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Perceiving signals, building networks, reprogramming germ cell fate

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ABSTRACT Germ cell development is a step-wise process that ensures the progression of the life cycle due to their unique ability to transmit their genome from one generation to the next. In the mouse, the precursors of germ cells, the Primordial Germ Cells (PGCs), arise at the onset of gastrulation. Here we discuss how PGCs acquire their fate in the epiblast and outline their development until their arrival into the gonads. Male germ cell tumors (GCTs) have a similar gene expression pattern to that of fetal germ cells and to pluripotent cells, suggesting that GCT originate from an alteration of gonocyte normal development. We evaluate coincidences and differences in germ cell development in mouse and humans and on this basis, we speculate future research perspectives.

KEY WORDS: *primordial germ cell, male germ cell tumor, signalling, reprogramming, cell fate*

Overview of mouse PGC development

"The establishment of a germ cell lineage must be about the most fundamental issue ever to have faced the Metazoa throughout their evolutionary history." (McLaren, 1999). During a mammal lifespan, there is a relatively short duration when development proceeds in the absence of germ cells. Male and female gametes fuse to generate a totipotent zygote, and development proceeds without germ cells until after implantation of the embryo (Fig. 1). In the mouse, new germ cells originate from cells of the proximal epiblast that lie close to already differentiated tissues around embryonic day 6.5 (E6.25) (Lawson and Hage, 1994; Ohinata *et al*., 2005). The precursors of primordial germ cells (PGCs, the precursors of gametes) are found among mesodermal precursors (Lawson and Hage, 1994; Ohinata *et al*., 2005). Active TGFβ signalling is essential for their specification as PGCs (Hayashi *et al*., 2002; Lawson *et al*., 1999; Ohinata *et al*., 2009). The expression of Blimp1 (also known as Prdm1) and Prdm14 around E6.25 – E7.0 marks the lineage restriction of the proximal epiblast cells towards the specification of the germ cell fate (Ohinata *et al*., 2005; Yamaji *et al*., 2008). Before the identification of Blimp1 and Prdm14, PGCs could only be recognized at E7.5 as a cluster of about 40 cells that are characteristically positive for alkaline phosphatase (TNAP) activity (Ginsburg *et al*., 1990). Blimp1 and Prdm14 expressing cells become specified as PGCs, which is followed by the expression of Stella (also known as Dppa3 and PGC7), Kit, Tnap, and Nanos3. Stella has a role in epigenetic asymmetry in the zygote as it apparently protects the erasure of imprints at the time of global DNA demethylation. Nanos3 and Kit have a pro-survival effect. Kit, in addition, guides PGCs away from the base of the allantois and towards the hindgut and the genital ridges. During this period, PGCs proliferate at a steady rate every 16 hours irrespective of their sex. PGCs are specified in an epigenetic context equivalent to their neighbouring mesodermal cells. While migrating and proliferating, PGCs reprogram their genome to a status that is equivalent to that associated with pluripotency. PGCs fate diverges from E13.5 onwards depending on the somatic environment that hosts them. In response to retinoic acid (RA), female PGCs, now oocytes (is the right terminology), will start entering the first meiotic division at E13.5 and progress through leptotene, zygotene, and pachytene stages to arrest after birth as diplotene oocytes within primordial follicles. In response to FGF9, male PGCs, from now on referred to as gonocytes, will remain in the G0/G1 cell cycle arrest up to birth and will not enter meiosis before postnatal day 7 (P7). Failure to establish the mitotic arrest is considered one of the main causes of male germ cell tumors (GCTs).

The molecular program, the signalling principle, and the epigenetic foundations of PGCs

The signalling principle

In vivo and *in vitro* evidence demonstrates that BMP4 is a

Abbreviations used in this paper: GCT, germ cell tumor; PGC, primordial germ cell.

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critical signal to induce PGC fate (Lawson *et al.*, 1999; Ohinata *et al.*, 2009; Okamura *et al.*, 2005). BMP4 signal emanates from the extraembryonic ectoderm (Lawson *et al.*, 1999) and a subset of epiblast cells respond by independently activating the expression of Blimp1 (Ohinata *et al.*, 2009; Ohinata *et al.*, 2005) and Prdm14 (Yamaji *et al.*, 2008). While Prdm1 appears responsible for the repression of Hoxb1, a marker of mesodermal precursors, and of slowing down the cell cycle, Prdm14 ensures the reacquisition of pluripotency factors that would otherwise be downregulated (Ohinata *et al.*, 2005; Yamaji *et al.*, 2008). Lineage restriction of the germ line among other cells of the epiblast is a dynamic interplay between signalling from extraembryonic tissues (extraembryonic ectoderm and primitive endoderm) and molecular determinants intrinsic to the future precursors of germ cells (Fig. 2).

Though the PGC-inductive effect of BMP4, BMP2, and BMP8b is widely accepted, there is no consensus about their mechanism of action in the developing mouse embryo. Within a restricted time window, the epiblast, isolated from its surrounding extraembryonic tissues, is able to induce the expression of Prdm1 and Prdm14 reporters and start lineage restriction towards the germ line (Ohinata *et al.*, 2009), suggesting thus that epiblast cells on their own meet all the requirements to respond to both classes of BMPs: Dpp (BMP4 and BMP2) and 60A (BMP8b). Studying whole embryos in culture around the same developmental window, or removing the differentiated germ layers and using feeder cells to sustain development, it has been shown that the visceral endoderm and the extraembryonic ectoderm are both required to mediate BMPs effect and direct PGC specification in the epiblast (de Sousa Lopes *et al.*, 2007; de Sousa Lopes *et al.*, 2004). Along with other studies that also indicated that epiblast responsiveness to BMP4 was tightly regulated to a restricted time window and to the integrity of cell – cell contact (Okamura *et al.*, 2005), the discrepancies of these observations are likely due to the highly dissimilar conditions in which they where obtained and highlight the need of still better understanding of the PGC precursors in their natural context: the developing embryo.

