Chapter 2
Pluripotent Stem Cells from the Postnatal Testis: Unlocking the Potential of Spermatogonial Stem Cells

Marco Seandel, Ilaria Falciatori, and Shahin Rafii

Abstract While embryonic stem (ES) cells are well known to give rise to tissues comprising all three germ layers, only recently was it shown that cells from the postnatal testis could produce embryonic-like stem cells in culture. The latter, arising *in vitro* from spermatogonia, can undertake most, if not all, the functions of ES cells. This chapter explores the potential predisposing factors for postnatal germ cells to become pluripotent, including expression of pluripotency-associated genes and epigenetic factors. The major published studies describing the production of ES-like cells from mice and human tissues are reviewed. Finally, we assess the data demonstrating functionality of the differentiated derivatives of ES-like cells. The possible uses of testis-derived stem cells for the study of pluripotency and for regenerative applications is also discussed in comparison to other approaches using ES cells and induced pluripotent stem (ips) cells.

Keywords Spermatogonia • Adult stem cells • Pluripotent stem cells • Testis • Cell transplantation

2.1 Introduction

More than one hundred years ago, it was recognized that testicular cells in adult men could give rise to outgrowths comprised of endoderm, mesoderm, and ectoderm, now well known as teratomas (Young 2005). Then, in the 1960s, Leroy

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Stevens made the startling observation that even transplantation of gonadal tissue out of the embryonic microenvironment and into the adult testis was sufficient to induce teratomas in mice of the proper genetic background (Stevens 1964). In the 1990s, a series of experimental conditions were established to efficiently obtain pluripotent clones, known as embryonic germ (EG) cells, by simply transferring murine primordial germ cells (PGCs) during a precise developmental window from the gonadal niche to an *in vitro* milieu defined by specific growth factors and feeder cells, as reviewed elsewhere in this volume (Matsui et al. 1992). These observations, in conjunction with the fact that the solitary task of the germline is to transmit the genetic and epigenetic information required for embryogenesis, all pointed to the possibility that postnatal germ cells could be predisposed to pluripotency. Data supporting this hypothesis has now been published by multiple groups of investigators, following a landmark study from T. Shinohara's laboratory in 2004 (Kanatsu-Shinohara et al. 2004).

In this chapter, we first introduce the mammalian spermatogonial stem cell (SSC), the cell type from which pluripotent stem cells are believed to arise, and discuss the technology that has facilitated investigation of this phenomenon. The unique properties of SSCs are highlighted in comparison to somatic cells and embryonic stem (ES) cells. We then address the factors that may predispose SSCs to pluripotency and review the studies in which murine and human germ cells have been observed to become pluripotent spontaneously *in vitro*, a phenomenon that is not observed with somatic cells in culture. Finally, we discuss the implications of the most recent findings related to male germline stem cells and we compare the properties of the germline-derived pluripotent cells with those of pluripotent cells generated from somatic cells through the delivery of exogenous pluripotency factors [induced pluripotent stem (iPS) cells].

2.2 The Putative Precursors: Spermatogonial Stem Cells (SSCs)

The SSC, responsible for maintaining near life-long spermatogenesis in mammals, is contained within the population of undifferentiated spermatogonia, along the basement membrane of the seminiferous tubule, but represents only about 0.03% of germ cells in mice (Tegelenbosch and de Rooij 1993). While morphologic criteria were previously used to define these stem cells, the advent of technology to transplant and later to expand them in culture has allowed a series of investigations into the molecular features that define SSCs, as reviewed elsewhere in this volume (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al. 2003). The notion that postnatal testicular cells are predisposed to pluripotency remained untestable prior to the advent of technology to accurately identify and propagate SSCs. In 2003, the Shinohara group described a set of culture conditions that allowed long-term culture of SSCs, by employment of mouse embryonic fibroblast (MEF) feeder cells, in conjunction with a rich culture medium supplemented with several recombinant

growth factors, including glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) (Kanatsu-Shinohara et al. 2003). As opposed to immunoselection, only a negative selection was required to remove the majority of somatic cells via binding to gelatin. However, the efficiency of deriving long-term SSC lines from adult mouse testis was only 20–50% in these culture conditions (Kanatsu-Shinohara et al. 2004; Ogawa et al. 2004). This could be due to a relative decline in the number of functional stem cells in older animals or in the self-renewal capacity of such cells. Nonetheless, multiple studies have subsequently confirmed that the SSCs could be passaged over many generations and retain the ability to restore fertility in animals with deficient spermatogenesis (Kanatsu-Shinohara et al. 2003, 2005a, b; Ryu et al. 2005; Kubota et al. 2004a, b). However, it has been estimated that only 1–2% of cultured SSCs exhibit testicular repopulation capacity (Kanatsu-Shinohara et al. 2005b). Conversely, recent data suggest that differentiating germ cells can display plasticity, potentially reverting back to the stem cell phenotype *in vitro* or *in vivo* (Nakagawa et al. 2007; Barroca et al. 2009). As our understanding of the nature of SSCs has evolved, the tools to study them have become increasingly sophisticated, revealing a number of unique properties as discussed below.

2.3 Molecular Features that Could Predispose SSCs to Pluripotency

It is reasonable to suppose that some of the same characteristics of male germ cells that facilitate initiation of embryogenesis at the time of fertilization could also play a role in spontaneous cellular reprogramming that would lead to formation of pluripotent stem cells *in vitro*. But what are these special molecular characteristics? Both in terms of gene expression and chromatin structure, SSCs have been found to share certain features (but also notable differences) with pluripotent stem cells. In the sections below, we first review the current understanding of the normal expression levels of the core pluripotency genes (particularly *Oct4, Nanog*, and *Sox2*) in the testis and in cultured SSCs then examine data describing the unique state of chromatin and its modifications in the germ lineage (see Fig. 2.1).

