Turning germ cells into stem cells

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Primordial germ cells (PGCs), the embryonic precursors of the gametes of the adult animal, can give rise to two types of pluripotent stem cells. *In vivo*, PGCs can give rise to embryonal carcinoma cells, the pluripotent stem cells of testicular tumors. Cultured PGCs exposed to a specific cocktail of growth factors give rise to embryonic germ cells, pluripotent stem cells that can contribute to all the lineages of chimeric embryos including the germline. The conversion of PGCs into pluripotent stem cells is a remarkably similar process to nuclear reprogramming in which a somatic nucleus is reprogrammed in the egg cytoplasm. Understanding the genetics of embryonal carcinoma cell formation and the growth factor signaling pathways controlling embryonic germ cell derivation could tell us much about the molecular controls on developmental potency in mammals.

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Abbreviations

ALV	avian leukosis virus
bFGF	basic fibroblast growth factor
CDK	cyclin-dependent kinase
EC	embryonal carcinoma
EG	embryonic germ
ES	embryonic stem
FGFRs	FGF RTKs
ICM	inner cell mass
JAKs	Janus kinases
KL	Kit ligand
LIF	leukemia inhibitory factor
PGC	primordial germ cell
PI3K	phosphatidylinositol 3-kinase
RTK	receptor tyrosine kinase
STAT	signal transducers and activation of transcription
TNAP	tissue non-specific alkaline phosphatase

Introduction

Pluripotent stem cells have two remarkable qualities: first, they can be grown indefinitely in laboratories as stem cells and maintain a normal karyotype making them an infinitely renewable resource; second, they can be induced to differentiate into every cell type in the body. These two properties make them an incredible reagent for the treatment of human disease, for studying development and for toxicological and teratological risk assessment. In mammals, three types of pluripotent stem cell types have been isolated into culture. Embryonic stem (ES) cells are derived by culturing the inner cell mass (ICM) of the pre-implantation blastocyst. Embryonic germ (EG) cells are derived from cultured primordial germ cells (PGCs), the embryonic precursors of the gametes of the adult animal. Embryonal carcinoma (EC) cells are derived by culturing testicular tumors (teratomas and teratocarcinomas) and represent the stem cells of those tumors and are also derived from PGCs (see [1] for review). The pluripotency of EC and EG cells has been demonstrated in widely-used assays and demonstrate that they share many properties with pluripotent ES cells. Curiously, when the PGCs from which EC and EG cells are derived are themselves tested in assays of developmental potency they cannot give rise to any other cell types and are described as 'nullipotent'. Therefore, unlike ES cells — which are derived from the pluripotent ICM — EC and EG cells are derived from a nullipotent PGC.

The conversion of PGCs into either EC or EG cells is a similar process to nuclear reprogramming [2], in which a somatic cell nucleus is reprogrammed in egg cytoplasm, converting the nucleus from a nullipotent to a totipotent state capable of recapitulating embryonic development [2,3]. The cytoplasmic factors present in the egg cytoplasm that allow reprogramming are not completely understood. The conversion of a PGC into a pluripotent stem cell provides a unique window into such reprogramming events. Here, we focus on two aspects of pluripotent stem cell formation from PGCs. First, we discuss the genetics of EC cell formation, which gives important clues as to genes regulating developmental potency. Second, we discuss growth factors required for EG cell formation, which provide an entrée into signaling pathways required for pluripotent stem cell formation.

Germ cells: taking the road less traveled

During development, PGCs are found in the epiblast adjacent to the extra-embryonic ectoderm [4,5]. They express tissue non-specific alkaline phosphatase (TNAP) and the POU domain transcription factor Oct4, both of which are also expressed by pluripotent cells of the ICM and ES cells [6]. PGCs also begin to express genes, such as *Stella* and *Fragilis*, that distinguish them from other epiblast cells and which may regulate germline development [7••,8••]. Once determined, PGCs migrate to the embryonic gonad [9] and proliferate to establish the

population of cells that will form the gametes. PGC growth during this period is controlled by multiple factors including the c-Kit receptor tyrosine kinase (RTK) and its ligand, Kit ligand (KL) [10]. PGCs also begin to express other markers that distinguish them from the ICM and ES cells such as the murine homolog of the *Drosophila vasa* gene, *mVasa*, an RNA helicase [11]. PGCs that have entered the gonad will then stop proliferating and begin differentiation. Male PGCs enter mitotic arrest whereas female PGCs enter directly into meiosis in the embryo and then arrest at meiotic prophase [9]. Some of these events can be recapitulated in culture because PGCs proliferate *in vitro* for about as long as they do *in vivo* and they can enter meiosis [12–14]. Precise control over PGC proliferation and differentiation *in vivo* ensures that appropriate numbers of cells are present in the gonad to form gametes in the adult. If the numbers of PGCs in the embryonic gonad are too small, animals can be infertile. Similarly, if PGC proliferation goes unchecked, especially in males, tumors can result.