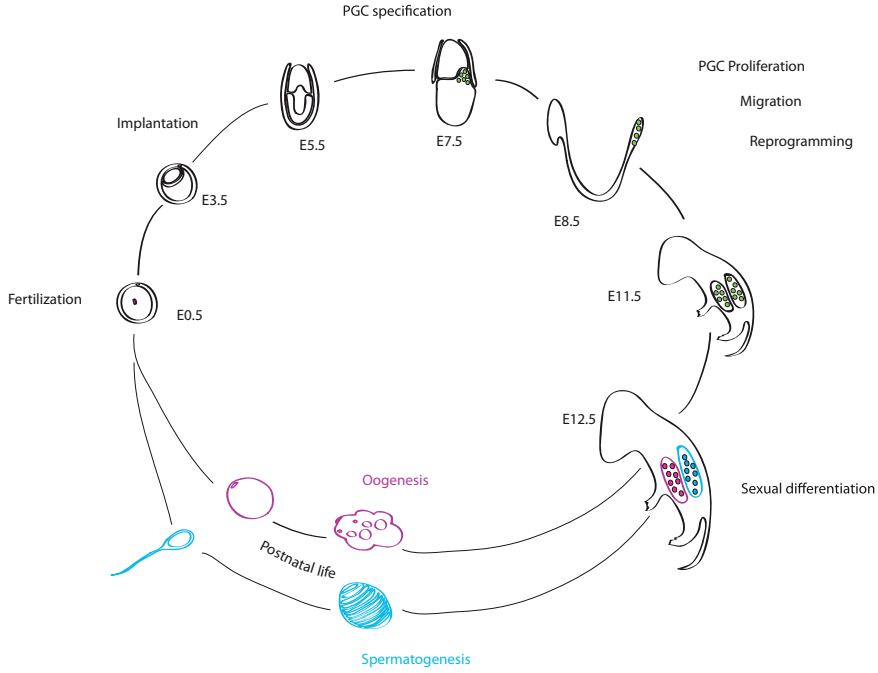

Fig. 1. Germ cells ensure the continuation of the life cycle. *In the mouse embryo, germ cell precursors (PGCs) appear after its implantation between E6.25 and E7.5. PGCs proliferate and reprogram their genome while they migrate towards the gonadal ridge. Morphological differentiation between female and male gonads is first observed at E12.5. Oocytes will enter meiosis at E13.5 and oogenesis will start from this point to be completed after birth. Spermatogenesis starts when gonocytes enter mitotic arrest at E13.5 but meiosis in the male germline is initiated after birth. Adult animals produce mature gametes, either oocytes or spermatozoa, which can generate a new organism upon fertilization.*

Lineage tracing and clonal analysis have identified PGCs precursors in a subset of epiblast cells among the closest to the extraembryonic ectoderm, at the exact embryonic – extraembryonic boundary and mostly situated towards the posterior side of the embryo (Lawson and Hage, 1994; Lawson *et al.*, 1991; Ohinata *et al.*, 2005). Likely, in about 24 hours, these cells are trailed towards the extraembryonic mesoderm as a consequence of the morphogenetic movements that govern gastrulation and will be found immediately below the visceral endoderm (Ginsburg *et al.*, 1990; Ohinata *et al.*, 2005). This window is a hallmark of murine development (Fig. 2). At this moment, only few cells will avoid the route to somatic fate despite being immersed in a rich signalling environment that has just established the anterior and posterior axis of the embryo. Since recent observations demonstrate that silencing of BMP signalling occurs in the endocytic compartment of the proximal visceral endoderm between E5.5 and E6.5 (Aoyama *et al.*, 2012), it could be that the highly restricted location of precursors of primordial germ cells to the most inner row of epiblast cells (Ohinata *et al.*, 2005), is due to an attenuation of BMP signalling immediately underneath the visceral endoderm in contrast to the propagation of the signal around cells in a deeper position.

In addition to BMPs, other signalling pathways have also been implicated in PGCs development. Wnt3 is likely required not just to provide the competence for PGC lineage restriction but to commit epiblast cells to a mesodermal fate. Wnt3-/- embryos fail to upregulate HoxB1, T, and to downregulate Oct4, signatures that appear to be a prerequisite for PGC lineage restriction. The expression of Bmp4 in the ExE of Wnt3-/- embryos is neither sufficient to induce mesodermal fate nor PGC lineage restriction. This may also be consequence of the lack of mesodermal BMP4 after E6.5 in Wnt3-/- embryos as a result of impaired phosphorylated Smad1, Smad5, and Smad8 activity (Liu *et al.*, 1999; Ohinata *et al.*, 2009). Wnt3 may thus be able to induce a positional and kinetic memory in epiblast cells that may then render them competent to differentiation.