Oct4 is a homeobox transcription factor that is crucial for pluripotency in embryonic stem cells (Nichols et al. 1998; Niwa et al. 2000). Oct4 is part of a core network of molecules, including Sox2 and Nanog, that both autoregulate and co-regulate downstream factors that maintain self-renewal and block differentiation (Boyer et al. 2005). Studies revealing the expression of Oct4 in the postnatal testis have relied both on immunological methods and genetic reporter systems with varying results, though no study has documented levels in postnatal germ cells comparable to those observed in ES cells. Pesce et al. (1998) found diffuse Oct4 protein by immunohistochemistry in spermatogonia up to 7 days postnatally but in adult animals only a subset of spermatogonia (type A) were positive (Pesce et al. 1998). However, in a report using transgenic mice that expressed GFP under control of an 18 kilobase

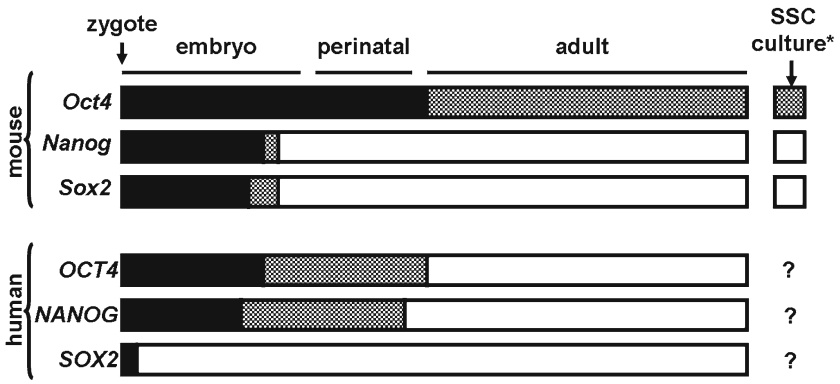

Fig. 2.1 Expression of core pluripotency-associated genes *OCT4, SOX2*, and *NANOG* in the testis. Relative levels are denoted by *black bars* (high), *gray bars* (down-regulated but detectable), and *white bars* (absent) for mouse and human testis *in vivo*. *Boxes* at right denote expression in long-term SSC culture. This data represents a summary of findings from multiple studies (Kanatsu-Shinohara et al. 2004, 2005b; Pesce et al. 1998; Yoshimizu et al. 1999; Ohbo et al. 2003; Ohmura et al. 2004; Tadokoro et al. 2002; Buaas et al. 2004; Tokuda et al. 2007; Looijenga et al. 2003; Rajpert-De et al. 2004; Seandel et al. 2007; Dann et al. 2008; Imamura et al. 2006; Avilion et al. 2003; Western et al. 2005; Perrett et al. 2008; de Jong et al. 2008; Shi et al. 2006; Chambers et al. 2003; Yamaguchi et al. 2005; Hoei-Hansen et al. 2005; Yeom et al. 1996) (see Addendum). *Question marks* indicate absence of published data

Oct4 gene fragment containing both the distal enhancer and the epiblast-specific proximal enhancer, the investigators were unable to directly visualize GFP expression more than 10 days after birth (Yoshimizu et al. 1999). Using similar *Oct4/GFP* transgenic reporter mice, Ohbo et al. (2003) identified *Oct4*-expressing spermatogonia (containing the majority of stem cell activity) in mice up to 14.5 days postnatally, after which point expression decreased (Ohbo et al. 2003). A subsequent study revealed *Oct4* expression by RT-PCR in the Ep-CAM$^+$ fraction of adult Oct4/GFP$^+$ cells (Ohmura et al. 2004). However, detection of endogenous Oct4 protein in histologic sections of the adult testis required significant amplification in the latter study, due to low levels of antigen compared to what was seen in the spermatogonia of younger animals. Nonetheless, Tadokoro et al. (2002) confirmed Oct4 protein expression in a substantial subpopulation of adult undifferentiated spermatogonia in progeny-deficient strains of mice and proposed that Oct4 expression is reversible in SSCs, depending on microenvironmental conditions, although the functional significance of this result was not clear. Buaas et al. (2004) demonstrated co-expression of Oct4 and Plzf, a marker of undifferentiated spermatogonia, in adult mouse testes, while Tokuda et al. (2007) found co-expression of Oct4 and Cdh1 in adults (Buaas et al. 2004; Tokuda et al. 2007). Taken together, these studies suggest that a subset of murine spermatogonia maintain Oct4 expression into adulthood, albeit at relatively low levels under normal (i.e., nonpathological) conditions.

In the human testis, though fewer data are available, OCT4 expression in the male germline appears rapidly down regulated after ~20 weeks of gestation (Looijenga et al. 2003; Rajpert-De et al. 2004). A small number of OCT4-positive cells are

detectable at 3–4 months postnatally but these normally disappear thereafter (Rajpert-De et al. 2004).

A similar picture has emerged for *Oct4* expression in cultured SSCs. Multiple studies confirmed expression by RT-PCR in neonatal SSCs (Kanatsu-Shinohara et al. 2004, 2005b). However, a quantitative analysis of neonatal SSCs later demonstrated sharply lower Oct4 expression by either mRNA or protein, compared to ES cells (Imamura et al. 2006). Not surprisingly, the same pattern was seen in adult SSCs in long-term culture (Seandel et al. 2007). Notably, this heterogeneous and relatively low magnitude of Oct4 expression has recently been found to be functionally important in self-renewal and survival of cultured SSCs (Dann et al. 2008).

A second major pluripotency gene studied in the male germ line is *Sox2* (SRY [sex determining region Y] – box 2, which, like *Oct4* is expressed in the germline) (Avilion et al. 2003). In mice, germline expression of *Sox2* is lost by E15.5 (Western et al. 2005). In humans, *SOX2* mRNA was detected in adult testis in two studies (Gure et al. 2000; Schmitz et al. 2007). However, using more rigorous methods SOX2 was later shown to be absent even in human PGCs and also absent in adult testis by both message and protein (Perrett et al. 2008; de Jong et al. 2008). In cultured neonatal murine SSCs, no Sox2 protein was detectable despite significant transcript levels (Imamura et al. 2006; Shi et al. 2006). In contrast, Seandel et al. (2007) found that adult SSCs in culture did not even express *Sox2* message (Seandel et al. 2007). Therefore, in both mice and human functional SOX2 protein is not likely to be present beyond an early developmental window, and this decline in expression maybe paralleled in cultured SSC lines derived from mice of increasing age.