Embryonal carcinoma cells: the 'first' pluripotent stem cells

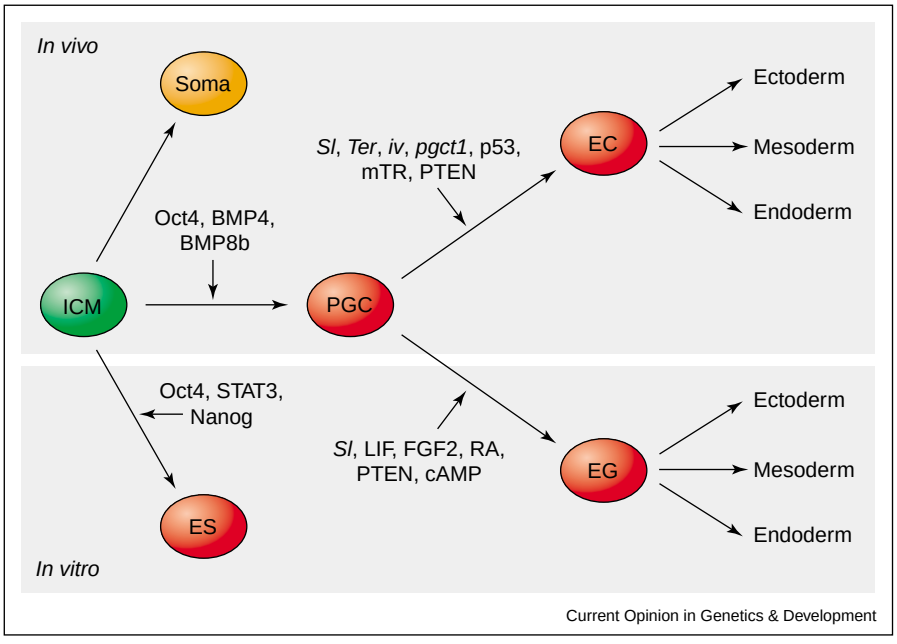
Testicular teratomas are highly unusual benign tumors containing derivatives of the three primary germ layers [15]. Roy Stevens was the first to note that in strains of mice that develop testicular teratomas there are small nests of proliferating cells in the developing gonad at E15 (reviewed in [15,16]). Eventually these cells, which he called EC cells, rupture the seminiferous tubules and enter the interstitial spaces. Here they differentiate into vesicle-like structures that resemble normal embryonic ectoderm, mesoderm and endoderm. After birth, these structures become disorganized and the embryonic-like cells differentiate into a wide variety of cell types and tissues [16]. Assays of developmental potency show that isolated EC cells are pluripotent stem cells but when they lose the ability to differentiate they form malignant teratocarcinomas. A beautiful study [17] showed that EC cells are themselves derived from PGCs (Figure 1).

The key issue is how PGCs give rise to EC cells. Interestingly, although testicular cancer is the most common type of cancer in young men, the disease is rare in mice. The strain developed by Stevens, 129/Sv, has an increased incidence (1–2%) of testicular teratoma [18]. However, when embryonic testes of 129/Sv mice are grafted to ectopic sites in adult hosts, ~66% of those grafts develop into tumors (see [15,16] for review), suggesting that environmental factors, together with genetic conditions, influence PGC growth and teratocarcinogenesis.