FGFs induce the formation of the primitive streak in the developing embryo and in the differentiation of mESC. The addition of FGFs to epiblast cultures did not enhance the derivation of PGCs. However, a significant increase in the number of PGCs was observed when EGF was added to the epiblast in culture (Ohinata *et al.*, 2009). However, there is no *in vivo* data suggesting that EGF has a role in modulating the quantity of PGCs that are specified in the mouse embryo. Interestingly, EGF counteracts BMP

signalling since it promotes retention of pSmad1 in the cytoplasm (Kretzschmar *et al.*, 1997). EGF is perceived in the cell surface through a receptor tyrosine kinase (RTK) that through Erk-MAPK phosphorylates Smad1 in a different residue than that made by BMP receptors. So the simultaneous addition of BMP4, BMP8b, and EGF (along with SCF and LIF) to epiblast in culture may have a "balancing effect" that regulates the recruitment of PGC precursors and their almost physiological propagation. The "attenuated" Smad signalling may be mimicking the *in vivo* microenvironment in which PGC precursors acquire their identity, which is not solely composed by BMPs but is instead a complex signalling network. Some RTK and receptor Serine / Threonine (RS/TK) are able to direct protein glycosylation within the Golgi and this is suggested to link cell signalling and glycomic diversity (Moremen *et al.*, 2012).

Interestingly, glycosylation of cell surface molecules is known to be involved in many developmental processes that include cell-cell interactions, tumorigenesis, and maintenance of stem cell niche in adults. The differential expression of cell surface carbohydrates in teratocarcinomas and cancer, make these molecules markers of transformed cells. In the gonadal ridges, PGCs entering meiosis express a distinct repertoire of cell surface carbohydrates distinct from those entering mitotic arrest (De Felici *et al.*, 1985). On the intracellular side, increasing evidence shows that carbohydrate modifications are associated with different epigenetic states. It will be interesting to investigate how these modifications occur in the developing germ line. The addition of *O*-linked β-D-*N*-Acetylglucosamine (*O*-GlcNAc) to Ser and Thr residues of proteins serves as a nutrient sensor and is directed at all aspects of cellular functions, including signalling and chromatin remodelling (Hanover *et al.*, 2012). Histones H2A and H2B have *O*-GlcNAcylation sites close to the DNA contact points, and on histone H3 the modification has been associated with both active and repressive marks. The gene encoding the enzyme responsible of *O*-GlcNAcylation is in close proximity to the Xist locus (both in mice and humans) and thus the levels *O*-GlcNAcylation are directly linked to the maintenance of stem cell pluripotency through the regulation Polycomb – dependent remodelling of the X chromosome and by influencing the Oct4 transcriptional network (Hanover *et al.*, 2012).

The molecular programme and the epigenetic foundation

The transcriptional mechanisms regulating Blimp1 and Prdm14 expression in the precursors of PGCs in the mouse epiblast are not yet elucidated. Likely, Smad1, Smad5, and Smad8, are key factors in the induction of PGC fate but their cis-acting sequences in the regulatory regions on the determinants of germ cell fate are not characterized.

Blimp1 and Prdm14 start being expressed in a subset of epiblast cells that are indistinguishable from their neighbours. Specification of the germ lineage seems to occur in a genome wide epigenetic context equivalent to that of somatic cells of the postimplantation epiblast. DNA methylation, X-inactivation, and histone modifications are globally the same as in somatic neighbours. At this time, germline specific genes such as deleted in azoospermia-like (Dazl) and synaptonemal complex protein 3 (Scp3) have their CpG dense promoters methylated. But, as soon as the germ line specific transcriptional network is established, it is imperative to prevent the continuation of the somatic program, erase repressive hallmarks, and start the unique reprograming towards the totipotent epigenome. The first step towards a pluripotent embryonic stem cell (ES) – like epigenetic state is signed by the loss of histone H3 lysine 9 dimethylation (H3K9me2) at E7.75 and a global increase in histone H3 lysine 27 trimethylation (H3K27me3) by E9.5 (Fig. 3). In concomitance, pluripotency genes are up regulated. The inactive X-chromosome (Xi) escapes the genome wide tendency of increasing H3K27me3 and the downregulation of this mark in this specific location leads to its re-activation to reach a status equivalent to the one in ES. The interaction between Blimp1 and the arginine methyltransferase 5 (Prmt5) determines an additional modification characteristic of migrating PGCs that contributes to the maintenance of the germ cell identity. Prmt5 is responsible for the symmetrical methylation of histones H2A/H4 (H2A/H4R3me2). DNA is also modified during the migratory phase and this may contribute to the start of the reprogramming events in PGCs. It is subject of active investigations whether at this stage DNA demethylation involves a passive or active loss of 5-methyl-Cytosine (5mC), perhaps through 5-hydroxy-methyl-Cytosine (5hmC) intermediate. The complete erasure of imprints will occur upon entry into the gonads at E10.5. It may be that the epigenetic modifications that occur in migrating PGCs are a prerequisite for the subsequent genome-wide erasure of DNA methylation and extensive chromatin remodelling that PGCs undergo once they reach the genital ridges. By E13.5, parental imprints and promoter CpG methylation at germline-specific genes are almost