The third canonical pluripotency-associated transcription factor is Nanog, another homeodomain-containing protein strongly expressed in ES cells (Chambers et al. 2003). Beyond the mouse blastocyst stage, *Nanog* expression is present in the male germ lineage, and the protein is detectable through E16.5, at which time it is largely down-regulated coincident with mitotic arrest (Chambers et al. 2003; Yamaguchi et al. 2005). No Nanog protein was detected in the adult mouse testis (Hoei-Hansen et al. 2005). Similarly, in the human testis, NANOG protein is present through 19 weeks of gestation, but the rare positive cells that remain at 3–4 months postnatally are completely absent by childhood and also in adults (Hoei-Hansen et al. 2005). Perhaps not surprisingly cultured murine SSCs from neonatal or adult stages do not express Nanog (Kanatsu-Shinohara et al. 2004; Seandel et al. 2007).

As mentioned above, OCT4, SOX2, and NANOG are thought to form a core regulatory network in ES cells (Boyer et al. 2005). Moreover, both OCT4 and SOX2 are key transcription factors in the cocktail of genes used to generate iPS cells starting from somatic cells, with OCT4 being the most critical of the two (Takahashi and Yamanaka 2006; Kim et al. 2009c). However, based on the studies described above, these proteins, with the exception of OCT4, are not present either in postnatal germ cells or in cultured SSCs (Fig. 2.1). Therefore, it is unlikely that a resident subpopulation of ES-like cells in the postnatal testis could give rise to pluripotent stem cells *in vitro*. Furthermore, based on their absence in the precursor population, it seems that neither SOX2 nor NANOG-driven signals are likely to be the most proximal mediators in the signaling pathway leading to the conversion of

SSCs into pluripotent stem cells. Of note, however, other pluripotency associated genes have been found to be expressed in the adult testis. For example, *Lin28*, previously associated with regulation of let-7 precursor microRNA processing, was found to induce pluripotency of somatic cells (when introduced ectopically in conjunction with *Oct4*, *Sox2*, and *Nanog*) (Viswanathan et al. 2008; Yu et al. 2007). Recently, *Lin28* was found to be expressed in adult undifferentiated spermatogonia (Zheng et al. 2009). It is not known whether *Lin28* can constitute an upstream signal leading to expression of other core pluripotency genes.

2.4 Epigenetic Factors that Could Predispose to Pluripotency

The state of chromatin in the development of the male germline represents a key distinguishing feature from somatic cell types and one that is crucial for reproductive success. Deficiencies of genes that drive chromatin modifications such as DNA methylation can result in male sterility (Kaneda et al. 2004). Likewise, the unique chromatin state could also represent a predisposing factor for premature acquisition of pluripotency. Murine PGCs, unlike somatic lineages, undergo erasure of recently acquired DNA methylation in both imprinted and nonimprinted loci around the time of entry into the gonads by about E12.5 (Hajkova et al. 2002). Subsequently, male imprinting patterns become reestablished during the remainder of the prenatal period and into early postnatal life (Davis et al. 1999; Li et al. 2004; Schaefer et al. 2007; Oakes et al. 2007). However, Farthing et al. (2008) recently found unexpected similarities in the global promoter methylation status between ES cells, EG cells, and sperm, suggesting that male germline cells could activate transcription of pluripotency-associated genes more easily than somatic cells (Farthing et al. 2008). ES cells have recently been shown to exhibit characteristic sets of histone methyl marks, linked to their pluripotent status (Bernstein et al. 2006). The histone methylation profile of postnatal SSCs is poorly characterized but a distinctive pattern of perinuclear histone H3 lysine 9 and H4 lysine 20 tri-methylation was recently described on postnatal undifferentiated spermatogonia, although the patterns at specific loci were not examined (Payne and Braun 2006). Recently, the acquisition of pluripotency in mouse PGCs at E8.5 was linked to DNA demethylation, with subsequent loss of pluripotency following histone replacement after E11.5 (Hajkova et al. 2008).

Since pluripotency is acquired *in vitro*, the chromatin status of cultured SSCs could affect the stability of lineage commitment and predispose the cells to pluripotency. The Shinohara Laboratory found that neonatal SSCs bear the expected androgenetic pattern of methylation at imprinted genes, which was stable in long-term culture (Kanatsu-Shinohara et al. 2004, 2005b). When pluripotency-associated genes were examined specifically, both sperm and cultured SSCs exhibited relative hypomethylation of regulatory regions in a number of such genes, although this was not the case for specific key genes, such as *Sox2*, and did not necessarily correlate with the presence of the corresponding protein (Imamura et al. 2006). The authors

concluded that, for certain key pluripotency genes, post-transcriptional mechanisms could be very important in controlling the phenotype. Thus, the extent to which the preexisting epigenetic profile of germ cells contributes to the observed acquisition of pluripotency is a matter of speculation.

2.5 Culture-Induced Pluripotency in Mice

While multiple laboratories have demonstrated the acquisition of pluripotency by germ cells *in vitro*, the procedures and conditions used are remarkably different (see Figs. 2.2 and 2.3 and Table 2.1). In 2004, it was discovered that SSCs derived from the neonatal testis could reproducibly give rise to pluripotent embryonic-like stem cells within 4–7 weeks of initiation of the stem cell culture in ~20% of experiments but at a very low rate relative to the number of cells plated (Kanatsu-Shinohara et al. 2004). These ES-like cells not only physically resembled ES cells but also bore a similar gene expression profile, marked by the presence of mRNA for *Nanog*, *Rex1*, *Utf1*, *Esg1*, and *Cripto*, among others. When wild-type testes

