Primer DNA sequencing of DNA templates for ivT reaction

TGGTAGTGTGGGGACTC
### Primer pairs for gene expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>ACTB</td>
<td>CCAACCGCGAGAAGATGAC</td>
<td>TAGCAGCAGCTGGATAGCAA</td>
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<td>CTNNB1</td>
<td>AGCTCTTACACCCACCATCC</td>
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<tr>
<td>GAPDH</td>
<td>ACACCATGGGGAAGGTGAAG</td>
<td>GTGACCAGGCGCCCAATA</td>
</tr>
<tr>
<td>GUSB</td>
<td>CATCAGAAGAGCGGAGATCAA</td>
<td>ACAGCTGATTTGAGCATTCAC</td>
</tr>
<tr>
<td>HPRT1</td>
<td>GCTTTTCCCTGGTACAGCAGTA</td>
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<tr>
<td>HSP90AB1</td>
<td>CCTACTAATGACTGGAAGAC</td>
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<td><strong>Pluripotency</strong></td>
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<td></td>
</tr>
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<td>DPPA4</td>
<td>CCAGGAGGACTGGAATCTCC</td>
<td>TGCTGAGGAGGACCTTTTCAC</td>
</tr>
<tr>
<td>FGF4</td>
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<td>ACAGGCGAGATGCTCAC</td>
</tr>
<tr>
<td>LIN28A</td>
<td>CATCGAGAAGAGCGGAGATCAA</td>
<td>GCTGAGGAGGAGGAGCTCACC</td>
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<td>NANOG</td>
<td>TGGCGAGGAAGAAGGGAGAAGACA</td>
<td>GCTGGAGGAGGAGGAGCAGAAA</td>
</tr>
<tr>
<td>NODAL</td>
<td>TGGCAACCAACCATGCAATA</td>
<td>TCTTCTGAGGAGCAACAAC</td>
</tr>
<tr>
<td>POU5F1</td>
<td>GGGGACACTGATCGCTTTC</td>
<td>GGGGAGGAGGAGCAGGAGTA</td>
</tr>
<tr>
<td>PRMT5</td>
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<td>ATGGTCTGCATCGCAGAAGAC</td>
</tr>
<tr>
<td>SALL4</td>
<td>CATCGAGAAGAGCGGAGATCAA</td>
<td>GCTGAGGAGGAGGAGCTCACC</td>
</tr>
<tr>
<td>SOX2</td>
<td>CATCGAGAAGGCGACCAGGATTA</td>
<td>GGGGCGAGTCTGTCTTATCC</td>
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<tr>
<td>TERT</td>
<td>TACGCGACATGGAGAAGAAGA A</td>
<td>GACAGAAGAAGAAATCATCACC</td>
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<td>UTF1</td>
<td>CCGGCCGCTACAGGATCTTAAAA</td>
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<td>ZFP42</td>
<td>CCTCTTTCCTGGGATATCCAGACC</td>
<td>TTCTGTTCACACAGGGCTCA</td>
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<tr>
<td><strong>Germ line</strong></td>
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<td></td>
</tr>
<tr>
<td>DAZ2</td>
<td>ACCGCAGTGCCCTGTT</td>
<td>GGATTAACAGACAGATACCA</td>
</tr>
<tr>
<td>DPPA3</td>
<td>CATGTTACTCGAGGAGGATTC</td>
<td>ACTCCCTGAGGCTCTTTGT</td>
</tr>
<tr>
<td>GFRA1</td>
<td>TCTCCTGGGCAACCTGCTAC</td>
<td>ACTGAGGTCTGACGCAATCC</td>
</tr>
<tr>
<td>NANOS2</td>
<td>TGCCCTCCTCTGAGGCACCTA</td>
<td>ACCGTTAACGCAGGCAGTAC</td>
</tr>
<tr>
<td>NANOS3</td>
<td>CTTGACAGGGGAGGAAGACACA</td>
<td>ACTTCCCGAGCAGTCCTGA</td>
</tr>
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<td>PRDM1</td>
<td>CCTGAGGAGCAGACGGAAGAAA</td>
<td>TGGAGGATGCTGCTGATGCTA</td>
</tr>
<tr>
<td>PRDM14</td>
<td>CACTGAGGAGCAGACGGAACCTACC</td>
<td>GAGTATGCTGGAGGCTCTGAA</td>
</tr>
<tr>
<td>RET</td>
<td>CCCAGTACCTACTCTCTCTCC</td>
<td>GCCTGGCAGTTTTCCACAC</td>
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Table B.1. Primer for gene expression analysis. Primer pairs for single cell gene expression analysis are undisclosed.
## B.5 Primer pairs for bisulfite sequencing