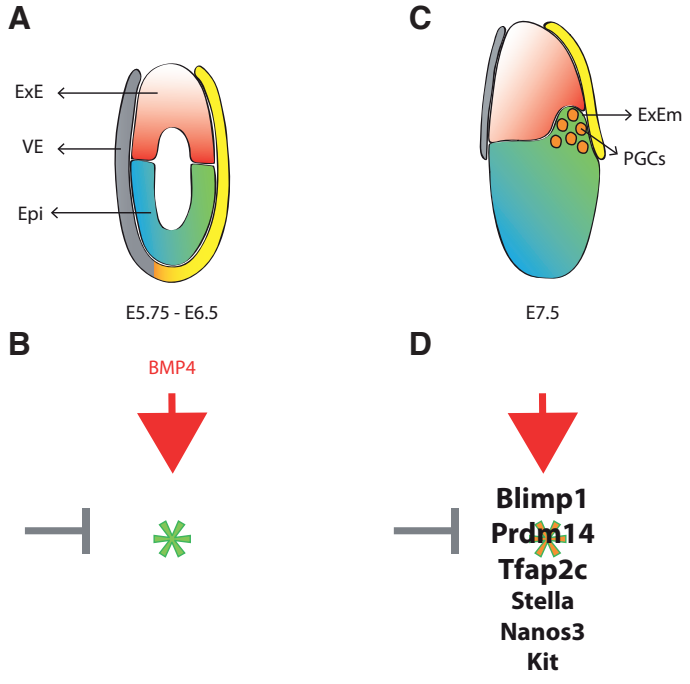

Fig. 2. Primordial germ cell (PGC) specification. (A,B) *Schematic representation of E5.75 – E6.25 mouse embryo. The visceral endoderm (VE) overlays the epiblast (Epi) and the extraembryonic ectoderm (ExE). The anterior VE (grey) is positioned to the left hand side of the scheme and is the source of inhibitory signals for PGC specification. BMP4 produced by the ExE (red) is the main inducer of PGCs. PGCs precursors are found in the proximal Epi towards the posterior side (green and asterisk, anterior Epi in blue).* **(C,D)** *Specified PGCs (orange, expressing Blimp1, Prdm14, Tfap2c, Stella, Nanos3, and Kit) are found in the Extraembryonic mesoderm (ExEm) at the base of the allantois and characteristically recognized by alkaline phosphatase staining.*

completely erased, in concomitance with a cascade of chromatin remodelling. Only intracisternal A particle (IAP) retrotransposons, partially evade DNA demethylation, and this could contribute to transgenerational epigenetic inheritance. Transposable elements (TE) are also demethylated, and, though potentially harmful, their activation could be useful to enable other silencing mechanisms mediated by small RNAs derived from the expressed TE. Overall, genome-wide reprogramming in PGCs has the function of erasing aberrant epigenetic information and thus prevent the inheritance of epimutations in the next generation. It is reasonable to consider that DNA demethylation and chromatin remodelling in PGCs occur through complementary pathways to provide a robust, efficient, and reliable process. The tightly controlled number of PGCs, particularly in the fetal ovary, must ensure that most, if not all, of the germ cells are efficiently reprogrammed before their entry into meiosis. Strikingly, even in the presence of multiple passive and active mechanisms controlling reprogramming, some germ cells can stochastically still escape from it. It is possible that reprogramming is a prerequisite to allow meiotic recombination. In line with this, it is worth highlighting that the generation of single-stranded DNA breaks could contribute to active demethylation mediated by the base excision repair (BER) pathway, XRCC1 and PARP (reviewed in detail by (Hackett *et al.*, 2012)).

Sexual bifurcation in development of male and female PGCs starts at E12.5 when the gonadal ridges first become differentially recognized as either testis or ovaries. By E13.5, oocytes enter meiosis and gonocytes enter mitiotic arrest. Proliferating germ cells and somatic cells do not seem to significantly differ in the mechanisms that govern cell cycle. However, particular cell cycle proteins are expressed in cycling mouse germ cells and not in quiescent germ cells, or vice versa. Male germ cells commit to spermatogenesis rapidly after somatic sex determination, which involves commitment of the Sertoli cell lineage and organization of the germ cells within the developing testis cords. At this developmental stage, Fgf9 expression is required to ensure somatic sex determination and promote germ cell survival in the testes (DiNapoli *et al.*, 2006). By E13.5, gonocytes start entering mitotic arrest, but male germ cell differentiation does not stop. Cell cycle arrest is accomplished both by transcriptional and translational control of gene expression. The absence of retinoic acid (RA) and the presence of FGF9 in the testis lead to the upregulation of the RNA-binding protein Nanos2 (Barrios *et al.*, 2010; Bowles *et al.*, 2010) which is critical for the establishment of the masculine traits in gonocytes (Saga, 2008; Suzuki and Saga, 2008). In proliferating PGCs the G1-S phase checkpoint retinoblastoma protein 1 (pRB1) is hyperphosphorilated (inactive) but becomes dephosphorilated (activated) in arresting germ cells. In quiescent cells, its expression is down-regulated and eventually abolished. So the transient activation of pRB1 in arresting germ cells may be related to the prevention of the G1/S transition. Its subsequent disappearance suggests that pRB1 activity is not necessary to maintain the quiescent state but just to induce it (Western, 2009a). Kit signalling is also downregulated when male germ cells enter mitotic arrest through the same transcriptional network that is observed in the postnatal testis (Barrios *et al.*, 2012; Filipponi *et al.*, 2007). Interestingly, supporting the notion that a failure to establish the mitotic arrest may underlie the origin of testicular GCTs (see below), pRB1 and other genes implicated in the mitotic arrest of gonocytes, are up-regulated in humans. This is the case for instance of p63, p53, and Atm, whose expression probably reinforces germline integrity controlling the DNA damage checkpoint (Spiller *et al.*, 2009a; Spiller *et al.*, 2009b; Western, 2009).

