The potency of human testicular stem cells

Chikhovskaya, J.V.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter VI

General discussion and implications for future research
Clinical approaches aiming at restoring male fertility require the understanding of biological mechanisms underlying reproduction including the maintenance of stem cell function. The homeostasis of adult testicular parenchyma is sustained by two distinct populations: germ and somatic stem cells. Whereas germ and somatic progenitors are occupying different niches within testicular tissue, their coordinated self-renewal and differentiation provide the most important input in the function of testis as an organ i.e. the formation of mature gametes and the production of sufficient androgen levels and finally, male fertility and procreation.

In vivo a complex of various regulatory signals and cell interactions is required to coordinate the proper function of tissue-specific stem cells and their progeny. Continuous spermatogenesis would not be possible without a correct balance between proliferation and differentiation of spermatogonial stem cells (SSCs). SSCs are supported by a specific microenvironment composed by the direct interactions with Sertoli cells, currently considered the central element of the SSC niche, as well as paracrine interactions with somatic cells occupying the interstitial space i.e. Leydig cells (LCs), peritubular stromal cells and endocrine factors via the microvasculature. Finally, constant maintenance of the testicular interstitial integrity and function of testis as an endocrine organ are provided by the balance between self-renewal and differentiation of cells from an interstitial somatic stem cell pool.

The application of stem cell therapy methods could theoretically become a novel approach for the restoration of reproductive function in diverse categories of patients. Since spermatogenesis is highly sensitive to chemotherapy and radiation treatment, infertility is a common consequence of cancer therapy (Ebata KT, et al.,2008). For example, high dose chemotherapy used as conditioning for bone marrow transplantation has been shown to result in a high incidence of azoospermia and oligozoospermia as well as to severe LC damage (up to the occurrence of premature hypogonadism) (Howell SJ, et al.,1999;Chatterjee R, et al.,2001). While for some patients, spontaneous post treatment restoration of gonadal function has been reported, the majority remains sterile. It is largely unpredictable a priori to determine in which patients recovery will take place and further studies evaluating effects of different treatment regimes and measures facilitating fertility restoration and overcoming post treatment gonadal dysfunction are eagerly awaited. For example, several recent studies on the longitudinal recovery of fertility after treatment for haematological malignancies have brought new insights into the understanding of this problem. The LC damage in case of graft-versus-host disease (GVHD) following hematopoietic stem cell transplantation was shown as one of the factors causing gonadal insufficiency in the posttransplantation period (Rovo A, et al.,2013). In this case the progressive loss of LCs is caused by infiltration of testicular interstitium by
donor alloreactive T cells and measures against GVHD may have additional positive effect on reversibility of testicular function (Wagner AM, et al., 2005).

A recent study on the gonadal function of male lymphoma survivors demonstrated hypogonadism in 30% of patients undergoing standard treatment and in 50% of patients undergoing high dose chemotherapy with autologous stem cell support (Kiserud CE, et al., 2009). Of course for adult patients sperm banking is a good option for fertility preservation, but some malignancies have negative influence on spermatogenesis themselves. For example, long periods of fever or tumor-released cytokines or negative effect on hypothalamus and pituitary function by central nervous system tumours may reduce sperm production (Tal R, et al., 2000; Dohle GR, 2010). For example, one study showed that most patients with malignant lymphomas and testicular germ tumours generally demonstrate diminished sperm quality; in 9,7% of cases no sperm sample could be banked due to absence of spermatozoa (van Casteren NJ, et al., 2009).

Another patient group where semen cryopreservation for fertility preservation becomes impossible is childhood cancer survivors. While the testicular tissue of these patients has resident SSCs, spermatogenesis has not commenced yet and recovery of mature spermatozoa is inapplicable. In this case, SSC cryopreservation for future transplantation is the only method for fertility preservation aiming to restore the testicular function and prevent severe consequences for quality of life (Ebata KT, et al., 2008; Struijk RB, et al., 2013).

In addition, in case of adult patients, in whom cryopreservation of ejaculated semen is possible and widely used, obtaining pregnancies with cryopreserved sperm nearly always requires the use of artificial reproductive techniques such as in vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI). These techniques thus require medical intervention of the unaffected female partner with relatively low pregnancy rates (between 24% for IVF and 33% for ICSI), high burden as well as high costs (van Casteren NJ, et al., 2009). In addition, recent data suggest that offspring obtained with ICSI is at increased risk for birth defects (Davies MJ, et al., 2012).

Development of fertility restoration methods based on germ and somatic testis stem cell transplantation would potentially allow recovery of spermatogenesis and subsequently natural conception in the future. Based on these clinical data on impairment of testicular functions and the biological knowledge of the association between different cell types within the testis tissue we conclude that restoration of testicular function with stem cell therapy can be applied for both germ and somatic cell lineages.