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>OCT3/4</td>
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<tr>
<td>Forward1</td>
<td>AAGTTTTTGTGGGGGATTTGTAT</td>
</tr>
<tr>
<td>Reverse1</td>
<td>CCACCCACTAACCTTAACCTCTA</td>
</tr>
<tr>
<td>Forward2</td>
<td>GTTAGAGGTTAAGGTTAGTGGGTG</td>
</tr>
<tr>
<td>Reverse2</td>
<td>AAACCTTAAAAAACTTAACCAAATCC</td>
</tr>
<tr>
<td>NANOG</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GAGTTAAAGAGTTTTTGTTTTTTAAATAATTAT</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCCCCAAATCTAATAATTTTATCATATCTTTTC</td>
</tr>
<tr>
<td>H19 DMR</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TGTTGAAGGTTGGGGAGATAGA</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCCAAACCATAACACTAAAACCCTC</td>
</tr>
<tr>
<td>H19 Promoter</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GGTATGGTGGTTTTTTGAGGGGAGAT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CATCCCAACCCTCCCTCACCCTA</td>
</tr>
<tr>
<td>KCNQ1OT1 DMR</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TGTTGAGGAGTTTTYGGGGAGGATTA</td>
</tr>
<tr>
<td>Reverse</td>
<td>CACCTCACACCCCAAACCATACCTCAT</td>
</tr>
<tr>
<td>PEG1/MEST</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TYGTTGTGTTGTTAGTTTGTAYGGTT</td>
</tr>
<tr>
<td>Reverse</td>
<td>AAAATAACACCCCTCCTCAAT</td>
</tr>
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</table>

Table B.2. Primer pairs for bisulfite sequencing.
Appendix C

Supplementary Figures

C.1 Supplementary Figure S1

Figure C.1. Gene expression validation of HPATs - three germ layers. Gene expression profiling of tissue-specific markers (ectoderm, endoderm, mesoderm).
Figure C.2. Gene expression validation of HPATs - additional markers. Gene expression profiling of pluripotency-associated markers, trophoderm markers and blastomere specific markers.
C.3 Supplementary Figure S3

Figure C.3. Validation of in vitro transcribed mRNA encoding for VASA. (A) Quantitative measurement of ivT product before (1100 ng/µl) and after dilution to working concentration (100 ng/µl). Ratio of A_{260}/A_{280} indicates pure product. (B) Denaturing formaldehyde agarose gel for size validation. Synthesized VASA mRNA had the correct size and little degradation was observed.
Figure C.4. Derivation of mRNA induced pluripotent stem cells in feeder- and xeno-free conditions. (A) Flowchart of cloning strategy. (B) Backbone sequence and DNA template cloning strategy for in vitro transcription of modified RNA. Sequence contains all essential features such as UTR regions, a multiple cloning site (MCS), a T7 promoter and specific restriction enzyme sites. (C) Detailed sequence of in vitro transcribed template encoding a gene of interest. Arrows indicate restriction enzyme cleavage sites. +1 indicates the first base incorporated into RNA during transcription. SpeI = restriction enzyme Z, NheI and AgeI = restriction enzyme X and Y, respectively. (D) Overview of feeder-free reprogramming with modified mRNA. (E) Morphology tracking of reprogrammed human fibroblasts during the course of 8 days. Fibroblasts show early epitheliod morphology (day 4), small cluster formation that form into small hES cell like colonies (day 4-7). Small colonies grow in size and become mature iPS cell colonies (day 8). Arrow indicates forming colony. Mock transfected cells proliferated until 100% confluent by day 8. (F) Live staining against TRA-1-60 and TRA-1-81 during reprogramming for colony identification. Scale bar, 150 µm. Abbreviations: HBA, hemoglobin alpha; IVT, in vitro transcription; ORF, open reading frame; PCR, polymerase chain reaction; UTR, untranslated region.
C.5 Supplementary Figure S5