Overview of testicular germ cell tumours

Origins

Germ cell tumors (GCTs) represent 60% of the malignancies diagnosed in men between the ages of 17 and 45 years. Human testicular GCTs are classified into two major groups: seminomas and non-seminomas (Fig. 4). Typically, non-seminomas are composed of a mixture of undifferentiated stem cell-like components (embryonic carcinoma cells, EC), and differentiated derivatives that

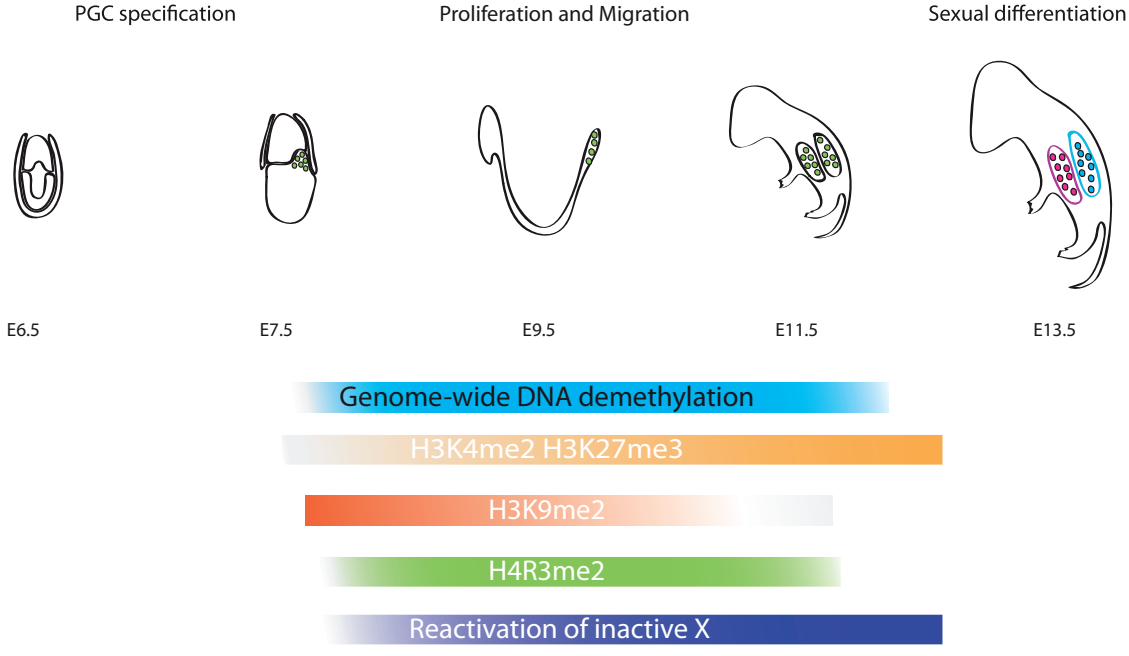

Fig. 3. Reprogramming events in the developing germline. *Specified PGCs undergo genome-wide epigenetic reprogramming. DNA methylation is gradually reduced from E8.5 onwards. The H3K9me2 mark is gradually reduced after PGCs specification while H3K4me2 and H3K27me3 have a complementary pattern, being gradually increased. The characteristic H4R3me2 is observed after specification until PGCs arrive into the gonads. The inactive X chromosome is progressively reactivated after specification.*