Recent studies showed interesting possibilities for development of SSCs transplantation techniques (Sadri-Ardekani H, et al., 2009; Lim JJ, et al., 2010; Sadri-Ardekani H, et al., 2011; Hermann BP, et al., 2012). This could potentially be performed in two different ways: testicular tissue grafting (Van Saen D, et al., 2009; Baert Y, et al., 2012; Jahnhukainen K, et al., 2012) and germ cell transplantation (Nagano M, et al., 2002; Sadri-Ardekani H, et
General discussion and implications for future research

al., 2009; Hermann BP, et al., 2012). In case of germ cell transplantation, resembling the transplantation assay applied for SSCs identification in rodent, the clinical application of SSCs requires their isolation and propagation in vitro. Being cultured in vitro, these cells lose specific microenvironment and their subsequent adaptation to the new in vitro conditions may potentially affect their stem cell properties and perhaps even induce differentiation or trans-differentiation into another cell type. These possible changes in stem cell properties will disturb the proposed clinical application. In this thesis, we evaluated the potential of the in vitro transition of testicular stem cells during propagation and established a protocol for enrichment, in vitro expansion and differentiation of human somatic testicular progenitors.

Multipotent human testis-derived stem cells in vitro

The derivation of pluripotent stem cells in primary mouse germ cell cultures has been demonstrated by several groups (Kanatsu-Shinohara M, et al., 2004; Guan K, et al., 2007; Ko K, et al., 2010). Mouse SSCs are, in vivo, unipotent stem cells responsible for maintenance of spermatogenesis. When propagated in vitro, during serial passaging, these cells undergo spontaneous transition into the pluripotent cell state during a process called culture-induced reprogramming. The obtained pluripotent mouse cells possess basic features of embryonic stem cells (three germ line differentiation in vitro, teratoma formation, and contribution to life born chimeras).

Initially this phenomenon was shown for mouse neonatal testis cell and later the derivation of pluripotent stem cells from adult mouse testis became a reproducible technique (Kanatsu-Shinohara M, et al., 2004; Guan K, et al., 2007; Ko K, et al., 2010).

The successful propagation of mouse SSCs raised the interest for similar possibilities for in vitro amplification and culture-induced reprogramming manipulations of human germ stem cells (Sadri-Ardekani H, et al., 2009; Lim JJ, et al., 2010). Recently, our group has demonstrated successful propagation of both adult and prepubertal human SSCs opening up new perspectives of fertility preservation for young cancer patients (Sadri-Ardekani H, et al., 2011; Sadri-Ardekani H, et al., 2013; Struijk RB, et al., 2013).

The attempts to derive pluripotent cells from human testis have proven to be more difficult. This so called “culture-induced” reprogramming, previously observed in rodent testicular cell culture systems, will raise pluripotent cells that might be a source for a broader range of clinical cell therapies (Seandel M, et al., 2007; Jaenisch R, et al., 2008; Kanatsu-Shinohara M, et al., 2008). On the other hand, the phenomenon of possible spontaneous transition of spermatogonia to the pluripotent cell state during propagation forms a significant concern in case of propagation of human SSC for a future clinical stem cell transplantation application as it might cause teratomas post transplantation.

Previously several research groups claimed the derivation of pluripotent stem
cells from human testis tissue based on the spontaneous transition of propagated germ cells. These authors used different methods aiming at enriching the initial testicular cell suspensions for SSCs: CD49F+ cell sorting combined with matrix selection (Conrad S, et al., 2008), enrichment for GFRα1 positive cells (Kossack N, et al., 2009) or starting with non-enriched cell suspensions (Golestaneh N, et al., 2009). Using also quite different plating protocols and propagation media, all these research groups reported the spontaneous arising of htES-like cell colonies resembling human ESC colonies. Subculture of these colonies resulted in generation of ES-like human cell lines possessing all common features of human pluripotent stem cells: expression of pluripotency associated markers (POU5f1 (OCT4), Nanog, Sox2, SSEA4, TRA-1-60, TRA-1-81 e.t.c.), a normal karyotype, and the capacity for in vitro differentiation toward derivatives of the three germ lineages.

Our study aimed at evaluating possible approaches resulting in the derivation of pluripotent stem cells from primary human testicular cultures in analogy with the well-established mouse culture system (Kanatsu-Shinohara M, et al., 2004; Guan K, et al., 2006; Kanatsu-Shinohara M, et al., 2008). In this study we also identified the spontaneous arising of htES-like colonies in human primary testicular cell cultures (Sadri-Ardekani H, et al., 2009). While a range of markers typical for pluripotent stem cells could be detected in these subcultured htES-like cells, formation of teratomas upon transplantation into immunodeficient animals was not demonstrated, even though they were performed in parallel with successful teratoma inducing hESC transplantation experiments. Based on these results, we designated these htES-like cells as multipotent stem cells (Chapter II).