Figure C.5. Morphology and gene expression of lentiviral/mRNA derived iPS cell lines (HUF1, BJ) with OSKM and OSKMV. (A) No differences in morphology, AP staining nor the ability to form embryoid bodies detected in OSKM and OSKMV derived clones. (B) Gene expression of endogenous VASA was examined by immunocytochemistry in undifferentiated OSKMV cells. Scale bar, 15µm. (C) Gene expression of markers associated with pluripotency in lentiviral/mRNA derived HUF1 and BJ iPS cells and in lentiviral HUF1 iPS cells. A great subset of markers were significantly decreased in OSKMV derived clones compared to OSKM. (student t-test, mean ±s.d.; n ≥ 16 for each gene and sample *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). (D) Gene expression of markers associated with germ cell lineage in iPSC BJ and lentiviral derived HUF1 iPSC cells. Ectopic lentiviral VASA expression was confirmed in OSKMV cells. (student t-test, mean ±s.d.; n ≥ 16 for each gene and sample *p ≤ 0.05).
C.6 Supplementary Figure S6

Figure C.6. Pluripotency assessment of lentiviral/mRNA derived iPSC cell lines (HUF1, BJ) with OSKM and OSKVMV. (A) Immunostaining showing expression of all three lineage markers after in vitro differentiation of iPSC.HUF1 cells derived with OSKM and OSKVM. (B) Bisulfite sequencing of H9, HUF1 fibroblast and iPSC.HUF1 derived with OSKM and OSKVM. (C) Normal karyotype of iPSC.HUF1.OSKM line. (D) Derived teratomas showing ectoderm (neural rosettes, epidermis), mesoderm (cartilage), and endoderm (gut-like endothelium) of iPSC.HUF1 cells derived with OSKM and OSKVM.
C.7 Supplementary Figure S7

Figure C.7. Molecular and functional characterization of HUF1 iPS cells derived with OSKM and OSKMV. (A) Morphological changes of iPSC.HUF1 clones upon BMP4 differentiation. (B) Gene expression analysis of pluripotency associated genes during PGC differentiation. (student t-test, mean ± s.d.; n ≥ 16 for each gene and sample *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) (C) Qualitative analysis of meiotic progression. Meiotic spreads were prepared for all samples followed by immunostaining against the meiotic specific SCP3 (green or red), and counterstained with DAPI. Each image is representative. Staining patterns were classified as negative, punctuated or elongated. Scale bar, 10 µm. (D-E) Differential methylation analysis at four imprinted genes with bisulfite sequencing. D: Bisulfite sequencing of four loci in five different samples. Unique sequences of each DNA clone are represented as rows of circles, with each circle symbolizing the methylation state of one CpG (black = methylated, white = demethylated). E: Quantitative calculation of bisulfite sequencing results. Percentage of methylated CpG islands was calculated for each sample and imprinted gene (Fisher’s exact test, mean ± s.e.m.; n ≥ 14 for each gene and sample, ****p ≤ 0.0001).
C.8 Supplementary Figure S8

Figure C.8. RNAseq to assess global gene expression of OSKM and OSKMV cells. (A-D) Pairwise comparison between all four samples using scatter plots, volcano plots, JS distance, and significant genes overview matrix. (E-F) Principal component analysis (PCA) and multi dimensional scaling (MDS). (G-H) Hierarchical clustering and heatmap of differential expressed genes of RNAseq data. See Materials and Methods for explanation.
C.9 Supplementary Figure S9