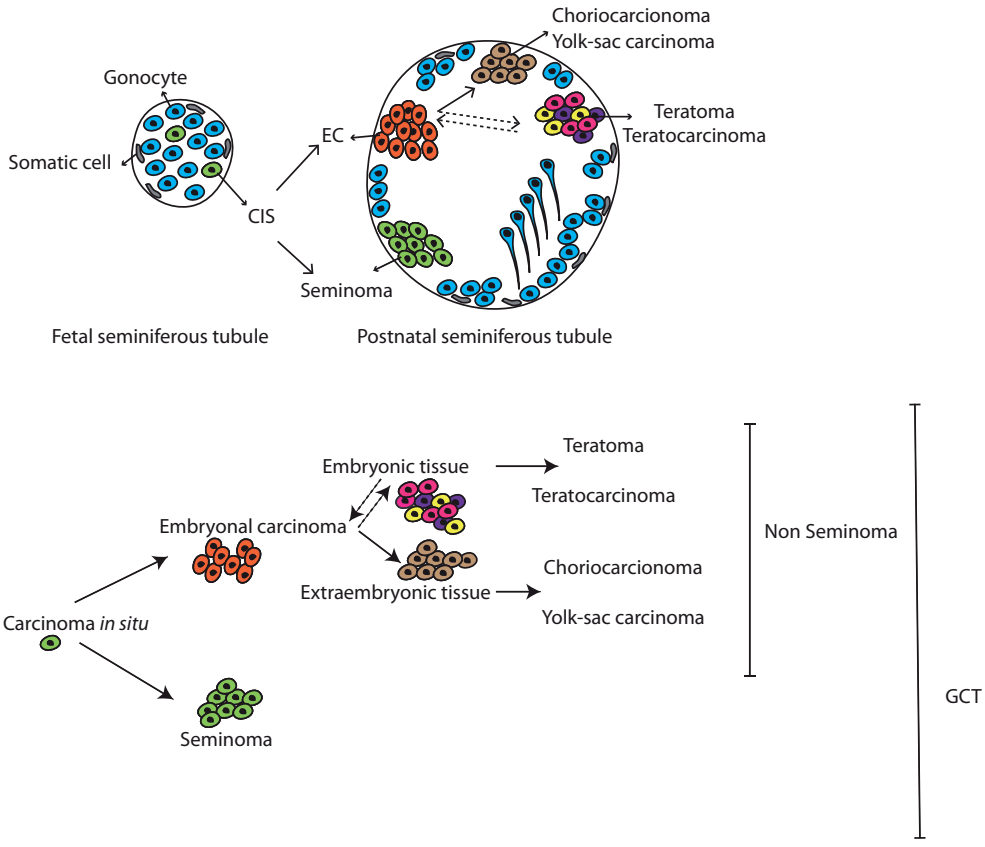

Fig. 4. Origin of Male Germ Cell Tumours (GCTs). *Schematic representation of a seminiferous tubule during fetal development and during adult life. Blue represents healthy male germ cell development. Green represents impaired male germ cell development. The first step in the transformation of PGCs or gonocytes within the seminiferous tubule is thought to be the Carcinoma* in situ *(CIS). CIS can generate seminomas, with a gene expression pattern highly similar to PGCs and gonocytes. It is speculated that CIS can differentiate into Embryonal Carcinomal cells (EC, in orange) able to generate derivatives similar to early embryonic and extraembryonic tissues. Teratomas and teratocarcinomas contain derivatives of the three germ layers (endoderm, mesoderm, and ectoderm, represented in green, pink, and violet). Choriocarcinoma and Yolk-sac carcinoma have a gene expression pattern that resembles extraembryonic tissues (represented in brown). The relationships presented in this scheme are based on similarities of gene expression patterns and imprinting profiles of human (GCTs). In mice seminomas do not occur. The dashed lines evidence differentiation pathways not precisely elucidated.*

can include all three germ layers, recognized as teratocarcinoma. Both seminomas and teratocarcinomas appear to arise, initially, as abnormal embryonic germ cells that form an intratubular germ cell neoplasia known as carcinoma *in situ* (CIS) within the seminiferous tubules (Skakkebaek, 1972). Morphological similarities and common gene expression pattern support the hypothesis that human testicular GCTs develop as a misregulation of normal PGC and/or gonocytes development. PGCs would first transform into a CIS and gradually gain invasive ability. If during this process they retain the general features of PGCs, they constitute a seminoma. Alternatively, PGCs may convert into a cell type resembling pluripotent cells from the inner cell mass (ICM) of the pre-implantation embryo or their *in vitro* derivatives, the embryonic stem cells (ESC), and are then called EC. In this event, EC cells further differentiate into all of the various somatic and extraembryonic cell types that together constitute a teratocarcinoma (Gilbert *et al.*, 2011; Oosterhuis and Looijenga, 2005) (Fig. 4).

Most of cases of seminoma and non-seminoma show gain of material from the chromosome arm 12p by generating an isochromosome. The isochromosome is a chromosome that has lost one of its arms and replaced it with an exact copy of the other arm, or through other rearrangements that result in multiple identical chromosomal arms. It is speculated that isochromosomes in GCTs might be generated either during the proliferative phase of PGC development or also later on during meiosis, as a failure to successfully separate sister chromatids (Gilbert *et al.*, 2011; Oosterhuis and Looijenga, 2005).

Interestingly, successive passages of human ES cells also accumulate alterations in 12p and 17p chromosome arms and this has been associated to an *in vitro* selective advantage (Draper *et al.*, 2004). Not surprisingly, some key genes for pluripotency and cell cycle regulation are localized in the chromosomal arm 12p. These include STELLA, NANOG, early development regulator 1 (EDR1; also known as PHC1) and growth/ differentiation factor 3 (GDF3), in addition to KRAS and cyclin D2 (CCND2), which are associated with malignant transformation and proliferation. In particular, cyclin D2 amplification and expression and the inactivation of the PTEN tumor suppressor gene might be important for the CIS and early seminoma to progress into invasive GCTs (Di Vizio *et al.*, 2005).

In mice, testicular GCTs can be induced by transplantation of the genital ridge to ectopic sites between E11.5-E13.5 of embryonic development. Embryonic gonads without PGCs do not develop into tumors when transplanted to recipients, suggesting that the PGCs are responsible for the testicular teratomas in mice (Stevens, 1967). Interestingly, in mouse, seminomas do not occur and the earliest intratubular GCTs observed already contain EC cells. It is unknown at the moment why humans may develop seminomas but mice do not.

Signalling, transcription, and imprinting alterations associated with GCTs

In line with the cumulative evidence that proposes that GCTs originate from PGC or gonocytes, many of the signalling pathway, transcriptional regulators, or epigenetic markers of normal germ cell development during fetal life, appear to be 're-used' to support survival of GCTs. For example, the seminoma cell line TCam-2, expresses many receptors for the TGFβ superfamily and its downstream transcription factors. BMP4 and RA each

support TCam-2 survival and/or proliferation through upregulation of KIT expression (Young *et al.*, 2011). Activation of KIT signaling is strongly implicated in testicular GCTs. High expression levels of KIT are generally seen in seminomas and are found in up to 30% of non-seminomas. Activating mutations of KIT are present in 9% of all testicular GCTs but are more frequently observed in seminomas (20%). Small interfering RNA (siRNA) knock down of KIT expression in the seminoma cell line TCam-2 reduced viable cell numbers (Goddard *et al.*, 2007).

