The potency of human testicular stem cells

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Chapter I

Introduction
Introduction

The two main characteristics of stem cells are their ability to self-renew and to differentiate towards specialized cells. During the last decades diverse sources of mouse and human stem cells have been identified and they have become the object of extensive investigation. This scientific interest is well explained by the broad variety of possible applications of stem cells in regenerative medicine and tissue engineering. Embryo-derived stem cells, pluripotent stem cells able to give rise to the cells of all three embryonic germ layers, remain the “gold standard” for research on stem cell therapy and regeneration of postnatal tissues. Nevertheless, the use of embryonic stem cells in clinical practice raises serious ethical and technical questions as it requires the use of preimplantation embryos which are not genetically similar to the patient. In order to overcome these problems many research groups focus their research on alternative sources of cells with basic features and differentiation capacities resembling pluripotent embryonic stem cells. One approach focuses on inducing transition of somatic cells towards the pluripotent cell state via somatic cell nuclear transfer, cell fusion or through generation of induced pluripotent stem cells with a defined set of transcription factors (Jaenisch R, et al.,2008;Lowry WE, et al.,2008).

Another way to derive stem cells for regenerative medicine is the use of endogenous adult stem cells. These cells lack the wide-ranging differentiation potential of embryonic stem cells, but show an extensive ability to self-renew as well as the ability to differentiate towards one particular lineage. Efficient methods for the isolation, in vitro propagation, enrichment and differentiation towards a specific lineage of such multipotent tissue-specific adult stem cells need to be established in order for these cells to become an alternative for patient-specific stem cell therapy. In this thesis we focus on stem cells from the human testis.
Background of the thesis

The testis as a source of stem cells

The testis is an interesting source of stem cells as it contains progenitors of both the germ cell- as well as the somatic cell-lineage. Coordinated self-renewal and differentiation of these distinct cell types provides tissue homeostasis, in particular spermatogenesis and hormone production. In addition, it has recently been demonstrated in rodents that in vitro propagated germline stem cells can acquire a pluripotent cell state making the testis a potential source of pluripotent cells for all applications of regenerative medicine (Kanatsu-Shinohara M, et al., 2004; Guan K, et al., 2006; Seandel M, et al., 2007; Ko K, et al., 2009). However, whether such cells can also be obtained from human testis is unknown. The identity of human testicular somatic stem cells still needs to be explored.

Germline stem cells

Spermatogonial stem cells (SSCs), the germline stem cells, are present in low numbers in both rodent and human testis, making their identification and further investigation difficult (Tegelenbosch RA, et al., 1993; He Z, et al., 2009). Several approaches for the identification, isolation and propagation of spermatogonial cells in vitro have been successfully established during the last decade (Shinohara T, et al., 2000; Kanatsu-Shinohara M, et al., 2003; Wu X, et al., 2009). Recently, several cell surface markers (CD9, CD49f, GFRα1, CD333) and transcription factors (MAGEA4, UTF1, PLZF) have been demonstrated to be competent tools in identifying human spermatogonia including SSCs. However, the lack of highly specific markers of SSCs hampers the investigation of characteristics and potential of these cells, and germ cell (xeno)transplantation remains the only definitive method for the detection of SSCs. Several techniques have been developed that allow the enrichment of spermatogonia including SSCs from testis cell suspensions, such as sorting for the presence (CD49f, CD90) and absence (c-kit (CD117), HLAI(ABC)/or H-2Kb) (multiparameter selection) (Shinohara T, et al., 2000; Geens M, et al., 2007) of uncommitted spermatogonia. Furthermore, a culture system for propagation of human adult and prepubertal SSCs has recently successfully been established by our group (Nagano M, et al., 2002; He Z, et al., 2009; Sadri-Ardekani H, et al., 2009; Sadri-Ardekani H, et al., 2011). Such in vitro propagation is an important prerequisite to obtain sufficient numbers of SSCs if one aims to use SSC transplantation to restore fertility in sterile childhood cancer survivors in the future.