Figure C.9. Transplantation of undifferentiated iPS cells into mouse testes. (A) Histology cross-sections of entire mouse testis with cell masses after OSKM cell transplantation. Testes were harvested two months after cell transplantation. Non-treated mouse testis is shown as negative control. Scale bar, 300 µm. (B) Immunohistochemistry of human and mouse tissue cross-sections and cells to validate NUMA human-specificity. NUMA antibody specifically recognizes human samples (nuclear). OCT3/4 stained positive in mouse iPS cells for positive secondary antibody control. (C) IgG control for NUMA primary antibody. Scale bar, 50 µm. (D) Representative images of OCT3/4 stained cross sections of iPS cell transplanted mouse testes. OCT3/4 co-localized with NUMA intra- and extra-tubular in an unorganized fashion. Specific OCT3/4 staining along the inside of the basement membrane of the mouse tubule appeared to be of mouse origin and did not co-localize with NUMA. Scale bar, 50 µm.
Figure C.10. **Functional characterization of iPS cell lines in GMP conditions.** Immunocytochemistry showing expression of three neuronal markers with NeuroD being the most mature marker in GMP converted iPS cell clones. Scale bar, 150 µm.
## Appendix D

### Supplementary Tables

#### D.1 Supplementary Table S1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Testes Site</th>
<th>Injected cell #</th>
<th>Outcome in whole mount</th>
<th>Testis structure</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human fetal testis – 22 weeks cells</td>
<td>R</td>
<td>1965950</td>
<td>Germ cell cluster</td>
<td>Intact</td>
<td>Cluster of germ cells</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>1965950</td>
<td>Intact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human fetal testis – 22 weeks cells</td>
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<td>1404250</td>
<td>no germ cells</td>
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<td>L</td>
<td>1965950</td>
<td>no germ cells</td>
<td>Intact, partially hard</td>
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<td>Human fetal testis – 22 weeks cells</td>
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<td>Intact</td>
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<td>Human fetal testis – 22 weeks cells</td>
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</tr>
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<td>L</td>
<td>1965950</td>
<td>Germ cell cluster</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>Human – undifferentiated iPSC.OSKM</td>
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<td>L</td>
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<td>Hard, enlarged</td>
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<td>Hard, enlarged</td>
<td></td>
</tr>
<tr>
<td>Human – undifferentiated iPSC.OSKM</td>
<td>R</td>
<td>2644490</td>
<td>no germ cells</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>2644490</td>
<td>no germ cells</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
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<td>R</td>
<td>2644490</td>
<td>Germ cell cluster</td>
<td>Intact</td>
<td>Cluster of cells</td>
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<tr>
<td></td>
<td>L</td>
<td>2644490</td>
<td>Germ cell cluster</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>Human – undifferentiated iPSC.OSKM</td>
<td>R</td>
<td>2644490</td>
<td>no germ cells</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>2644490</td>
<td>no germ cells</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>Human – undifferentiated iPSC.OSKM</td>
<td>R</td>
<td>1603789</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>1603789</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
<td></td>
</tr>
<tr>
<td>Human – undifferentiated iPSC.OSKM</td>
<td>R</td>
<td>1603789</td>
<td>no germ cells</td>
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<td></td>
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<td></td>
<td>L</td>
<td>1603789</td>
<td>no germ cells</td>
<td>Hard, enlarged</td>
<td></td>
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<tr>
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<td>1603789</td>
<td>Germ cell cluster</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
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<td>L</td>
<td>1603789</td>
<td>Germ cell cluster</td>
<td>Intact</td>
<td></td>
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<tr>
<td>H1 - hESC</td>
<td>R</td>
<td>1436200</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
<td>Subject to IHC analysis</td>
</tr>
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<td></td>
<td>L</td>
<td>2010680</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
<td></td>
</tr>
<tr>
<td>H1 - hESC</td>
<td>R</td>
<td>2010680</td>
<td>Cell cluster</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>2010680</td>
<td>Cell cluster</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>H1 - hESC</td>
<td>R</td>
<td>2010680</td>
<td>no germ cells</td>
<td>Hard, enlarged</td>
<td>Subject to IHC analysis</td>
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<td>L</td>
<td>2010680</td>
<td>no germ cells</td>
<td>Hard, enlarged</td>
<td></td>
</tr>
<tr>
<td>H1 - hESC</td>
<td>R</td>
<td>2010680</td>
<td>no germ cells</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>2010680</td>
<td>no germ cells</td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>H9 - hESC</td>
<td>R</td>
<td>1810085</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
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<td>L</td>
<td>2092122</td>
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<td>R</td>
<td>1103952</td>
<td>no germ cells</td>
<td>Intact</td>
<td>Testis filled with filled cysts</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>1287944</td>
<td>no germ cells</td>
<td>Intact</td>
<td>Testis filled with filled cysts</td>
</tr>
<tr>
<td>H9 - hESC</td>
<td>R</td>
<td>1551501</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
<td>Subject to IHC analysis</td>
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<td></td>
<td>L</td>
<td>1810085</td>
<td>Germ cell cluster</td>
<td>Hard, enlarged</td>
<td></td>
</tr>
</tbody>
</table>