Another signalling pathway frequently associated with germ cell tumors is the one involving the GTPase KRAS. KRAS is subject of activating mutations and is among loci contained in the chromosome arm 12p. KRAS acts downstream from KIT and, similar to KIT, activated KRAS increases survival of seminoma cells in vitro. KRAS leads ultimately to the phosphorylation of AKT, which also constitutes a downstream effector of Stem Cell Factor (SCF, also known as Kit Ligand, KL) in PGCs. Activated AKT is also evident in most TGCTs. Additional mechanisms such as the loss of the PI3K inhibitor PTEN, through loss of heterozygosity or mutation, have also been described in TGCTs (Di Vizio *et al.*, 2005; Kimura *et al.*, 2003; Teng *et al.*, 1997).

Pluripotent genes like POU5F1 and NANOG are expressed in ES cells PGCs and GCTs, CIS, seminomas, and EC. SRY-related HMG box 2 (SOX2/Sox2) is expressed ESCs, ICM, EC in mouse and human. Curiously, Sox2 is upregulated in concomitance with mouse PGC specification (Kurimoto *et al.*, 2008) but SOX2 is absent from germ cells in the human fetal gonads, CIS and seminoma. Genome-wide expression profiling revealed that the absence of SOX2 could be fulfilled by another SOX family member, the endoderm marker SOX17, which is present in early germ cells from 18-week-old fetus and their malignant counterpart (de Jong *et al.*, 2008; Perrett *et al.*, 2008).

Most interestingly, human PGCs, CIS and seminomas, but not non-seminomas, express BLIMP1 and TCFAP2C, two critical transcriptional regulators that determine mouse PGC specification (Hoei-Hansen *et al.*, 2004). As in mouse germ cells, BLIMP1 localizes to the nucleus and is associated to the H2A/H4R3me2 mark (Ancelin *et al.*, 2006; Eckert *et al.*, 2008). However, it is likely that in CIS and seminomas the H2A/H4R3 mark is established by PRMT7 and not by PRMT5, which is found mostly in the cytoplasm of these cells instead of the nucleus (Eckert *et al.*, 2008). In TCam-2 cells, knock down of BLIMP1 using siRNA resulted in a weaker expression of TFAP2C, loss of H2A/H4R3 dimethylation and de-repression of the somatic marker HOXB1. Also, siRNA-based reduction of TFAP2C lead to upregulation of mesodermal markers HOXA1, HOXB1, HAND1 and MYOD1. This suggests that BLIMP1 and TFAP2C determine the germ cell identity of seminomas through inhibition of somatic differentiation in CIS and seminoma (Weber *et al.*, 2010). In mice, Tfap2c is expressed in PGCs as soon as these are specified (Kurimoto *et al.*, 2008; Weber *et al.*, 2010; Yamaji *et al.*, 2008) so it will be interesting to determine how these two transcriptional regulators interact to establish the germ cell fate.

In humans, the global methylation pattern of male germ cells changes from hypomethylation in fetal spermatogonia to hypermethylation in mature sperm. Undifferentiated GCTs, seminomas and CIS, are globally hypomethylated, whereas more differentiated GCTs, teratomas, yolk sac tumours, and choriocarcinomas, show a higher degree of methylation. Demethylation of TCam-2 cells is associated with an increased expression of the pluripotency markers NANOG and POU5F1, as well as the germ cell-specific marker VASA (Wermann *et al.*, 2010). In line with the gain of pluripotent features, multiple X chromosomes in testicular GCTs were predominantly hypomethylated and active regardless of XIST expression. XIST expression is common in seminomas but not in non-seminomas. However, X chromosomal gain was consistently observed in both types of tumors. The biological significance of excess active X chromosomes in testicular GCTs was suggested by enhanced expression of the two X-linked oncogenes ARAF1 and ELK1 in the testicular GCT derived cell lines, suggesting the potential oncogenic implications of X chromosomal gain in testicular GCTs (Kawakami *et al.*, 2003).

Critical advances for the study of PGC specification

To discover novel genes involved in PGC development it was essential, in the first place, to gain rigorous access to the extra-embryonic mesoderm at the base of the allantois, and be able from there to collect single cells. Then a critical step was to find the molecular way of analysing the single cells obtained in order to search for candidates. This approach proved to be useful for the discovery of the first molecular programme for the specification of germ cell fate in mice (Saitou *et al.*, 2002) and set the bases for subsequent exploration of the transcriptional landscape of pluripotency and the germ line in single cells (Kurimoto *et al.*, 2008; Tang *et al.*, 2010).