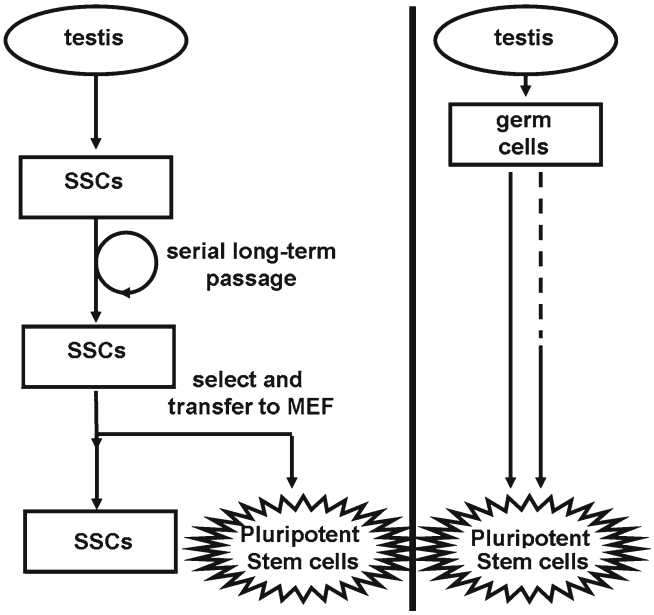

Fig. 2.2 Variations in experimental approaches for obtaining pluripotent stem cells from postnatal testis. *Left panel*: Some laboratories have employed long-term culture of SSCs prior to obtaining pluripotent stem cells that were then separated from the parental cells by selection (Kanatsu-Shinohara et al. 2004; Seandel et al. 2007; Ko et al. 2009; Yu et al. 2000). *Right panel*: Other groups have employed short-term culture without parallel propagation of the precursor (parental) cell population (Guan et al. 2006; Ko et al. 2009; Mizrak et al. 2010)

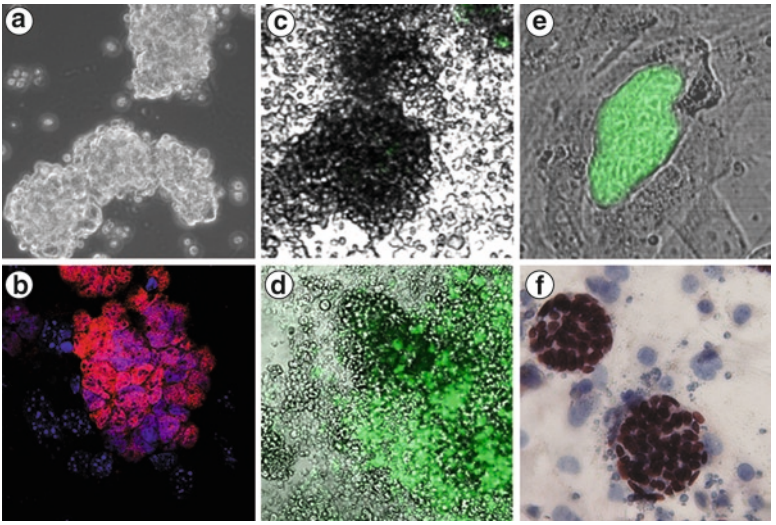

Fig. 2.3 Culture-induced up-regulation of OCT4 in spermatogonial stem and progenitor cells coincident with appearance of ES-like colonies. SSCs were derived from adult wild-type or OCT4-GFP reporter mice. (**a**) Phase contrast appearance of routine SSC cultures. (**b**) Specific nuclear labeling of SSCs using anti-PLZF antibody (*red*). (**c**) Fluorescence microscopy for OCT4-GFP reporter in routine SSC cultures. (**d**) Spontaneous up-regulation of OCT4-GFP (*green*) in long-term culture of SSCs. (**e**) Uniform OCT4-GFP expression (*green*) in ES-like colonies mechanically transferred to MEF feeder cells. (**f**) Immunohistochemistry demonstrating uniform endogenous OCT4 protein expression (*brown*) in ES-like cells derived from SSC cultures. Counterstain in (**b**) and (**f**) is *blue* (Seandel et al. unpublished data)

(3–8 weeks old) were the source of SSCs, no ES-like cells appeared, although the parental adult SSC lines could be derived in only 20% of experiments. Adult-derived *P53* knockout SSC, which could be derived at higher rate, also gave rise to ES-like cells. The investigators provided substantial evidence that the ES-like cells were not only distinct from the parental SSCs but that the ES-like cells could undertake most if not all of the functions of ES cells, including long-term self-renewal in culture, multi-lineage differentiation and formation of chimeric animals, including germline transmission.

A major distinction from the parental SSCs was that the ES-like cells formed teratomas in both subcutaneous teratoma assays and upon injection into the seminiferous tubules, indicating that the novel ES-like phenotype was stable and that the cells could not simply revert back to the SSC phenotype upon placement back into the normal SSC niche (see Table 2.1) (Kanatsu-Shinohara et al. 2004). This was in contrast to the parental SSCs that did not form teratomas at all, consistent with our own experience (Seandel et al. unpublished data). The authors proposed that a predisposition to pluripotency could be a general property of SSCs but that the somatic cells *in vivo* may help to suppress such aberrant cell phenotypes, in order to prevent teratoma formation in the normal testis. Furthermore, the mechanism of conversion of SSCs into ES-like cells appeared to be different from that in which

Table 2.1 Selected studies of germ cell-derived pluripotent stem cells

Parameter	Kanatsu-Shinohara et al. (2004)	Guan et al. (2006)	Seandel et al. (2007)	Izadyar et al. (2008)	Ko et al. (2009)	Conrad et al. (2008)	Kossack et al. (2009)
Age/species of donor	Neonatal/ mouse	Adult/ mouse	Adult/ mouse	Neonatal-adult/ mouse	Adult/ mouse	Adult/human	Adult/ human
Designation for pluripotent cell lines	mGS	maGSC	MASC	mGC	gPS	haGSC	hMGSC
Precursor population kept in long-term culture	Yes	No	Yes	No	Yes	No	No
Precursor population restores spermatogenesis[a]	Yes	Yes	Yes	No (c-kit+)	Yes	N/A	N/A
Precursor population forms teratoma	No	N/A	No	N/A	No	N/A	N/A
Pluripotent stem cells differentiate *in vitro*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pluripotent stem cells form teratoma	Yes	Yes	Yes	No	Yes	Yes	No[c]
Precursor population forms chimeric blastocysts	No	Yes	No[b]	N/A	N/A	N/A	N/A
Pluripotent stem cells form chimeric blastocysts	Yes	N/A	Yes	Yes	Yes	N/A	N/A