The general features of testis-derived pluripotent and multipotent cells reported by other research groups and cells derived in our center are summarized in Table 1. These results started a debate on the possibility to generate teratomas upon injection of human testis-derived ES-like cells into immunodeficient mice, making interpretation of their pluripotent stem cell state rather controversial (Ko K, et al., 2010; Ko K, et al., 2011). The reproducibility of the generation of human pluripotent testis-derived ES-like cells as well as their pluripotent cell state remained questionable (Conrad S, et al., 2008; Ko K, et al., 2010; Tapia N, et al., 2011; Warthemann R, et al., 2012).

In order to elucidate the exact stem cell state and the effect of the propagation conditions on their stem cell properties, selective expansion of htES-like colonies under various culture conditions designated for propagation human ESCs and rodent epiblast stem cells was performed (Chapter III). Cautious evaluation of htES-like cell immunophenotype by flow cytometrical analyses, using pluripotent NCCIT cell line (human embryonic carcinoma cells) as an internal control, revealed the presence of cells expressing pluripotency associated marker SSEA4. However, this was not accompanied with expression of other surface markers typical for pluripotent stem cells (TRA-1-60, TRA-1-81). Further cell surface characterization suggested that the detected immunophenotype did not resemble the phenotype of human ESCs, but corresponded to a surface marker.
expression profile of human mesenchymal stem cells (MSCs), lately so called multipotent stromal cells (Dominici M, et al.,2006). In order to further assess the stem cell state of the htES-like cells, the gene expression profile of the derived progenitors and the careful evaluation of pluripotency-associated genes expressed within testis tissue and isolated cells in culture was performed. Quantitative assessment of the main pluripotency-associated transcription factors POU5F1, NANOG and SOX2, including selective evaluation of POU5F1 isoform 1 avoiding isoform 2 and POU5F1 pseudogene transcripts revealed a thousand fold lower expression level compared with expression by bona fide human ESCs (Chapter III). These results are in line with previous observations showing that low levels of POU5F1 isoform 1 transcripts could be detected in non-pluripotent somatic stem cells; this low level of expression has no impact on their stem cell state and differentiation capabilities (Kaltz N, et al.,2008).

These results show very clearly that the search for pluripotent cells derived from human postnatal tissues requires additional caution and correct interpretation during detection of pluripotency-associated POU5F1(OCT4) isoform 1 as well as co-expressed POU5F1 pseudogene transcripts that are not related to the pluripotent cell state, but are recently reported as a protein encoding sequences (Kastler S, et al.,2010). In our hands, expanded htES-like cells were not able to spontaneously differentiate towards derivatives of three germ layers in vitro upon embryoid body induction and did not give rise to teratomas in vivo (Chapter III). Although htES-like cells did not differentiate spontaneously in embryoid bodies similar as ESCs, their differentiation towards mesodermal lineages (adipo-, osteo-, chondrogenic) could be prominently induced by application of conventional differentiation protocols. The interpretation of differentiation towards other lineages than mesoderm based on the detection of single ecto- and endodermal markers must be performed with caution, because some of these markers could be detected in this cell population even before the initiation of the differentiation assay and are therefore not related to specific roles of these markers in bona fide ecto- and endodermal differentiation (Bertani N, et al.,2005;Wislet-Gendebien S, et al.,2005).