Table D.1. Whole mount analysis. Log sheet for transplantation of OSKM and OSKMV cells into mouse testes with control cells. L=left, R=right.
## D.2 Supplementary Table S2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Karyotype</th>
<th>Age</th>
<th>Origin</th>
<th>Phenotype</th>
<th>Passage # of clones</th>
<th>Derived clones</th>
<th>Immuno-Phenotype</th>
<th>Gene expression</th>
<th>In vitro differentiation</th>
<th>In vivo differentiation</th>
<th>Successful GMP transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ</td>
<td>XY</td>
<td>Newborn</td>
<td>Foreskin</td>
<td>WT</td>
<td>1-35</td>
<td>10</td>
<td>OCT3/4, TRA-1-60, TRA-1-81, SSEA3, SSEA4</td>
<td>OCT3/4, LIN28A, NANOG, TERT, ZFP42, SALL4</td>
<td>✔</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>HUF1</td>
<td>XY</td>
<td>28 years</td>
<td>Dermis</td>
<td>WT</td>
<td>6-30</td>
<td>3</td>
<td>OCT3/4, NANOG, TRA-1-60, TRA-1-81, SSEA3, SSEA4</td>
<td>OCT3/4, LIN28A, NANOG, TERT, ZFP42, SALL4</td>
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<td>YES</td>
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</tr>
<tr>
<td>HUF58</td>
<td>XY</td>
<td>60 years</td>
<td>Dermis</td>
<td>Chromosome 2 pericentric inversion</td>
<td>4-30</td>
<td>4</td>
<td>OCT3/4, NANOG, TRA-1-60, TRA-1-81, SSEA3, SSEA4</td>
<td>OCT3/4, LIN28A, NANOG, TERT, ZFP42, SALL4</td>
<td>✔</td>
<td>YES</td>
<td></td>
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<tr>
<td>GM13325</td>
<td>XX</td>
<td>9 days</td>
<td>Dermis</td>
<td>DiGeorge Syndrome del(22)q11</td>
<td>5-22</td>
<td>4</td>
<td>OCT3/4, TRA-1-60, TRA-1-81, SSEA3, SSEA4</td>
<td>OCT3/4, LIN28A, NANOG, TERT, ZFP42, SALL4</td>
<td>✔</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>HUF9</td>
<td>XX</td>
<td>31 years</td>
<td>Dermis</td>
<td>Premature ovarian failure</td>
<td>1-20</td>
<td>6</td>
<td>OCT3/4, NANOG, TRA-1-60, TRA-1-81, SSEA3, SSEA4</td>
<td>OCT3/4, LIN28A, NANOG, TERT, ZFP42, SALL4</td>
<td>✔</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>HUF3</td>
<td>XX</td>
<td>30 years</td>
<td>Dermis</td>
<td>WT</td>
<td>1-22</td>
<td>6</td>
<td>OCT3/4, NANOG, TRA-1-60, TRA-1-81, SSEA3, SSEA4</td>
<td>OCT3/4, LIN28A, NANOG, TERT, ZFP42, SALL4</td>
<td>✔</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Table D.2. *Fibroblast cell lines used to reprogram into mRNA induced pluripotent stem cells.* Information regarding successful GMP transfer and clone number are indicated. N.D. = not determined.