[a] In spermatogonial stem cell-type transplantation assay

[b] Seandel et al. unpublished data

[c] Additionally, studies by Golestaneh et al. (2009) and Mizrak et al. (2010) did not yield large, complex teratomas from the ES-like cells. *N/A* not applicable

PGCs convert into EG cells, since the latter set of culture conditions were not successful for conversion of SSCs. These conclusions raised the intriguing question of whether SSCs should generally be considered capable of "dedifferentiation" and therefore inherently multipotent or alternatively whether a small primitive subpopulation of germ cells in the postnatal testis could be responsible for the production of ES-like cells in culture. To answer this, genetic marking was used to demonstrate that single SSC clones could give rise both to continually self-renewing SSC cultures and to ES-like cells (Kanatsu-Shinohara et al. 2008a). Of note, the partial androgenetic pattern of imprinting in SSCs was invoked as an indication that epigenetic instability in culture could contribute to the change in fate of SSCs, upon conversion into ES-like cells (Kanatsu-Shinohara et al. 2004).

Several studies subsequently confirmed the general concept that pluripotent stem cells could be derived from the testis and specifically found that even the adult testis harbors cells with such capability. Guan et al. (2006) found that, after a very brief period of culture, the Stra8^{+} population of adult testicular cells was able to generate ES-like cells (Guan et al. 2006). However, the published nomenclature provided did not unequivocally distinguish the identity of the parental population from the ES-like derivatives. Interestingly, the authors found that even the parental SSCs could directly contribute to chimerism in blastocyst injection assays, although no SSC transplantation data were shown to establish the purity and functionality of the starting population of germ cells, which were precultured for 1 week prior to initiation of ES-like induction culture conditions. The authors suggested that the presence of somatic factors such as GDNF could serve to inhibit plasticity both *in vitro* and *in vivo*. This hypothesis is in contrast with the results obtained by another group (Huang et al. 2009). The latter reported the derivation of alkaline phosphatase-positive pluripotent cells from unselected neonatal testicular cell suspensions in short-term culture (1 week), but they proposed that the testicular somatic cells present in the culture, specifically the Leydig cells, are responsible for the production of Igf1 which, through Akt signaling, maintains pluripotency of SSCs. In this study, there is no distinction between SSC and pluripotent cells, but rather they were proposed to be the same cell type able to contribute both to spermatogenesis after transplantation in busulfan-treated testes and to chimera formation upon blastocyst injection. Notably, these cells did not form teratomas when transplanted into the testis, whereas they did form teratomas when transplanted subcutaneously in NOD-SCID mice. Another group reported the development of pluripotent stem cells during short-term culture of neonatal and adult testicular cells from *Oct4/GFP*-reporter mice (Izadyar et al. 2008). This report also did not distinguish between the different populations of germline-derived cells in question (i.e., SSCs and ES-like cells) in the culture system, a similar semantic and experimental issue as in the Guan et al. (2006) study (Izadyar et al. 2008). In fact, both populations appeared to coexist (based on heterogeneous morphology of colonies in the images provided). Perhaps not surprisingly, the authors found a remarkably similar expression profile and imprinting pattern between germ cells before and after culture. This could be due to dilution of ES-like cells by a majority of SSC-like cells in the culture (which could also explain the absence of teratoma formation), although this issue was not specifically addressed.

Seandel et al. (2007) developed a means to derive adult SSC lines from mice up to 11 months of age with overall >90% efficiency, using feeders comprised of mitotically inactivated primary testicular stromal cells. We found that the novel G-protein coupled receptor Gpr125 was expressed in a population of cultured germ cells that contained SSC activity based on transplantation assays. These experiments were performed using engineered mice (and SSCs derived from such mice) in which *lacZ* was placed within the endogenous *Gpr125* locus (*Gpr125-lacZ*), representing an extremely sensitive and specific reporter system. After about 3 months following initiation of SSC cultures even without preselection of cells from the testes of Gpr125-lacZ mice (or from other strains), morphologically distinct colonies comprised of ES-like cells [referred to as multipotent adult spermatogonial-derived stem cells (MASCs)] appeared spontaneously (Fig. 2.3). These colonies were selected and transferred into ES culture conditions for long-term propagation, establishing new colonies that closely resembled mouse ES cells when plated upon inactivated MEFs. These adult-derived ES-like cells expressed Oct4 and Nanog protein and readily differentiated into derivatives of all three germ layers *in vitro*, including contractile cardiogenic tissue. Similarly, the ES-like cells produced tri-lineage teratomas in immunodeficient mice (Seandel et al. 2007), including foci of de novo germ cell differentiation (Seandel et al. unpublished data).

As more stringent evidence of pluripotency, the ES-like cells that had been cloned at the single cell level were found to be competent at forming chimeric embryos, with contributions to multiple organ systems. Of note, despite obtaining live-born chimeric mice from ES-like cells, we have observed generally low contributions of the adult ES-like cells in chimeras and a reproducible congenital abnormality comprised of hyperplasia and abnormal chondrogenesis of the anterior rib cage (Falciatori et al. unpublished data). This phenotype, reminiscent of androgenetic embryos, would be consistent with the partial androgenetic imprinting profile previously described for ES-like cells (Kanatsu-Shinohara et al. 2004; Mann et al. 1990). Also of great interest, the gene expression profile of the adult-derived ES-like cells was not identical to that of ES cells. Among the pluripotency genes markedly lower in the ES-like cells were *Rex1, Esg1,* and *Gdf3*, while *Nanog* was also lower but still expressed at absolutely high levels. Certain lineage commitment markers were present at substantially higher levels in ES-like cells than in bona fide ES cells, including the mesodermal gene *brachyury*.