Taking into consideration the specific surface marker expression profile, the differentiation potential restricted to mesodermal lineages and the inability to form teratomas, we hypothesized that the obtained htES-like progenitors represented a population of MSC-like cells. This hypothesis was confirmed by comparing the global gene expression profiles of htES-like cells with the profiles of reference cell lines of pluripotent human ESCs (HuES-1, GFP-HES-3), multipotent bone marrow-derived MSCs representing the so called “golden standard” MSCs and adult human fibroblasts. Microarray analysis revealed that the expression pattern of htES-like cells was distinct from hESCs and fibroblasts but more similar to MSCs (Chapter III). Consequently, due to the lack of features in common with human ESCs, this cell population was designated as multipotent human testis-derived stem cells (mhtSC).
Table 1. The general features of testis-derived pluripotent and multipotent stem cells reported by other research groups and cells derived in our center.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Mouse testis-derived ES-like cells</th>
<th>Human testis-derived ES-like cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definition</strong></td>
<td>ES-like cells from neonatal mouse tests</td>
<td>Human testis-derived ES-like cells from adult human testis</td>
</tr>
<tr>
<td><strong>Source of SSCs</strong></td>
<td>newborn (0–2 days old) ddY or DBA/2 mice, newborn Green mouse or p53 knockout mouse in ICR background</td>
<td>Testicular tissue of patients undergoing bilateral orchidectomy as part of prostate cancer treatment (aged between 60 and 79 years of age)</td>
</tr>
<tr>
<td><strong>Enrichment procedures</strong></td>
<td>Collection of floating cells after overnight plating of testis cell suspension on gelatin-coated plates</td>
<td>No</td>
</tr>
<tr>
<td><strong>Duration of culture before colony formation</strong></td>
<td>4-7 weeks</td>
<td>3-4 weeks and &gt;3 months</td>
</tr>
<tr>
<td><strong>Derivation rate</strong></td>
<td>ES-like cells were established in 4 of 21 experiments (19%). The overall frequency of forming ES-like cells was 1 in 1.5 × 10⁶ cells</td>
<td>2 lines out of 17 tests biopsies</td>
</tr>
<tr>
<td><strong>Expression of pluripotency-associated markers</strong></td>
<td>POU5F1 (OCT4)</td>
<td>SSEA-1*</td>
</tr>
<tr>
<td>POU5F1+</td>
<td>POU5F1+</td>
<td>SSEA1+</td>
</tr>
<tr>
<td>Nanog+</td>
<td>Nanog+</td>
<td>SSEA1+</td>
</tr>
<tr>
<td>Sox2+</td>
<td>Sox2 +</td>
<td>SSEA1+</td>
</tr>
<tr>
<td>UTF1+</td>
<td>UTF1+</td>
<td>SSEA1+</td>
</tr>
<tr>
<td>Pluripotency-associated surface antigens</td>
<td>SSEA-1+</td>
<td>SSEA4+/SSEA3+</td>
</tr>
<tr>
<td>SSEA4+</td>
<td>TRA-1-60+</td>
<td>TRA-1-81+</td>
</tr>
<tr>
<td>Propagation rate</td>
<td>Not specified</td>
<td>&gt;30 passages</td>
</tr>
</tbody>
</table>

---

Chapter VI

Seandel et al. 2006

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |

Chapter VI

MaGSC (multipotent adult germ line stem cells) | MASCs (multipotent adult spermatogonial derived stem cells) | gPS (germline derived pluripotent stem cells) | hiGSC (human adult germ line stem cells) | MGSC (multipotent germ line stem cells) | ES-like cells from adult human testis |
**General discussion and implications for future research**

### Differentiation in vitro

- **Differentiation according to protocols designed to induce ESC differentiation:**
  - Plating on OP9 layers resulted in formation of hematopoietic cells, vascular cells and spontaneously beating myocytes. Neurons or glial cells were formed after plating on gelatin-coated wells.
  - Differentiation in vitro
    - According to protocols designed to induce ESC differentiation:
      - Plating on OP9 layers resulted in formation of hematopoietic cells, vascular cells and spontaneously beating myocytes. Neurons or glial cells were formed after plating on gelatin-coated wells.

#### Differentiation towards derivatives of 3 germ layers upon embryoid body formation.

- Clusters of contracting cells were identified in 90% of embryoid bodies, cells isolated from these areas showed sarcomeric striations when stained for α-sarcomeric actin.
- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. Plated embryoid bodies differentiate into a variety of cell types including differentiated beating areas.
- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. "Embryoid bodies formation, qPCR analysis of transcripts indicating differentiation towards derivatives of three germ layers. No contracting embryoid bodies."

#### Differentiation towards derivatives of 3 germ layers.

- Differentiation towards multiple lineages including rhythmically contractile cardiogenic tissue.
- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. Plated embryoid bodies differentiate into a variety of cell types including differentiated beating areas.
- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. Plated embryoid bodies differentiate into a variety of cell types including differentiated beating areas.
- Embryoid bodies formation, qPCR analysis of transcripts indicating differentiation towards derivatives of three germ layers. No contracting embryoid bodies.

#### Differentiation towards derivatives of 3 germ layers.

- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. Plated embryoid bodies differentiate into a variety of cell types including differentiated beating areas.
- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. Plated embryoid bodies differentiate into a variety of cell types including differentiated beating areas.
- Differentiation towards derivatives of 3 germ layers upon embryoid body formation. Plated embryoid bodies differentiate into a variety of cell types including differentiated beating areas.
- Embryoid bodies formation, qPCR analysis of transcripts indicating differentiation towards derivatives of three germ layers. No contracting embryoid bodies.

#### Do not differentiate spontaneously within embryoid bodies.

- Induced differentiation towards mesodermal lineages (adipo-, osteo-, chondrogenic).
  - Spontaneous differentiation into adipocytes during propagation on MEFs.