Supplementary Table S1; Related to Figure 1. *Fibroblast cell lines used to reprogram into mRNA induced pluripotent stem cells.* Information regarding successful GMP transfer and clone number are indicated. N.D. = not determined.
D.3 Supplementary Table S3

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthemax</td>
<td>Corning, USA</td>
<td>3535XX1</td>
<td>Manufactured under cGMP</td>
</tr>
<tr>
<td>CELLstart</td>
<td>Invitrogen, USA</td>
<td>A10142-01</td>
<td>Manufactured under cGMP</td>
</tr>
<tr>
<td>Pluriton medium</td>
<td>Stemgent, USA</td>
<td>00-0070</td>
<td>Xeno-free</td>
</tr>
<tr>
<td>mTeSR1</td>
<td>StemCell Technologies, USA</td>
<td>05850</td>
<td>Manufactured under cGMP</td>
</tr>
<tr>
<td>TeSR2</td>
<td>StemCell Technologies, USA</td>
<td>05860</td>
<td>Manufactured under cGMP</td>
</tr>
<tr>
<td>Nutristem</td>
<td>Stemgent, USA</td>
<td>01-0005</td>
<td>Xeno-free</td>
</tr>
<tr>
<td>mRNA</td>
<td>Stemgent/in house</td>
<td>00-0067</td>
<td>Manufactured under defined SOPs</td>
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<tr>
<td>NuFF</td>
<td>Globalstem</td>
<td>GSC-3006C</td>
<td>Manufactured under defined SOPs, quality and sterility testing</td>
</tr>
<tr>
<td>B18R</td>
<td>eBioscience</td>
<td>34-8185</td>
<td>Manufactured under cGMP, ASR, or ISO CE-IVD regulatory requirements</td>
</tr>
</tbody>
</table>

Table D.3. **GMP-compliant reagents for iPS cell line derivation, culture and cryopreservation.**

Abbreviations: ASR, Analyte Specific Reagent; cGMP, current Good Manufacturing Practices; ISO, International Organization for Standardization; SOP, Standard Operating Procedure
### D.4 Supplementary Table S4

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sterility</th>
<th>Mycoplasma</th>
<th>DNA fingerprint</th>
<th>Gram+ bacteria</th>
<th>Gram- bacteria</th>
<th>Fungi</th>
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<td>RiPSC.HUF58</td>
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<td>neg.</td>
<td>passed</td>
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<td>passed</td>
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<td>passed</td>
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</tbody>
</table>

Table D.4. iPS cell line sterility and pathogen testing after GMP conversion.

**Table 3; Related to Figure 2.** RiPSC line sterility and pathogen testing after GMP conversion
Appendix E

List of publications

Parts of this thesis have been submitted for a patent application and peer-reviewed journals as listed here. Furthermore, data of this thesis have been shared with the public at conferences, retreats and poster sessions.

E.1 Patent application

A U.S. Provisional Patent Application with the title "Generation of Male Germ Cells" has been filed on July 11, 2013.

E.2 Peer-reviewed journals related to this thesis


Appendix E. List of publications.


### E.3 Peer-reviewed journals unrelated to this thesis


### E.4 Oral presentations at conferences, retreats and poster sessions


Durruthy Durruthy J., September 2013. Fate of Induced Pluripotent Stem Cells Following Transplantation to Murine Seminiferous Tubules. Progenitor Meeting, Stanford, CA and New York, NY (talk).


References


improves the differentiation potential of ips cells generated with a single excisable vector,” *Stem Cells*, vol. 28, no. 1, pp. 64–74, 2010.


Reference