Susceptibility loci and the hunt for genes

Testicular teratoma and teratocarcinoma development in mice is amenable to genetic analysis and modifier loci controlling testicular cancer incidence have been identified [19••]. Mutations at the *Steel* locus on mouse chromosome 10 (encoding KL), the *Teratoma* locus on mouse

Figure 1



Pluripotent cells of the ICM give rise to all the cells of the embryo including the somatic cell lineages (soma) and the germline (PGC). Cultured cells of the ICM can give rise to ES cells *in vitro* through the action of the indicated genetic pathways. PGCs give rise to EC cells *in vivo* through the activation or mutation of the indicated genes, or to EG cells *in vitro* by exposure to the indicated cocktail of growth factors. Both EC and EG cells are pluripotent stem cells that can give rise to cells of the three germ layers.

chromosome 18 and the Trp53 gene all increase the testicular tumor incidence [18,19••,20]. Curiously, both the *steel* and *teratoma* mutations actually impair PGC development but act to increase the incidence of teratocarcinogenesis [18]. The simplest explanation for this phenomenon is that conditions favoring PGC death effectively drive selection of cells that can survive, forcing the conversion of PGCs into EC cells. Indeed, recent studies (e.g. [21]) demonstrate that somatic cells derived from the gonads of 129/Sv*Teratoma* mice induce programmed cell death in PGCs perhaps because they lack a key PGC survival factor. Recent studies (e.g. [20]) have also identified a primordial germ cell tumor susceptibility locus (*pgct1*) on mouse chromosome 13. Interestingly, that region of mouse chromosome 13 is syntenic with a human chromosome 5q region implicated in human testicular tumor susceptibility. The ability of genetic approaches to identify tumor susceptibility loci has recently been improved and several germ cell tumor susceptibility loci on mouse chromosome 19 have been identified [19••,22]. Importantly, completion of the mouse and human genome sequences now allows candidate modifier genes to be quickly identified and tested.

In humans, testicular cancer seems to correlate with conditions that result in lower germ cell numbers [23], including cryptorchidism [24], androgen insensitivity syndrome [25], testicular atrophy [26], infertility [27], or gonadal dysgenesis [28]. All these symptoms, together with poor semen quality, might be part of one entity, newly termed testicular dysgenesis syndrome [29] that may result from disruption of embryonic gonadal development [30–32].

In men, testicular cancer arises from carcinoma *in situ* cells, which are thought to derive from PGCs that have escaped normal differentiation. Human testicular cancers demonstrate consistent abnormalities in chromosome 12, such as the presence (gain) of isochromosome 12p, i(12p) ([33]; for an updated review see [34]). Several genes map to this region, including cyclin D, the cyclin-dependent kinases (CDKs) 2, 4 and 6, Ras, p53 and mdm-2 [35], and indeed some of these genes are deregulated in testicular cancer [36–39]. A correlation has also been shown between altered expression of p53 and mdm-2 and testicular cancer incidence [40,41], consistent with observations on the role of p53 in mouse teratocarcinoma [42].

Recently, other known and novel genes amplified from gain of the entire short arm of chromosome 12 or amplification of 12p11.2-p12.1 found in testicular cancers were determined by microarray analysis [43]. Interestingly, the *Nanog* gene, which maintains ES cell pluripotency [44••,45••], maps to mouse chromosome 6, the syntenic region of human chromosome 12 [46].

Genes that localize to different genomic regions are also overexpressed in testicular cancers. For example, overexpression or activation of c-Kit in germ cells may lead to cellular transformation [47,48]. These are interesting observations given the important role that c-Kit plays in PGC growth. Elevated Myc expression or loss of expression of the RB tumor-suppressor gene are also correlated with germ cell tumors [49,50]. These data suggest an unusual deregulated G_1–S checkpoint in germ cell tumors.

Embryonic germ cells: the 'other' pluripotent stem cell

Derivation of EG cells provides a unique insight into the formation of pluripotent stem cells. PGCs are cultured by dissociating isolated embryo fragments containing PGCs and placing the resultant single-cell suspension onto preformed feeder layers of irradiated fibroblasts [12]. Feeder layers produce factors such as KL that are required for PGC survival and also factors that stimulate PGC proliferation [51–53]. In culture, PGCs are mortal, proliferate for 7–10 days, and then disappear either because they differentiate or die. They may differentiate *in vitro* over the same time period as they would *in vivo* [12–14]. But when PGCs are exposed to three polypeptide growth factors — KL, leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) — they continue to proliferate and form large colonies of cells that can be expanded indefinitely [54,55]. Those cells, which we termed EG cells, continue to express the PGC markers TNAP and Oct4, which are also ES and EC cell markers. Assays of developmental potential show EG cells to be pluripotent. Human PGCs exposed to the same growth factors also form EG cells that are pluripotent [56], suggesting that several of the pathways regulating germline development have been conserved throughout mammalian evolution. Like human ES cells, human EG cells are thought to have tremendous potential for treatment of human disease and for analysis of human development (reviewed in [1,6,57]).