### Table

<table>
<thead>
<tr>
<th>Teratoma formation</th>
<th>yes</th>
<th>yes</th>
<th>yes</th>
<th>yes</th>
<th>yes</th>
<th>no</th>
<th>yes</th>
<th>no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to chimeras</td>
<td>yes</td>
<td>ND</td>
<td>yes</td>
<td>yes</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Special features</td>
<td>Nog+ Brachyury+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TM4SF 1+ NFIB+</td>
<td></td>
</tr>
</tbody>
</table>

* Detectable transcripts, however 1000 times lower expression levels compared to human ESC

ND  not defined

NA  not applicable
The notions regarding the non pluripotent cell state of cells originating from mhtSC colonies that are spontaneously formed in human primary testicular cell cultures have been suggested previously (Ko K, et al., 2010; Tapia N, et al., 2011). Careful analysis of published microarray data revealed inconsistency in microarray profiles of cells initially claimed to possess the complete pluripotent state (Conrad S, et al., 2008; Ko K, et al., 2010). However, comparison of the described gene expression profiles of these cells with human ESCs did not show expression of pluripotency-specific genes, or genes suggesting their germ cell origin, while expression of markers typical for cells of somatic lineage and explicitly fibroblast-specific markers was quite prominent.

Our data further refined this statement by the comparison of gene expression profiles of mhtSCs with the specific comparison groups (ESC, MSC and fibroblasts) in the same microarray experiment (Chapter III). In agreement with published data, the expression profiles of mhtSC did not correspond to the profiles of pluripotent stem cells. In addition, the observed high levels of ACTA2 expression in combination with lack of MAGEA4 and DDX4 (VASA) suggested that they are not of germ cell origin (Ko K, et al., 2010). Our analysis of the specific gene expression pattern in mhtSCs revealed a closer relation to bona fide MSC than to fibroblasts. This fact most likely can be explained by the kinetics of the cell culture and the ways of colony generation applied in our study. While most of the studies describe colony formation shortly after testicular cell isolation (4-14 days) when the risks of nonspecific cell aggregation appear rather high, mhtSCs in our study were generated from single colonies appearing in the monolayer of primary testicular cell cultures at 30-50 days after the initiation of culture. In this situation, the appearance of cell colonies was caused by proliferation of specific progenitors, rather than aggregation of primary isolated cells. The cell population obtained after selective expansion of single colonies possesses high expression levels of MSC-defining markers (TM4SF1, NFIB, JAG1, NOTCH3), enabling us to distinguish these cells from a general somatic/fibroblast population (Chapter III).

This striking similarity of mhtSCs to bona fide MSC raised the question whether they originate from reprogrammed germ cells or from a subpopulation of uncommitted mesenchymal progenitors isolated from testicular tissue and are present already in the primary testicular cultures. Direct isolation of MSC from human testis has indeed been reported (Gonzalez R, et al., 2009). On the other hand, the chance that these multipotent cells arise as a result of ‘incomplete’ reprogramming of SSC in vitro still could not be excluded. In order to determine the exact origin of mhtSCs, we generated mhtSC colonies in primary testicular cell cultures from three different starting populations of testicular cells: germ cells only, germ cells together with somatic testicular cells and only somatic cells from complete SCO testis tissue. Typical mhtSC colonies could be generated only from the starting cell populations containing only somatic cells or germ cells with somatic cells, and did not arise from the highly purified human germ cell fraction containing a population of
General discussion and implications for future research

Uncommitted spermatogonia (Chapter IV). However, recognizing the presence or absence of spermatogonia in a testicular cell population requires cautious characterization. Recent studies have reported potential pitfalls in germ cell identification due to the use of markers that are co-expressed by germ as well as somatic testicular cells (Ko K, et al., 2010; Tapia N, et al., 2011). In order to avoid this misinterpretation, we used, besides identification of CD49f expression by cells in the starting testicular cell populations, simultaneous detection of VASA (DDX4) in combination with PLZF or MAGEA4 to detect the presence of germ cells (Eildermann K, et al., 2012).

However, distinguishing the uncommitted germ cell progenitors, which theoretically might be predisposed to acquire the pluripotent cell state, within the heterogeneous population of spermatogonia in testicular cell fractions also appeared rather challenging. Recently two biological markers, fibroblast growth factor receptor 3 (FGFR3, CD333) and undifferentiated embryonic cell transcription factor 1 (UTF1), have been suggested as new markers for uncommitted germ cells (von Kopylow K, et al., 2010; von Kopylow K, et al., 2012). In case of human adult testis, the expression of UTF1 has shown to be restricted to a subpopulation of A\textsuperscript{dark} and A\textsuperscript{pale} spermatogonia (von Kopylow K, et al., 2010; von Kopylow K, et al., 2012). In agreement with this report, our highly purified spermatogonial fraction demonstrated clear UTF-1 expression. However, these spermatogonia did not provide pluripotent stem cell generation. At the same time, the mhtSCs obtained in primary testicular cell cultures containing only somatic cells did not express this transcription factor (Chapter IV). An algorithm that can be applied for