Pluripotent stem cells derived from testicular tissue

SSCs are well known as unipotent cells responsible for the maintenance of
spermatogenesis. Yet, another unique feature of these progenitors has recently become clear upon the development of a method to propagate SSCs in vitro. Several research groups have described the spontaneous arising of pluripotent embryonic stem cell-like cells (ES-like cells) during long-term culture and serial passaging of postnatal mouse germ cells (Kanatsu-Shinohara M, et al., 2004; Guan K, et al., 2006; Seandel M, et al., 2007; Kanatsu-Shinohara M, et al., 2008; Ko K, et al., 2009). This spontaneous transition to the pluripotent state by unipotent stem cells in vitro was termed culture-induced reprogramming and emerged as a new promising source of pluripotent stem cells (Seandel M, et al., 2007). The pluripotent state of the obtained ES-like cells was confirmed by in vitro and in vivo differentiation, teratoma formation upon transplantation, and contribution to the soma and the germ line in chimeras after injection of these cells into mouse blastocysts. With the use of an OCT4-GFP reporter mouse model and clonal analysis it was further confirmed that the pluripotent stem cells that arise in vitro are indeed derived from adult unipotent germline stem cells (Ko K, et al., 2010). Derivation of a comparable cell population from human testicular cultures could open a new source of autologous pluripotent stem cells for regenerative medicine. In addition, the discovery of such cells in the human testis could shed new light on the mechanisms of testicular tumorigenesis (Clark AT, 2007; Looijenga LH, et al., 2007) (Figure 1). Further investigation is required in order to evaluate the ability

**Figure 1. Physiological and pathological stem cell fates of germ and somatic stem cells in vivo and in vitro.**

The physiological development of spermatooza from spermatogonial stem cells (SSCs) in vivo is shown in the central column. In parallel, somatic MSC-like adult Leydig cell (ALC) progenitors are responsible for physiological Leydig cell regeneration in vivo. In pathological situations (left side of figure), germ and somatic progenitors can turn into cancer stem cells in vivo leading to testicular germ cell tumors, testicular teratomas and testicular interstitial cell tumors. When taken out of their tissue-specific microenvironment and brought into cell culture (right side of figure), it is known that mouse SSCs spontaneously change their stem cell potential by means of culture-induced reprogramming. Our aim was to investigate the in vitro potency of germ and somatic stem cells derived from the human testis.
of human germ cells to overcome their lineage restriction and to obtain a pluripotent cell state.

**Somatic stem cells in the testis**

For several decades, the testis has for the most part been studied as a source of germ cells, but more recently focus has shifted towards somatic stem cells and their role in testis tissue homeostasis. Previously, development of techniques for germ cell enrichment and transplantation revealed heterogeneity in the stem cells purified from testis single cell suspensions based on side population (SP) phenotype using Hoechst 33342 exclusion criteria (Shinohara T, et al., 2011). Stem cells with SP phenotype have been isolated from multiple mammalian tissues (Zhou S, et al., 2001; Challen GA, et al., 2006). Spermatogonial stem cells enriched by this method could be “contaminated” by somatic stem cells also possessing SP phenotype (Lassalle B, et al., 2004).

Several recent studies in rodents have shown that this non-germ stem cell population contains progenitors for testosterone-producing Leydig cells (LC). These progenitors are responsible for maintenance of the adult LC subset during the life of the male individual and regeneration in case of damage. During development in pre- and postnatal life of a male individual testosterone production is provided by two distinct subpopulations of cells: fetal LC and adult LC. The functional properties and development of these subpopulations during testes organogenesis are mainly known from rodent models, but it is generally accepted that this development is also common for primates including human (Davidoff MS, et al., 2004). Fetal LCs originate from mesenchymal-like cells situated between the testis cords (Benton L, et al., 1995; Zirkin BR, 2010) and provide the androgen levels required for development of the male gonad.