Each of the growth factors required for EG cell derivation activates unique signal transduction pathways but there is also considerable overlap in the downstream effectors that are activated. Most likely KL and LIF act as survival factors and co-mitogens to control PGC survival and proliferation [51–53,58]. Activation of the signaling component of the LIF receptor, gp130, is required for PGC survival *in vivo* and *in vitro*. Binding of ligand to the LIF receptor complex causes gp130 to associate with the Janus kinases (JAKs), which in turn transduce intracellular signals via the signal transducers and activation of transcription (STATs). PGCs are severely depleted in gp130 knockout mice [59] and treatment of cultured PGCs with a blocking gp130 antibody causes apoptosis [60]. Therefore, gp130-mediated signaling is required for PGC survival and together with c-Kit signaling promotes PGC proliferation [61].

But KL and LIF together do not cause PGC conversion to EG cells. Therefore, the factor that seems to deserve

most attention is bFGF. bFGF action is mediated by high affinity FGF RTKs (FGFRs) and low affinity binding heparan sulfate proteoglycans [62,63]. Binding of bFGF to cells causes FGFR dimerization and receptor autophosphorylation, in turn leading to recruitment of cytoplasmic FGFR binding proteins [62,63]. How might bFGF affect PGC growth? A critical issue is whether bFGF acts directly on germ cells or whether it acts via the feeder layer. Interpreting bFGF function in culture is complicated because cultures contain PGCs, the feeder layer and embryonic somatic cells derived from the gonad. PGCs themselves express FGFRs [64] but probably many cell types in culture do also. Our own feeling is that bFGF acts directly on PGCs to effect conversion into EG cells because exposure of PGCs to bFGF for as little as 12 hours is sufficient for EG cell formation (PJ Donovan, MP de Miguel, unpublished observations). Moreover, once EG cells are established, bFGF is then no longer required for their growth ([54]; PJ Donovan, MP de Miguel, unpublished observations). Some studies suggest that bFGF can be replaced by retinoic acid or agents that activate cAMP such as forskolin [60]. The significance of these observations is unclear but if we can understand how they substitute for bFGF that might provide important clues about the molecular mechanisms underlying the PGC to EG cell conversion.