<table>
<thead>
<tr>
<th>CD49f ((\alpha_6) INTEGRIN)(^+)</th>
<th>Primary testicular cell suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germ cells</td>
<td>Somatic cells</td>
</tr>
<tr>
<td>HLA ABC (MHC Class I) –</td>
<td>HLA ABC (MHC Class I) +</td>
</tr>
<tr>
<td>ACTA 2 ((\alpha) SMA) –</td>
<td>ACTA 2 ((\alpha)SMA) +</td>
</tr>
<tr>
<td>Spermatogonia</td>
<td>Testis-derived MSC</td>
</tr>
<tr>
<td>- Morphology</td>
<td>(heterogenous trilineage potential/ mesodermal lineages)</td>
</tr>
<tr>
<td>- VASA (DDX4) +/PLZF+/MAGEA4+</td>
<td>- Morphology</td>
</tr>
<tr>
<td>Uncommitted spermatogonia</td>
<td>- CD73+/CD90+/CD105+/CD31-/D34-/</td>
</tr>
<tr>
<td>A dark/ A pale</td>
<td>CD45-/HLADR-</td>
</tr>
<tr>
<td>UTF1 +</td>
<td>mhtSCs</td>
</tr>
<tr>
<td>- FGFR3+ (CD333+)</td>
<td>(uniform trilineage potential/ mesodermal lineages)</td>
</tr>
<tr>
<td>- JAG1+</td>
<td>- CD146 +</td>
</tr>
<tr>
<td>- TM4SF1+</td>
<td>- NOTCH3+</td>
</tr>
</tbody>
</table>

Figure 1. An algorithm for determining human germ and somatic testicular cell based on expression of specific biomarkers.
determining human germ and somatic testicular cell based on expression of specific biomarkers is represented in Figure 1.

The efficiency of colony-formation in vitro did not differ between mhtSCs obtained from tissue specimens of patients with full spermatogenesis and patients with idiopathic Sertoli cell-only syndrome. This fact further supported the hypothesis of a somatic origin of mhtSCs and confirmed that colonies do not originate from propagated ‘incomplete’ reprogrammed germ cells, but from proliferating somatic progenitors with multilineage differentiation potential (Chapter IV). MhtSC derived from a variety of tissue samples possess uniform MSC-defining characteristics including a specific MSC immunophenotype, expression of MSC-specific transcripts (TM4SF1, NFIB) and ability to differentiate towards mesodermal lineages in vitro after the application of conventional induction protocols. The presence of somatic mesenchymal progenitors within the mhtSC subpopulation was further confirmed by the detection of the markers CD146 (MCAM/Melanoma Cell Adhesion Molecule) and CD200, both known to be specific for uncommitted MSCs with multipotent characteristics, distinguishing between tri- and unipotent MSC–like cells (Russell KC, et al., 2010). Based on this data, we conclude that mhtSC colonies, that spontaneously form in human primary testicular cell cultures, represent a population of testis tissue specific MSCs and therefore they do not possess the pluripotent stem cell state. These results finally allowed us to designate mhtSCs obtained in our laboratory as testis-derived MSCs (Chapter IV).

Further investigation is required in order to evaluate the ability of human germ cells to overcome the lineage restriction and obtain pluripotent cell state, if at all possible for human SSCs. The current state of this problem seems to focus on finding the appropriate culture conditions and methods to establish homogeneous human unipotent germ stem cell lines from isolated spermatogonia similar as in the case of rodent germ cells. It has become clear that besides having some comparable characteristics with rodent germ line stem cells, human SSCs seem to hold their unique features in culture including specific gene expression and the mechanisms controlling self renewal and differentiation. Therefore, a direct transition of methods and culture conditions efficient for the induction of the pluripotent cell state of rodent germ cells are not applicable for human germ cells. Further evaluation of differences between cell properties of rodent and human spermatogonia might uncover new mechanisms responsible for the maintenance of their stem cell state in vivo and in vitro. These mechanisms will lead to suggestions for new culture approaches and modification with specific growth factors, small molecules, and/or specific gene delivery to induce the reprogramming of human SSCs to the pluripotent cell state.

The assumption that human SSCs, in line with rodent germ stem cells, could be “predisposed” to become pluripotent after minor interventions in vitro remains to be tested. This would mean that compared to somatic cells (fibroblasts, lymphocytes,
keratinocytes), commonly used for iPS generation, they may require application of less reprogramming factors for inducing their transition back to the ES-like state or may have higher frequency of reprogramming. Finally, beside all controversy at this moment, the possibility of derivation of pluripotent patient-specific stem cells from propagated human SSC remains an interesting point for further investigation for regenerative medicine and still could uncover additional biological features of human germ cells.