More recently, the ability of adult SSCs in culture to produce pluripotent stem cells was confirmed by Ko et al. (2009). These authors not only demonstrated the ability of authentic SSC clones to acquire pluripotency (including a germline contribution) but also showed that the initial plating density of SSCs was crucial for efficient conversion (estimated at about 0.01% of cells). Furthermore, this study also compared methylation patterns at imprinted genes to demonstrate the origin of the pluripotent stem cells from spermatogonia. The latter analysis showed that the ES-like cells exhibited an androgenetic imprinting pattern at the differentially methylated region of the H19 gene, similar to what was seen in spermatogonia but dissimilar to the somatic pattern. These data argued against a possible origin from a somatic stem cell. As with the prior studies, the authors demonstrated that derivatives

of the pluripotent stem cells were functional in various assays. Together, these results highlight the conclusion that the adult spermatogonial-derived ES-like cells should not be considered equivalent to ES cells, despite their common ability to form functional tissues, since differences were found in both gene expression by Seandel et al. (2007) and in imprinting status by Ko et al. (2009), respectively.

2.6 Culture-Induced Pluripotency in Humans

Recent provocative studies have found evidence for pluripotent stem cells derived from the adult human testis (Conrad et al. 2008; Kossack et al. 2009; Golestaneh et al. 2009; Mizrak et al. 2010). Conrad et al. (2008) employed the following steps to obtain highly enriched germ cells from fresh tissue: 4 days of culture of mixed enzymatically dispersed testicular cells, followed by immunoselection using alpha6 integrin, and differential matrix selection for collagen-non-binding and laminin-binding cells. It is quite remarkable that within 4 days of culture the selected germ cells activated expression of OCT4 protein in the nucleus and cytoplasm, even though no OCT4 is found subsequent to embryonic stages in the human testis (Rajpert-De et al. 2004; Conrad et al. 2008). After several additional weeks of incubation, cultures were obtained containing fibroblast-like monolayers that surrounded discrete multilayered colonies of cells with ES-like properties. Subsequent culture in the presence of LIF resulted in generation of new colonies of such stem cells, although it is not clear whether these represented de novo conversion from the precursor spermatogonial cells.

The ES-like cells expressed *OCT4, SOX2,* and *NANOG*, demonstrated by RT-PCR, while both OCT4 and NANOG protein were present by immunofluorescence, flow cytometry, and Western blot analysis. Microarray expression profiling detected expression of all three of these pluripotency genes not only in the ES-like stem cells but interestingly also in the precursor spermatogonial cells, implying that transcriptional activation also of *SOX2* and *NANOG* (in addition to *OCT4* and other pluripotency associated genes such as *REX1*) must take place during the initial four-day culture period. While this study actually detected *SOX2* by RT-PCR in normal adult testis, neither *SOX2* nor *NANOG* were found expressed after embryonic stages by other investigators (Perrett et al. 2008; de Jong et al. 2008; Hoei-Hansen et al. 2005; Conrad et al. 2008). The reason for these discrepancies between laboratories is unclear but may be due to technical differences. While the global expression profile of the human ES-like cells was generally similar to ES cells, the retention of a germline signature could be seen in high levels of expression of *DAZL* and *POU6F1*. To demonstrate pluripotency, the authors formed teratomas from independent ES-like cultures from eight normal samples in 23 of 32 attempts, using cells with normal karyotype. These data portray a relatively robust system for obtaining pluripotent stem cells.

Kossack et al. (2009) reported the derivation of two ES-like cell lines from testicular biopsy-derived cells (Kossack et al. 2009). These lines were obtained

after a 1-week culture of mixed testicular cells followed by a manual selection and transfer of putative stem cell colonies onto MEF feeders. While these ES-like cells lacked *NANOG* expression, they did express *OCT4* and *SOX2*. In addition, the ES-like cells exhibited high levels of telomerase and normal karyotype. The ES-like cells exhibit an intermediate pattern of methylation at both imprinted and non-imprinted loci, similar, in general, to that described by (Kossack et al. 2009; Conrad et al. (2008)). Although functional during *in vitro* differentiation assays, the ES-like cells did not form teratomas in immunocompromised mice. This contrasts to a third report in which ES-like cells derived from biopsy-sized quantities of human testicular tissue from organ donors were able to give rise to small teratomas (Golestaneh et al. 2009). In this study, total testicular cell suspensions were plated in defined human ES cell culture medium in order to generate ES-like colonies within 1 week after testicular cell isolation. Another report also describes the generation of pluripotent cells from testicular biopsies but with slower kinetics (3–8 weeks) (Mizrak et al. 2010). When transplanted into immunodeficient mice, these cultured testicular cells were also able to give rise to tissues containing some differentiated human cells but not complex teratomas (which were produced, in contrast, using either hESC or iPS cells). Since neither of these latter studies used preselection of germ cells, the exact origin of the pluripotent cells derived is unclear.

Thus, the aforementioned pluripotent cell lines derived from postnatal testis share certain important properties, including expression of pluripotency genes, trilineage differentiation ability, and variations on the expected androgenetic methylation profile at imprinted genes, in conjunction with intermediate levels of methylation at other important loci (Table 2.1). The functionality of human pluripotent cell lines is generally somewhat more difficult to assess due to technical and ethical limitations. Nonetheless, data initially generated using the murine system appear to be relevant for the human testis. Unfortunately, different approaches were used to generate the pluripotent lines in each study, precluding their direct comparison (see Fig. 2.2). For example, multiple different culture media were used for induction conditions, in the presence or absence of fetal bovine serum, GDNF, bFGF, EGF, and LIF, with somewhat conflicting results about their respective effects (de Rooij and Mizrak 2008). Furthermore, the timeframe for conversion of unipotent germ cells into pluripotent stem cells is also quite variable. These observations suggest that more than one mechanism could theoretically be responsible. It should be noted, however, that spontaneous teratomas occur only very rarely in males, with an incidence of <1 in ~11,000 in wild-type laboratory mice and <1 in ~16,000 in the human testis (Krausz and Looijenga 2008; Stevens and Mackensen 1961). In comparison, the experimental rate of emergence of pluripotent cells observed in the aforementioned studies is much higher in aggregate and no such similar process is known to occur in somatic cells without experimental delivery of pluripotency factors. Nonetheless, it is difficult to formally rule out the possibility that a spontaneous genetic change, as opposed to an epigenetic or culture-induced phenotypic change, contributed the observed results. While the mechanism of conversion will be extremely important to determine and may have relevance for acquisition of pluripotency by other cell types, the crucial criteria in

considering such cells for eventual clinical implementation is their functionality and safety, as discussed below.