PTEN ways to make pluripotent stem cells

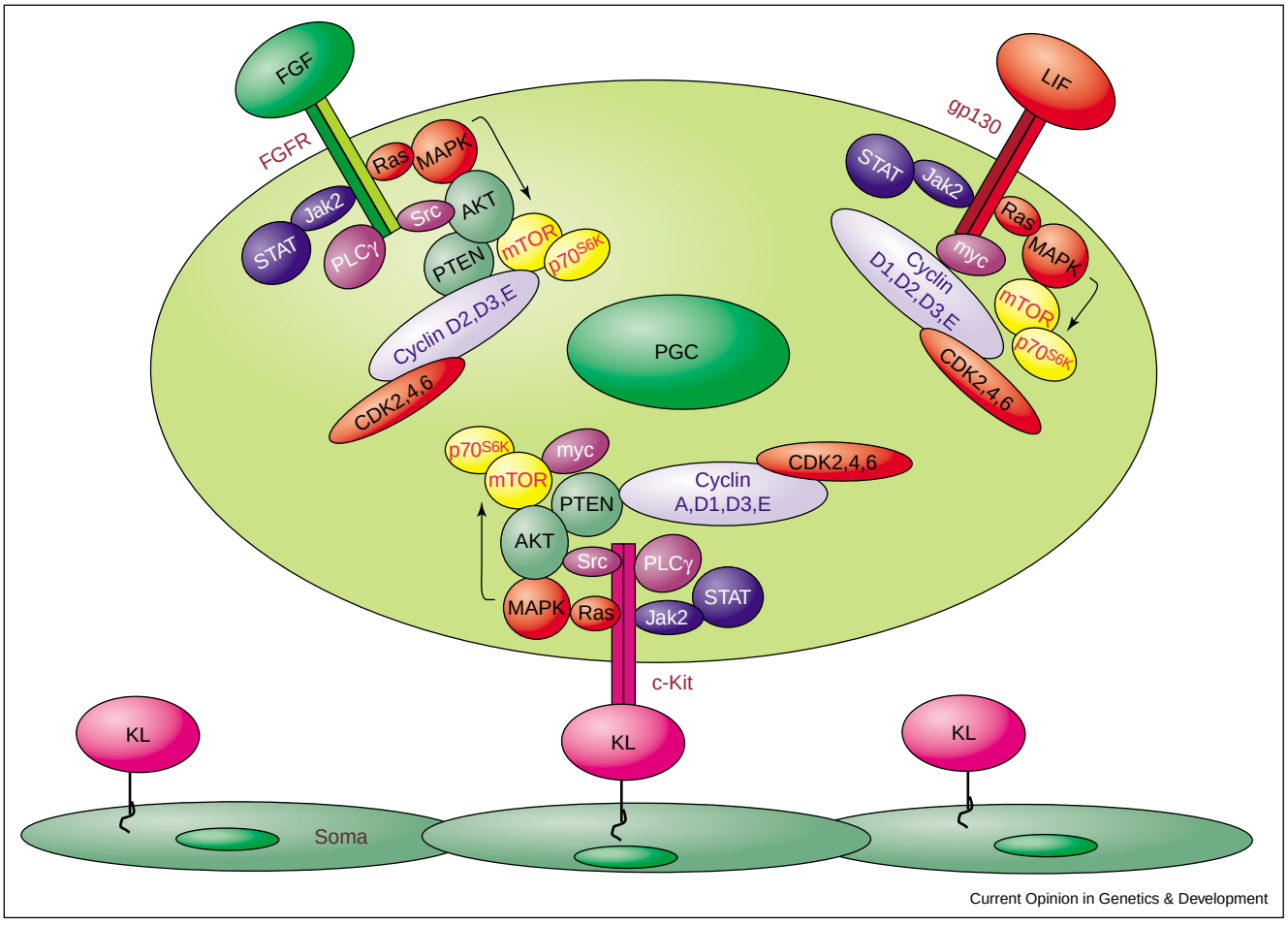
If bFGF does act directly on PGCs, how might it act to effect EG cell formation? The signal transduction pathways activated by KL, LIF and bFGF have been studied in many cell types and numerous downstream targets have been identified. Interestingly, several proteins in these signaling pathways — Myc, Ras and cyclin D/CDK4 — are overexpressed in human germ cell tumors, perhaps suggesting shared mechanisms of stem cell origin. Clearly there is considerable overlap in signaling downstream of the KL, bFGF and LIF receptors (Figure 2). Activation of these receptors leads to activation of pathways including phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR/$p70^{S6K}$ [65,66], Ras/mitogen-activated protein kinase kinase (MEK)/mitogen activated protein kinase (MAPK) [67], the JAK/STAT [68] and the Src signaling pathways [67]. Despite this overlap, the ultimate downstream targets of these three pathways may be distinct. Signal transduction pathways may be constructed in a modular fashion, utilizing scaffolds that effectively separate and distinguish each of the pathways while at the same time allowing interpolation of various growth and differentiation signals [69,70]. What might distinguish the different signaling pathways from each other may be their more downstream targets such as cyclins and CDKs (for a review, see [71]). Cyclin/CDK complexes could be critical in regulating PGC growth (Figure 2). Thus, the decision of whether a PGC either survives, proliferates or differentiates might involve integration of signals from multiple inputs. A critical problem, therefore, is to identify the downstream targets of the various signal transduction pathways.

To dissect signaling pathways in PGCs we have developed a novel system of retrovirally-mediated gene transfer. We used mice expressing the receptor for the avian leukosis virus (ALV) [72••]. PGCs from these mice can be infected with ALVs and will express genes efficiently from the ALV promoter. We dissected the role of the AKT kinase in c-Kit signaling. These data suggest an important role for AKT in mediating the survival effects of c-Kit signaling. Expression of AKT promoted PGC growth in the absence of KL and a dominant-negative form of AKT inhibited PGC growth in the presence of KL [72••]. These studies also point to a critical role for mTOR/FRAP and $p70^{S6K}$ in mediating PGC survival downstream of AKT. Our data also suggest that, in PGCs, signaling via PI3K is not important for their survival [72••] consistent with studies showing that mice expressing a c-Kit receptor with a mutation in the PI3K binding site have normal PGC numbers [73,74]. Therefore, in PGCs, AKT may be activated by an unconventional mechanism as in some other cell types. In many cell types, the tumor suppressor PTEN — phosphatase and tensin homolog deleted from chromosome 10 — is a critical regulator of signal transduction pathways including AKT. To investigate the role of PTEN in PGC development, Kimura *et al.* [75••] knocked out the PTEN gene in PGCs using a Cre-*lox* strategy. Three important effects of PTEN deletion were observed. First, PGCs derived from PTEN null animals proliferate more extensively. Second, PGCs from PTEN null mice were able to make EG cells more efficiently. Third, mice lacking PTEN in PGCs developed a higher incidence of testicular teratocarcinoma. Therefore, signaling downstream of PTEN seems to have a critical role in regulating both PGC proliferation and conversion to EG and EC cells [75••].