**Testicular somatic cell population harbors Leydig cell progenitors**

Impaired testicular interstitium commonly appears as a serious complication of high dose chemotherapy leading to an irreversible loss of testicular function (Howell SJ, et al.,1999). A recent study among a large cohort of male patients undergoing allogenic hematopoietic stem cell transplantation (259 patients) showed the effect of conditioning regimes as well as GVHD following the transplantation on spermatogenesis and impairment of testicular somatic cells (Rovo A, et al.,2013). In this case donor alloreactive T cells infiltrating parenchimas of multiple organs, including testis, cause severe multiple cell damage typical for GVHD. Accumulation of alloreactive T cells within the testis interstitium results in a direct target cell injury and Leydig cell (LC) depletion (direct T-cell mediated cytotoxicity) as well as an indirect effect on LCs via antigen-nonspecific mechanisms due to production of inflammatory cytokines (IFN-γ and TNF-α) (Wagner AM, et al.,2005). Interestingly, experimental studies on GVHD demonstrated the absence of a direct T-cell infiltration of seminiferous tubules in a GVHD animal model, suggesting a possible indirect effect of GVHD on spermatogenesis almost exclusively via loss of LC function (Wagner AM, et al.,2005). The results of these experimental studies indeed find conformation in clinical settings; a recent cohort study showed that for patients not conditioned as well as conditioned with total body irradiation, ongoing chronic GVHD is the prominent adverse factor for sperm recovery (Rovo A, et al.,2013).

These data indicate the clinical importance of a possibility to preserve LC function. In this case the search for human Leydig stem cells and the development of the techniques for their isolation, cryopreservation and propagation would be an ideal way to preserve and restore testicular endocrine function.

It is generally accepted that testicular MSCs harbour the progenitors of adult LC. Several studies that attempted to identify the adult LC stem cells in vivo within the human tissue indeed revealed expression of typical MSC/pericyte- and oligodendrocyte-specific markers by testis interstitial cells associated with microvasculature (Davidoff MS, et al.,2004;Davidoff MS, et al.,2009). However, the identification of this testicular interstitial cell population has only been determined by application of immunocytochemical staining techniques on testis tissue sections, and effective methods for isolation or enrichment, long-term *in vitro* propagation to prove their differentiation ability towards LC remained
to be determined.

Stem cells possessing the fundamental features of bone marrow-derived (*bona fide*) MSCs have been isolated from various postnatal organs and have been characterized by their ability to self-renew and differentiate towards various cell lineages, mostly mesodermal derivatives (Friedenstein AJ, et al., 1974; Prockop DJ, 1997; Pittenger MF, et al., 1999). The wide distribution of MSC-like cells within different organs suggested the tissue specific niche of these cells being closely related to blood vessels. This hypothesis was proven to be correct by a study describing CD146+/CD34-/CD45-/CD56- pericytes and confirming the MSC-like cell from various organs (Crisan M, et al., 2008). These new facts presented a prospective look on MSCs/pericytes as a kind of “universal” stem cell that is located within the interstitial compartment within multiple organs and is playing a role in tissue homeostasis. It has recently been shown that MSCs could be isolated from placenta, muscle, lung, skin, adipose tissue, synovial membrane and testis (Crisan M, et al., 2008; Dominici M, et al., 2009; Gonzalez R, et al., 2009).

The resemblance of LC progenitors to MSCs could be traced in a number of examples from clinical practice. The occurrence of Leydig cell tumors with adipose differentiation and/or calcification with ossification in patients of 28-70 years of age have been reported (Ulbright TM, et al., 2002). The adipose cells found in these tumors with adipose metaplasia are usually positive for LC markers (INHIBIN-A, CALB2 and MELAN-A) and are considered originating from neoplastic LCs. This reported cell plasticity and spontaneous differentiation towards two mesodermal differentiation lineages well known for *bona fide* MSCs further suggests a close relation between LCs and MCSs within testis tissue.

Based on the MSC-specific immunophenotype characteristics of the mhtSC cells, we developed an isolation approach for progenitors of steroidogenic human LCs and discovered an uncommitted progenitor subpopulation in conjunction with a population of early committed progenitors. We conclude that the population of CD146+/CD34-testicular somatic cells co-expressing NESTIN and CALB2, but not luteinizing hormone receptor (LHR), represent LC stem cells. We were able to perform cell isolation followed by propagation and differentiation towards cells expressing LHR and the specific steroidogenic enzyme 3-β-hydroxysteroid dehydrogenase 2 (3β–HSD2) *in vitro* (Chapter V). This population of LC progenitors is different from the previously described rodent PDGFRα+ LC progenitors that also show the capability to differentiate towards mature LC in vitro (Ge RS, et al., 2006; Stanley E, et al., 2012). Indeed, an isolated population of human PDGFRα+ (CD140A+)/CD34- testicular somatic cells represents a population of low-proliferating, 3β–HSD2 negative but steroidogenic acute regulatory protein (*StAR*) and LHR expressing committed progenitor cells that most likely already partially progressed in the differentiation towards adult LC (Chapter V).
Remarkably, induced differentiation of the sorted CD146+/CD34- cell \textit{in vitro}, with the same protocol already described for the PDGFRA+ progenitors, was accompanied by consequent down regulation of CD146 surface expression and \textit{de novo} appearance of PDGFRA-expression with corresponding upregulation in expression of steroidogenic enzymes. These observations enables us to propose a model for human LC differentiation accompanied by successive changes in cell phenotypes during transition from LC stem cell to committed progenitors and finally mature LC (Chapter V) (Figure 2).

