Published in final edited form as:
Cell Stem Cell. 2009 July 2; 5(1): 3–4. doi:10.1016/j.stem.2009.06.012.

gPS navigates germ cells to pluripotency

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Abstract

The establishment of pluripotent stem cell lines from explanted testes has been hampered by the lack of robust derivation protocols and a poor understanding of the cellular origin of pluripotent cells. In this issue, Ko et al. present improved culture conditions to reproducibly generate pluripotent cell lines from murine testes and provide unequivocal evidence for their origin from spermatogonial stem cells.

Pluripotent cell lines can be established from different sources, including embryonic stem cells (ESCs) from blastocyst-stage embryos, embryonic germ cells (EGCs) from primordial germ cells (PGCs) and induced pluripotent stem cells (iPSCs) from somatic cells upon overexpression of defined transcription factors (Hochedlinger and Plath, 2009). In addition, several groups have succeeded in deriving ESC-like cells from explanted murine and human testis cells (Conrad et al., 2008; Guan et al., 2006; Kanatsu-Shinohara et al., 2004; Kanatsu-Shinohara et al., 2008; Kossack et al., 2008; Seandel et al., 2007). However, due to the low derivation efficiency of ESC-like cells from testes, and the lack of robust derivation protocols, the precise cellular origin of testis-derived pluripotent cells remained elusive. A functional and molecular comparison of the reported cell lines could not conclusively ascertain whether they originated from rare, residual PGCs, or rather from more committed spermatogonial stem cells (SSCs). In this issue, Schöler and co-workers (Ko et al., 2009) use clonal analyses to demonstrate that germline-derived pluripotent stem cells (gPSCs) arise from unipotent SSCs. In addition, the authors define specific cell culture parameters to consistently induce the switch from unipotent to pluripotent cells.

Robust derivation of pluripotent cells from adult testis

In their study, Ko et al. cultured single Oct4-expressing, c-kit-negative testis cells isolated from an Oct4-GFP reporter mouse, and derived stable SSC lines at a frequency of ~0.6%. Of note, the fraction of Oct4-GFP+ c-kit- cells (0.023%) in the testes is very similar to the assumed frequency of spermatogonial stem cells (0.02-0.03%), suggesting that this marker combination might enrich for an almost pure population of GSCs. Importantly, the resulting, cultured SSCs restore spermatogenesis upon transplantation into the testes of infertile mice,

confirming their germline properties. Furthermore, when seeded at low plating densities, SSC cultures gave rise to spontaneous gPSC colonies at a reproducible frequency of ~0.01% within 4 weeks. I*n vitro* differentiation of gPSCs yielded cardiomyocytes or neural lineages with the potential to undergo spontaneous contraction, action potential and myelin formation upon *in vivo* engraftment. In addition, gPSCs were able to generate low-degree chimeras capable of germline transmission (Table 1), thus fulfilling all the requirements for a *bona fide* pluripotent cell type. Interestingly, SSCs maintained their germ cell character and failed to form gPSC colonies at high seeding densities, perhaps because the cultures reached confluence too quickly, thus providing insufficient time for the conversion to a pluripotent state. Alternatively, dense SSC cultures might secrete inhibitory paracrine signals that prevent pluripotent conversion of adjacent cells.

Prior to the present report from Scholer and colleagues (Ko et al., 2009), the cellular origin of gPSCs remained controversial. Previously, Seandel et al attempted to address this question by demonstrating that rare pluripotent cell clusters emerged spontaneously within long-term cultured (3 months) SSCs (Seandel et al., 2007). However, the presence of a rare contaminating pluripotent cell type could not be excluded in this setting. Kanatsu-Shinohara and colleagues performed a more definitive experiment by comparing the genomic integration pattern of vector DNA introduced into SSCs with that of a derivative pluripotent cell line (Kanatsu-Shinohara et al., 2008). The authors found that a single SSC line and a resultant pluripotent line carried apparently identical integration marks, suggestive of a clonal origin. Since the genomic marking was performed with a targeting construct with significant homologous arms, the rare possibility of two independent, identical integration events in two unrelated cells could not be formally excluded. An important advance of the current study is the demonstration that SSC lines can be established from single Oct4+c-kit- GSCs, and that gPSCs can be clonally derived from SSC lines, thus providing unequivocal evidence that unipotent SSCs are the cells of origin for pluripotent cells (Ko et al., 2009).

Characteristics of testis-derived pluripotent cells

In their seminal report demonstrating the derivation of so-called multipotent germline stem cells (mGSCs) from neonatal testes, Kanatsu-Shinohara and colleagues used unfractionated testes cells and selected reprogrammed colonies solely on the basis of their ESC morphology (Kanatsu-Shinohara et al., 2004) (Table 1). mGS cells formed differentiated teratomas and supported the development of chimeric mice. However, this methodology did not allow the derivation of mGS cells from adult mice unless p53-deficient animals were used, which are more prone to develop teratocarcinoma.

Neonatally-derived mGSCs exhibited a genomic imprint pattern reminiscent of PGCs with largely erased imprints and some male-specific (androgenetic) imprints. Because imprinted DNA methylation marks are thought to be established around birth, it is likely that mGSCs were derived from relatively primitive germ cells that had not yet fully established their androgenetic imprints. Alternatively, the derivation procedure for mGSCs itself could have resulted in the erasure of some of the imprints, as is often seen in cultured ESCs. The current report revisits this issue and finds that male-specific imprints are maintained following conversion into gPSCs, suggesting that gPSCs may originate from a different type of germ

cell than mGSCs (Ko et al., 2009). Since unbalanced genomic imprinting can result in tumor formation in mice, caution is warranted in a potential therapeutic application of human germ cell-derived pluripotent cells.