Signaling stemness

Some features of EG cell derivation might provide clues as to how they are formed. First, the age of the PGCs seems to be important. PGCs isolated from young (E8.5) embryos make EG cells readily, whereas PGCs isolated from older (E12.5) embryos make either few or no EG cells [54,55,76–79]. Second, EG cell derivation efficiency is affected by serum, because culture of PGCs in serum replacement medium improves both PGC proliferation and EG cell formation efficiency [80]. Third, loss of the tumor suppressor PTEN increases PGC proliferation and EG cell formation efficiency [75••]. Together, these data suggest that EG cell formation is related in some way to PGC mitotic status. Perhaps extended PGC proliferation makes them susceptible to conversion into EG cells or sustained proliferation could inhibit PGC differentiation. Intriguingly, the observation that EG cell formation is associated with extended PGC proliferation is reminiscent of the original description that EC cells arise from

Figure 2



Schematic representation of the putative signal transduction pathways downstream of KL, LIF and bFGF in PGCs. KL is expressed as a transmembrane growth factor by the feeder cells (fibroblastic somatic cells) in the culture. Activation of the respective receptors by specific ligand binding results in a cascade of signal transduction that results in activation of specific cyclin/CDK complexes. For simplicity, inhibitory interactions between cytoplasmic molecules downstream of the ligand receptors are not depicted but these contribute to their levels of activation.

small nests of cells that continue to proliferate in embryonic gonads [17,81].

How, then, does this combination of growth factors act on PGCs to effect their conversion to EG cells? The factors that control developmental potency in mammals are still being elucidated and include the following: the Oct4 POU domain transcription factor (reviewed in [82]); the STAT3 transcriptional activator (reviewed in [83]); the HMG-box protein SOX2 [84•]; the forkhead transcriptional regulator FoxD3 [85•]; and the recently-described Nanog protein [44••,45••]. But both Oct4 and Nanog are expressed in PGCs as they are in pluripotent stem cells such as ES and EG cells [44••,45••,86]. So PGC potency cannot be regulated simply by manipulating the expression of these two proteins. Activation of gp130 is required for PGC survival both *in vivo* and *in vitro* [59,60]. Because STAT3 is one of the key downstream targets of gp130-signaling, it could function in germline development. But the role of STAT3 in PGC growth remains unresolved. In ES cells, the balance between the STAT3 and MAPK pathways plays an important role in regulating the choice between either self-renewal or differentiation [83,87,88]. An important issue is whether interplay between different growth factor signaling pathways in PGCs influences choices between either self-renewal or differentiation.

Two other factors that can regulate developmental potency are SOX2 and FoxD3 but little is known about their role in PGCs. Another interesting question is how molecules that distinguish PGCs from ES, EC and EG cells such as the RNA helicase *mVasa* might be regulated during conversion of PGCs to stem cells. Indeed, growing evidence suggests that an important aspect of mammalian germline development involves controlling RNA

localization and translation (e.g. see [89]). If and how those processes are altered during formation of EG and EC cells remains to be determined. Recent technological developments have improved the ability to manipulate PGCs and test such hypotheses. Transgenic and 'knock in' technology have allowed either the expression or deletion of genes exclusively in the germline [75••,90,91,92••]. Retroviral vectors have been shown to work efficiently in PGCs and can be used to manipulate gene expression *in vitro* [72••]. *In vivo* electroporation techniques have been developed that allow gene expression in PGCs in organ cultures of developing gonads [93]. These techniques should allow the signal transduction pathways regulating PGC growth to be comprehensively dissected.

Are embryonic germ cells and embryonal carcinoma cells one and the same?