2.7 Potential Applications of Germline-Derived Pluripotent Cells: A Comparison with ES Cells and iPS Cells

Pluripotent stem cells derived from the adult testis could serve two distinct and important functions in the future. First, this alternative stem cell type provides a useful research tool in parallel with ES cells and iPS cells to probe the mechanisms by which pluripotency is acquired and maintained. Second, patients affected by a plethora of pathological conditions (including heart failure, neurodegenerative diseases, diabetes, etc.) could theoretically benefit from an alternative source of self-renewing pluripotent stem cells. These cells could be expanded and differentiated *in vitro* into the desired cell type before being transplanted to the patient. ES cells represent the paradigm of such a stem cell. However, the use of ES cells in clinical practice is hindered by two major problems. First, ethical concerns arise from the fact that the derivation of human ES cells requires the destruction of human embryos. Moreover, it is impossible to derive patient-specific ES cells and therefore immunological rejection is a major issue for their use in regenerative medicine. If pluripotent stem cells could be derived directly from adult tissues, both issues could be solved.

It has already been discussed in this chapter that pluripotent ES-like stem cells can be spontaneously derived from the adult testis, perhaps from SSCs. Theoretically, similar cells could be derived directly from a male patient, serving as a tool for regenerative therapy without ethical or immunological concerns. We and others demonstrated that upon *in vitro* differentiation ES-like cells can originate different cell types, as contractile cardiomyocytes, endothelial cells, neural cells, pancreatic cells, and others (Kanatsu-Shinohara et al. 2004; Seandel et al. 2007; Guan et al. 2006; Izadyar et al. 2008; Conrad et al. 2008; Kossack et al. 2009). We also found that the endothelial cells formed by the ES-like cells participate in the generation of blood vessels that are connected with the host circulation and therefore are functional (Seandel et al. 2007).

More recently, two research groups have independently demonstrated that murine pluripotent stem cells derived from either adult or neonatal SSCs can be efficiently differentiated into cardiomyocytes using the protocols already available for ES cells. In the first study, pluripotent cells derived from mouse neonatal SSCs were compared to other pluripotent cells (ES and EG cells) in terms of their ability to differentiate into cardiomyocytes and endothelium (Baba et al. 2007). Notably, ES-like cells and EG cells were actually more efficient than ES cells in generating cardiomyocytes, suggesting that germ cell-derived pluripotent cells might be somewhat biased toward mesodermal differentiation. This is consistent with the fact that, even in the undifferentiated state, ES-like cells may express higher levels of mesodermal markers than ES cells (Seandel et al.

unpublished data; Seandel et al. 2007). The ability of ES-like cells to differentiate into functional cardiomyocytes exhibiting spontaneous action potentials, as well as electromechanical coupling between cells, have been confirmed by another study (Guan et al. 2007). Moreover, when undifferentiated ES-like cells were transplanted directly into the hearts of normal mice they proliferated in the site of engraftment, gradually lost their pluripotency, and differentiated into vascular endothelial and smooth muscle cells. Unfortunately, no evidence of differentiation into cardiomyocytes was found (Guan et al. 2007). This could be due to the fact that the ES-like cells were implanted into normal hearts and failed to be efficiently recruited to the cardiomyocyte fate, as occurs in infarcted hearts (Singla et al. 2006). The generation of cardiomyocytes could also be improved by pre-differentiating the cells *in vitro* before transplantation (Zeineddine et al. 2005). It is quite interesting that the transplantation of undifferentiated ES-like cells did not result in tumor formation, since the ability to form teratomas is an intrinsic property of pluripotent cells (and a key criteria for demonstrating pluripotency) (Damjanov and Solter 1974; Evans and Kaufman 1981; Wobus and Boheler 2005). However, it has been reported previously that intramyocardial transplantation of undifferentiated ES cells in both mice and rats did not result in tumor formation either (Singla et al. 2006; Min et al. 2002). More recently, it has been found that the formation of teratomas in the heart depends on the number of undifferentiated ES cell transplanted (Behfar et al. 2007).

Using conditions already established for ES cells, germ cell-derived ES-like cells have been induced towards neural differentiation (Glaser et al. 2008). Similar to ES cells, they formed different kinds of neurons (GABAergic, glutamatergic, serotonergic, and TH-positive) and glial cells (astrocytes and oligodendrocytes). During the differentiation process multipotent neural stem cells were formed that could be propagated as stem cells for many passages and also differentiate into both neurons and glia. The neurons derived from ES-like cells showed action potentials and were organized in functional synaptic networks. Interestingly, oligodendrocytes derived from ES-like cells were able to home and form myelin in slices of central nervous system tissue of myelin-deficient rats, suggesting that ES-like cells could be useful to treat demyelinating disorders (Glaser et al. 2008). More recently, ES-like cells have been differentiated in hepatocytes at a level comparable to ES cells. However, the amount of hepatocytes generated with the protocols described from both ES and ES-like cells is still too little to be considered useful in clinical setting (Loya et al. 2009).

The studies above demonstrate that mouse ES-like cells can be differentiated *in vitro* using the same protocols already established for mouse ES cells. Although still very preliminary in this regard, some of these studies tried to evaluate both the ability of ES-like cells to improve pathological conditions and their safety after transplantation (Guan et al. 2007; Glaser et al. 2008). It appears likely that further studies with the same rationale will support the ability of transplanted ES-like cells to rescue disease models in a manner comparable to that of ES cells.

In addition, it has also been reported that is possible to correct a genetic defect in mouse ES-like cells by using a human artificial chromosome (HAC). The delivery

of a HAC has been used to restore P53 expression and function in P53 null ES-like cells. These cells retained the ability to differentiate along several lineages both *in vitro* and *in vivo*. This report constitutes the first indication that ES-like cells can be used for gene therapy (Kazuki et al. 2008).