Most likely, physiological differentiation of the LC progenitor subpopulation \textit{in vivo} is progressing via several stages and associated with changes in their cell immunophenotypes and upregulation of LC specific markers. The maturation of adult LC progenitors is accompanied by gradual decrease in CD146 expression, appearance of surface PFGFRα and upregulation of markers related to cell steroidogenic activity (\textit{StAR}, \textit{CHOLESTEROL SIDE-CHAIN CLEAVAGE} enzyme (\textit{CYTP450scc/CYP11A}), 3β-HSD2) as well as specific LC markers: \textit{LHR} and \textit{INSULIN-LIKE FACTOR 3} (\textit{INSL3}), also known as \textit{RELAXIN-LIKE FACTOR} (\textit{RLF}). Although we observed high cell proliferation during subculture of an initially purified CD146+/CD34- somatic stem cell population, at the same time we found a rapid decrease of cells with the CD146+/CD34- progenitor-specific immunophenotype already at passage 3. Methods facilitating \textit{in vitro} maintenance of these LC stem cell properties through multiple passaging are eagerly awaited. We cultured these CD146+/CD34- cells for the first time and used culture conditions previously described for maintenance of PDGFRA+ cell and that might not be the most optimal for this particular cell population (Ge RS, et al., 2006). Therefore, further studies are required for optimization.
of the *in vitro* propagation conditions for enriched CD146+/CD34- somatic stem cell population. Optimization of propagation conditions will facilitate in vitro amplification of progenitors after cell sorting and help to provide sufficient cell quantities for possible cell transplantation techniques.

Although the obtained population of human LC progenitors described in our study differentiated *in vitro* with relatively low efficiency (only 2-5% of the total CD146+ cell population) and further optimization is required, these LC stem cells have the potency to propagate and differentiate *in vitro* and therefore could potentially contribute in the restoration of testis steroidogenic function by stem cell therapy.

**Implications for future research and clinical practice**

Stem cell therapy based on isolation of testicular stem cells could become an alternative for complex treatments of patients with gonadal dysfunction (germ cell damage and Leydig cell insufficiency) such as patients after hematopoietic stem cell transplantation and chemotherapy.

The clinical application of stem cell therapies involving cryopreservation may require *in vitro* propagation of germ stem cells. Results from animal studies have raised concern that this brings several additional risks, including the spontaneous transition of SSCs towards the pluripotent state during propagation. Our observation suggests that in contrast to rodent SSC culture systems, the spontaneous formation of pluripotent cell lines does not take place in our described human testicular cell culture system. The observed spontaneously-formed colonies in our culture system represent testis-specific MSCs the testis specific somatic stem cells and therefore do not raise the potential risk for teratoma formation in case of clinical application. However, the epigenetic stability of human SSCs during in vitro propagation and subsequent possible risks to the SSC generated offspring when preparing these SSCs for fertility restoration remains an important point for further research. Additional studies exploring the effect of the *in vitro* microenvironment on germ stem cell properties and repopulation capabilities after their transplantation in vivo are required before initiation of pilot clinical application trials.

Somatic stem cells present within the human testicular interstitium represent another interesting area for investigation. A subpopulation of MSC-like cells isolated from human testicular tissue on the bases of a specific MSC immunophenotype contains uncommitted progenitors of adult human LCs, ie LC stem cells. These LC stem cells are able to self-renew and differentiate towards steroidogenic cells *in vitro*. Follow up studies are required to evaluate their ability to colonize recipient testis and to determine the number of progenitor cells within the interstitium of different individuals in order to estimate the optimal size of testicular biopsies allowing the successful cell isolation. Additional studies are required in order to optimize the methods of in vitro propagation of isolated
General discussion and implications for future research

LC stem cells and efficient maintenance of their undifferentiated cell state prior to cell transplantation. The end point of this work is the establishment of a clinical application of LC stem cell autotransplantation for restoration of testis endocrine function as alternative for androgen-replacement therapy.

References


49. Van Saen D, Goossens E, De Block G, Tournaye H. Regeneration of spermatogenesis