Are the EG and EC cell formation processes related in some way? When embryonic gonads are grafted to ectopic sites they make teratomas but only if the gonad is isolated before E12.5 [94]. Similarly, EG cells can be formed from PGCs up to E12.5 but not later. Therefore, the time window during which either EC or EG cells can be made from PGCs is remarkably coincident. Both EC and EG cells are also pluripotent and express many of the same markers [1]. Although EG cells contribute to all lineages in chimeras [76], EC cells typically do not contribute to the germline (reviewed in [1]). Most likely this reflects EC cell aneuploidy that perhaps prevents them from completing some aspect of gametogenesis. It would be intriguing to test if EC cells, freshly isolated from the embryonic gonad, would be able to make germline-competent chimeras. Finally, loss of PTEN leads to testicular tumor formation *in vivo* and also EG cell formation *in vitro* [75••], suggesting that the route from a PGC to either an EC or a EG cell shares at least one genetic pathway.

Another important issue is whether growth factors required for EG cell formation *in vitro* would drive EC cell formation *in vivo*. Although it would be difficult to expose PGCs in embryonic gonads to factors directly, other approaches could test this idea. Various gene promoters can efficiently drive transgene expression in germ cells or gonadal somatic cells [75••,90,91,92••,95,96]. Moreover, retrovirally-mediated gene delivery or electroporation can now be used to express factors in a variety of gonadal cell types including PGCs [72••,93] allowing hypotheses about PGC conversion to stem cells to be tested.

Embryonic germ cells, embryonal carcinoma cells and epigenetics

How might increased PGC proliferation *per se* drive the conversion to EG cells? Continued proliferation could either impair or delay ongoing differentiation events, providing a window for reprogramming the developmental potential of PGCs. During gametogenesis, PGCs must erase imprints that mark chromosomes as to their parent of origin [97–100]. That process likely involves covalent modifications to histones, DNA and widespread chromatin remodeling. At this time, PGCs might be most susceptible to modifications of imprints. Perhaps extracellular signals driving PGCs prematurely through the cell cycle prevent correct re-establishment of imprinting marks in those cells. This, in turn, might allow genes that are temporarily silenced to be re-activated or genes that are active to be silenced. Such alterations in gene expression likely underlie the changes in cell potency observed in the conversion of PGCs to either EC or EG cells. Studies of imprinting in EG cells demonstrate that, in many cases, they show evidence of partial imprinting consistent with the idea that imprints had either not been fully erased or re-established in the PGCs from which they were derived [101,102].

Could the same events influence EC cell formation? Using chromosome substitution strains of mice in which entire chromosomes are transferred from one strain to the other, it was demonstrated that mouse chromosome 19 could contain several genes with additive and epistatic effect on EC cell formation. Alternatively, Youngren *et al.* [19••] proposed that epigenetic modifications might be imposed by the 129/Sv background on the donor-derived chromosome 19. Epigenetic effects could silence any gene on the donor-derived chromosome involved in PGC development or in the process of testicular differentiation and determination [19••]. Interestingly, the majority of human infantile testis tumors exhibit biallelic expression of imprinted genes, again suggesting that EC cells are derived from PGCs that had erased imprints [103]. It will be interesting to determine whether the differences in the imprinting status of EC and EG cells are either the cause or consequence of their origin.

Conclusions

The conversion of PGCs into pluripotent stem cells may be linked in some way with their deregulated proliferation. How extended PGC proliferation leads to development of EC or EG cells remains to be determined. The development of new and rapid methods to identify testicular cancer susceptibility genes, coupled with genomic information, will accelerate our understanding of the genetic pathways regulating PGC differentiation and developmental potency. Similarly, technological developments have improved our ability to identify genes expressed in PGCs and to manipulate their expression. These advances should allow us rapid dissection of signaling pathways that regulate PGC growth and their conversion into pluripotent stem cells. That information could impact our thinking about the etiology of testicular cancer, stem cell plasticity in general, and the molecular mechanisms underlying nuclear reprogramming.

Update

A recent study [104] shows that there is a statistically significant reduction in male PGC numbers at E13.5

when a floxed allele of gp130 is deleted in PGCs by TNAP-Cre-mediated excision. Given that the TNAP-Cre allele is reported to be only 60% efficient at excision of a reporter gene, these data suggest that gp130 does have a role in PGC development.

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