The observation that genomic imprinting appears conserved in adult germ cell-derived pluripotent cells raises questions about the origin of the previously reported multipotent adult germline stem cells (maGSCs) derived from the testes of a Stra8-GFP reporter mouse (Guan et al., 2006) (Table 1). In contrast to adult SSCs and gPSCs, maGSCs displayed a somatic imprinting pattern. Another difference compared with the other studies is the observation that Stra8-GFP mGSCs have the ability to both restore spermatogenesis upon transplantation into testes and form teratomas and chimeras upon injection into immunocompromised mice and blastocysts, respectively. This is a puzzling result, as testicular engraftment and teratoma/chimera forming potential are usually mutually exclusive properties. Clearly, further work is needed to resolve the apparent discrepancies between the various studies.

Seandel and co-workers used an alternative spermatogonial marker, GPR125, to identify a population of germ cells that can give rise to multipotent adult spermatogonial-derived stem cells (MASCs) (Seandel et al., 2007) (Table 1). MASCs exhibited multilineage differentiation potential *in vitro* and in the context of teratomas and generated low-degree fetal chimeras *in vivo*. Interestingly, MASCs appeared to share several features with the recently reported epiblast stem cells (EpiSCs), which are pluripotent stem cells derived from murine post-implantation embryos (Brons et al., 2007; Tesar et al., 2007). These features include colony morphology and an epiblast-like gene expression pattern that excludes several ESC markers. Like MASCs, EpiSCs can generate teratomas and show negligible chimera contribution, demonstrating that these two cell types share both molecular and functional characteristics, suggestive of a similar pluripotent state. Notably, Kanatsu-Shinohara et al. also comment on the epiblast-like morphology of emerging mGSC colonies, and the gene expression data of mGSCs seems to support an epiblast-like signature of these cells (Kanatsu-Shinohara et al., 2004; Kanatsu-Shinohara et al., 2008).

Different pluripotent states of testis-derived stem cells?

In summary, several groups have reported the derivation of pluripotent stem cells from murine and human testes, which display different epigenetic, molecular and functional properties. Murine pluripotent stem cells can exist in a variety of distinct pluripotent states depending on the stage of donor embryo, strain background and derivation conditions. While the molecular and functional properties of previously described testis-derived stem cell lines appear consistent with an EpiSC-like pluripotent state, the gPSCs reported here by Schöler and colleagues display morphological and molecular characteristics akin to ESCs, including their ability to generate germline chimeras. It remains to be seen, however, whether these differences reflect different pluripotent states or result from different culture conditions, strain backgrounds, or reporter alleles, which are known to influence the pluripotent state of murine ESCs (Chou et al., 2008; Hanna et al., 2009; Hochedlinger and Plath, 2009). For example, the reporter alleles that have been used to isolate GSCs may mark different subpopulations that give rise to pluripotent cells with different properties.

That is, germ cell markers such as *Stra8* and *GPR125* may mark more differentiated subpopulations of spermatogonia compared with *Oct4*, which may identify more primitive cells. Consistent with the notion that different markers give rise to pluripotent cells with different properties is the observation that iPSCs selected for *Fbx15* are partially reprogrammed and developmentally less potent compared to iPS cells selected for *Oct4* or *Nanog* expression (Hochedlinger and Plath, 2009). The advances reported by Ko and colleagues greatly facilitate further exploration of the influence of genetic background and microenvironment on the frequency and dynamics of converting SSCs into pluripotent cells. The system may also serve as a valuable paradigm for studying cellular reprogramming.

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Table 1

Summary of reports of murine testis-derived pluripotent cell lines.

	Kanatsu- Shinohara et al.	Seandel et al.	Guan et al.	Ko et al.
Donor cells	Spermatogonial cell line established from total testis	GPR125-lacZ+ spermatogonial cell line	Stra-GFP+ spermatogonial cells	Oct4-GFP+ spermatogonial cell line
Age of donor mouse	Neonates or p53-deficient adult mice	Up to 1 year of age	4-6 weeks	5-8 weeks
Strain of mice	ddY and DBA/2	Various strains	Mixed 129/Ola, C57Bl/6, FVB	129Sv, C57Bl/6, FVB
Resultant pluripotent cell line	mGSCs	MASCs	maGSCs	gPSCs
Conversion frequency	1 in 10^7 testis cells (0.00001%)	N.D.	4 maGSC lines from 15 mice	0.01%
Teratomas	Yes	Yes	Yes	Yes
Chimeras (stage)	Adult	Midgestation	Adult	Adult
Germline transmission	Yes (tested by transmission of GFP transgene upon ICSI)	N.D.	Yes (tested by PCR and lacZ staining)	Yes (tested by PCR)
Imprint status	Erased imprints with partially re-established androgenetic pattern	N.D.	Somatic	Androgenetic
Gene expression pattern	Epiblast-like	Epiblast-like	ESC-like?	ESC-like

N.D. not determined; ICSI, intracytoplasmic sperm injection