The recent demonstration that pluripotent stem cells can also be derived from human testis demonstrates the feasibility of using a testis biopsy to derive patient-specific ES-like cells for the use in regenerative medicine (Conrad et al. 2008; Kossack et al. 2009). These studies showed that human ES-like cells can be differentiated *in vitro* into myogenic, osteogenic, pancreatic, and neural cells via spontaneous differentiation or using the protocols currently available for human ES cells. The developmental plasticity of the human ES-like cells was generally comparable to that of human ES cells, which were actually *less* efficient than ES-like cells in forming differentiated pancreatic cells (Conrad et al. 2008).

Perhaps the most important consideration in the use of pluripotent cells in clinic is safety. Teratomas are benign tumors that contain self-renewing stem cells, as well as their differentiated progeny, and can be induced experimentally through injection of pluripotent stem cells in different sites. The ES-like cells, as a pluripotent stem cell type, are generally able to form teratomas, with certain exceptions (see Table 2.1). Therefore, it is possible that teratomas could arise even after therapeutic transplantation. Since only the undifferentiated stem cells produce teratomas, one way to circumvent this problem, already exploited for ES cells, is to efficiently differentiate the pluripotent cells before transplantation (Blum and Benvenisty 2008). There is no a priori reason to believe that the risk of teratoma formation associated with the ES-like cells would be higher than the risk associated with ES cells.

The pioneering work of S. Yamanaka's group introduced a promising alternative to obtain pluripotent cells from adult tissue (Takahashi and Yamanaka 2006). Using viral transduction of pluripotency-related genes (*Oct4, Sox2, Myc, Klf4*) differentiated cells were reprogrammed into iPS cells. The latter approach has been since exploited by numerous research groups, and it is now possible to produce both mouse and human iPS cells (Okita et al. 2007; Takahashi et al. 2007; Park et al. 2008). The safety of the first generation of iPS cells for clinical use has been a major concern. Besides the previously discussed risk of teratoma formation, which is intrinsic to any pluripotent cell, iPS cells also have an increased risk of oncogenic transformation due to multiple stable integrations of the oncogenes typically used to trigger the reprogramming process (like *Myc* and *Klf4*) and that can be reactivated over time (Okita et al. 2007). Therefore, while terminally differentiated cells produced from ES cells or ES-like cells should be safe in theory, those derived from iPS cells still bear a high risk of oncogenic transformation. Recently, different strategies have been applied to reduce the oncogenic potential of iPS cells, for example, by reduction of the number of exogenous genes necessary for obtaining the reprogramming. This can be achieved by using somatic cells that endogenously express some of reprogramming factors, like neural stem cells or by substituting some of the reprogramming factors with small molecules (Kim et al. 2009b, c; Shi et al. 2008). Additionally, nonintegrating means of gene delivery have been

exploited (Okita et al. 2008; Stadtfeld et al. 2008). The most promising strategy toward the clinical application of iPS cells is the direct delivery of the reprogramming factors as recombinant proteins instead of DNA, which has been demonstrated very recently to be feasible in both mouse and humans and which may decrease the possibility of oncogenic transformation of these iPS cells (Kim et al. 2009a; Zhou et al. 2009). Nevertheless, germline-derived stem pluripotent stem cells, iPS cells and ES-like cells should all be regarded as good model systems to study the reprogramming process involved in the reacquisition of pluripotency. The comparison between these systems can shed light on the underlying mechanisms of pluripotency and on why germline cells are able to be reprogrammed to pluripotency simply by the culture environment whereas somatic cells require the delivery of cocktails of pluripotency-related factors.

2.8 Conclusions

The spontaneous conversion of unipotent germ cells into pluripotent ES-like cells constitutes an ideal model with which to unravel the mechanisms necessary to switch a lineage-committed cell into a pluripotent cell. While the pluripotent stem cells derived from conversion of SSCs produced in different laboratories all share several major characteristics with ES cells, including their ability to self-renew and differentiate into many cell types, there are potentially important and revealing distinctions (Kanatsu-Shinohara et al. 2008b). Elucidation of these differences could reveal the answer to the intriguing question of whether more than one set of signals can lead to the pluripotent phenotype. Functional data indicate that ES-like stem cells derived from the testis represent at least a good research tool for probing the acquisition of pluripotency and perhaps even a viable alternative to ES cells and iPS cells for *in vitro* production of tissues for use in regenerative applications. However, extensive work needs to be done to improve the efficiency of production of ES-like cells, as well as to demonstrate their safety, before they can be implemented in clinical trials.

One might hypothesize that the reason why SSCs can convert to pluripotent cells spontaneously is that they are the only adult cells that maintain expression of the most important pluripotency factor OCT4. In this scenario their conversion to pluripotent cells would just simply follow the same processes that are involved in the conversion of somatic cells in iPS cells. In fact, it has already been demonstrated that somatic cells that express endogenously some of the pluripotency factors (like neural stem cells) can be reprogrammed by the expression of only the missing ones (Kim et al. 2009b). However, at the present stage it cannot be excluded that the set of molecular changes that take place to convert a SSC to a pluripotent cell could be different from those necessary to convert a somatic cell to a pluripotent stem cell. We believe that the comparison of the two conversion processes at the molecular level would shed light on the basic requirements for pluripotency as well as on how many possible pathways exist to regain pluripotency after differentiation.

2.9 Addendum

Since this chapter was originally prepared, two recent publications merit brief mention. First, Kuijk et al. (2010) have demonstrated that NANOG is present at the level of mRNA and protein in the adult testis of various species, particularly in meiotic and post-meiotic germ cells but also at lower levels in spermatogonia (Kuijk et al. 2010). Second, using an experimental approach, Ko et al. (2010) have strongly questioned the results of Conrad et al. (2008) and suggested that the cells produced in the latter study were not actually pluripotent but rather more similar to fibroblasts (Conrad et al. 2008; Ko et al. 2010).

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