The stem cell properties of adult LC progenitors in adult murine testis have previously been proven by selection of “Hoechst dim” SP containing LC progenitors that proliferate and differentiate towards mature adult LC after transplantation into the testis of an LC depleted recipient mouse (Lo KC, et al., 2004). However, the molecular characteristics of the uncommitted Leydig cell progenitors are still unclear. Several research groups have investigated adult LC regeneration after complete depletion of existing cell populations induced by administration of ethane dimethanesulphonate (EDS) in rats. This approach provided essential information about the localization and specific markers expressed by differentiating LC precursors (Teerds KJ, et al., 1999; Davidoff MS, et al., 2004). EDS exposure studies revealed close relation of MSC-like adult LC progenitors to testicular microvasculature and tissue-specific pericytes such as connection of newly formed cell clusters with the blood vessels, coexpression of several pericyte-specific markers (aSMA, NG2, nestin e.t.c.) and basement membrane fragments traceable on the cell surface of adult LC (Davidoff MS, et al., 2004).
Although it is generally thought that similar adult LC progenitors exist in human testis based on the MSC characteristics of neoplastic LC in testicular tumors (Ulbright TM, et al., 2002), consequential studies on human testis-specific undifferentiated MSC/pericyte-like cells are required to identify this specific Leydig cell progenitors. Their identification would facilitate development of the efficient methods for isolation, enrichment, propagation and differentiation to androgen-producing cells in vitro. These Leydig stem cells could potentially be considered as candidates for regenerative medicine by autotransplantation to restore testis steroidogenic function in case of testosterone insufficiency after chemotherapy or other conditions associated with hypogonadism requiring androgen replacement (Figure 1).

**Aim and outline of the thesis**

The aim of this thesis is to translate recent findings from animal studies on the potency of testicular stem cells to the human and to determine whether stem cells from human testis can provide a new stem cells source for regenerative medicine. We have elucidated this issue by investigating the presence, origin and differentiation abilities of such cells derived from human testicular tissue.

The main questions addressed by this thesis are:

1. Do cells with embryonic stem cell-like (ES-like) properties arise spontaneously from *in vitro* human primary testicular cell cultures?

2. How close do these ES-like cells resemble pluripotent embryonic stem cells?

3. What is the origin of these ES-like cells in human primary testicular cell culture system?

4. Could testicular somatic progenitors be identified and applied for generation of steroid-producing Leydig cells?

In **Chapter II** we describe the derivation of ES-like cells from primary testicular cultures started from testis tissue of four adult men undergoing bilateral orchidectomy due to prostate cancer. The obtained human testis-derived ES-like cells (htES-like cells) were propagated under culture conditions commonly used for propagation of hESCs and characterized for expression of pluripotency-associated markers (POU5f1, NANOG, SOX2), directed differentiation potential *in vitro* and *in vivo*, chromosomal stability and epigenetic
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In Chapter III the stem cell state of htES-like cells is further studied, by means of selective expansion of ES-like colonies under culture conditions known to be supportive for propagation of different types of pluripotent stem cells (hESCs, mouse epiblast stem cells and human naïve ESC). Furthermore, combinations of growth factors and small molecules in the culture media were used to trigger diverse mechanisms known to be involved in the maintenance of the human pluripotent cell state. Microarray analysis was applied to compare the gene expression profiles of the in vitro propagated htES-like cells with that of pluripotent hESCs, multipotent somatic stem cells and fully differentiated fibroblasts to evaluate their actual stem cell state.

Chapter IV defines htES-like cells as multipotent human testis-derived stem cells (mhtSCs) rather than ES-like cells and describes their derivation in vitro by selective isolation of different testicular cell populations, i.e. a mix of somatic and germ cells or pure somatic and germ cells, aiming to determine their origin.

Chapter V focuses on the isolation and characterization of somatic MSC-like progenitors from testis interstitium as possible precursors of adult Leydig cells. Their characteristics and ability to differentiate towards androgen-producing cells in vitro is evaluated.

Chapter VI summarizes the obtained results and discusses the possible application of testis-derived stem cell in regenerative medicine.
References

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