Recently, novel *in vitro* systems capable of recapitulating PGC specification and early development have been described opening doors for detailed genetic and biochemical analysis of this process (Hayashi *et al.*, 2011; Ohinata *et al.*, 2009). Compared to previous attempts to obtain bona fide PGCs in culture (Geijsen *et al.*, 2004; Yoshimizu *et al.*, 2001), these new systems stand out by the use of chemically defined conditions and by meeting the gold standard of *in vivo* generation of functional gametes able to give rise to male and female fertile offspring (Hayashi *et al.*, 2011; Ohinata *et al.*, 2009). Excitingly enough, longstanding questions of how the germ line fate is acquired in the epiblast and which could be the *in vitro* requirements to generate a functional oocyte still remain open to investigation. Moreover, it is not known yet if these same *in vitro* conditions established for mouse epiblast, murine ESC or mouse iPS cells, would be useful for other mammals, humans in particular. Some attempts have been made to obtain PGCs from human ESCs (hESCs) or human induced pluripotent stem (hiPSCs) cells. Either the generation of embryoid bodies (Clark *et al.*, 2004) or adherent cultures of hESCs (Chen *et al.*, 2007) induces the expression of some germ cell markers, including VASA, SYCP3 and/or GDF9. hESCs were also cultured on mouse embryonic fibroblasts in the presence of bFGF without passaging causing differentiation into putative germ cells. The majority of these cells expressed the meiotic markers SYCP3 and MLH1 after 16 days, suggesting that PGC-like cells were formed capable of undergoing meiosis (West *et al.*, 2008). The first report of germ-like cells from hiPSCs suggests that it may be easier to derive germ cell-like cells from human iPSCs than from hESCs (Park *et al.*, 2009). The use of co-culture systems or conditioned medium, increases the efficiency of human PGC-like cells derivation suggesting that yet unknown factors are required to develop germline cells from pluripotent stem cells (Geens *et al.*, 2011; Park *et al.*, 2009; Richards *et al.*, 2010; West *et al.*, 2008). In an attempt to define the most suitable conditions

for the differentiation of germ cells from hESCs/iPSCs, various cytokines and signaling molecules have been tested. BMP4 alone or in combination with BMP7 and BMP8b are added to cultures to promote PGC-like differentiation from hESCs/iPSCs (Kee *et al.*, 2006; Panula *et al.*, 2011; Richards *et al.*, 2010; West *et al.*, 2011). In contrast, when hESCs were differentiated as monolayer to SSEA1+ / VASA + cells, the presence of BMP4 decreased VASA expression, suggesting a downregulation in the numbers of PGC-like cells formed (Tilgner *et al.*, 2008). Whether this difference is due to differences in the respective hESC lines used or in the differentiation method (monolayer versus EB formation) is unclear. RA has been used to stimulate meiosis (Eguizabal *et al.*, 2011; Richards *et al.*, 2010). FGF2, LIF, SCF, forskolin, (Richards *et al.*, 2010, Eguizabal *et al.*, 2011), are also used to enhance germline differentiation from hESCs/iPSCs (Eguizabal *et al.*, 2011; Richards *et al.*, 2010; West *et al.*, 2008).

Finally, an alternative route to induce differentiation of germ cells from hESCs or hiPSC has been the manipulation of gene expression. Surprisingly, notwithstanding the critical role that transcription factors have in the specification of germ cell fate, the overexpression of RNA-binding proteins like DAZL (deleted in azoosperma-like) and/or VASA proved to be critical in the derivation of PGC from hESCs/iPSCs and generation of haploid gametes. It is remarkable that some of these putative germ cells can erase imprints in the genome-wide and locus-specifc context and recapitulate meiotic progression of the male germline (Kee *et al.*, 2009; Medrano *et al.*, 2012) but no evidence of successful oogenesis has been observed in the same conditions. The generation of male and female gametes competent to create a totipotent zygote from hESCs or hiPSCs is still open to investigation.

Perspective

Loosing cellular identity and gaining a less differentiated phenotype, or a pluripotent-like phenotype, can be achieved *in vitro* by many means (somatic cell nuclear transfer (SCNT), cell fusions, introduction of exogenous transcription factors, growth factors). In vivo, malignant cellular growth has been associated to a genetic and epigenetic pattern different from the one observed healthy cells.

Reprogramming is defined as the conversion of a differentiated cell to another cell type, either to another differentiated cell type (transdifferentiation) or to a progenitor (dedifferentiation) (Holmberg and Perlmann, 2012). In this perspective, reprogramming of healthy cells *in vivo* could be due to alterations intrinsic to the cell that will change fate or could be induced by alterations in the niche in which this cell resides. In either case, the predetermined or stochastic nature of *in vivo* reprogramming remains unclear.

iPS studies suggest a critical upstream role for a network of few transcription factors in governing cell fate decisions. In line with heterokaryon experiments, transcription factors seem to have an instructive effect in the regulation of cellular identity. Conditional ablation of selected transcription factors in differentiated cells can lead to changes in cellular identity, highlighting thus that specific transcription factors ensure the maintenance of a certain identity. In the adult mouse gonad, sexual reversion can be achieved by selective ablation of transcription factors specific to the granulosa or Sertoli cells. In the hematopoietic lineage, one of the mechanisms by which a transcription factors may maintain the differentiated state is by binding to target genes that are not enriched in repressive chromatin marks like H3K27me3. This would facilitate a certain degree of plasticity that then allows the cell to be reprogrammed upon deletion of a lineage-specific transcription factor after a specific cell fate had been achieved (Holmberg and Perlmann, 2012; Matson and Zarkower, 2012). Interestingly, there is no indication whether the same regulation holds true for the germ lineage. Due to its critical in role in the transmission of the genome to the following generation, it would be reasonable to speculate that several mechanisms act in concomitance to ensure that the germ cell identity is maintained and is not plausible of ectopic reprogramming once its fate has been established. In fact, to date, no conditional deletions in germ cells have led to the loss of the germ cell fate. During fetal development, germ cells can revert their sexual identity (for example in Nanos2 knock out mice (Suzuki and Saga, 2008) or FGF9 knock out mice (DiNapoli *et al.*, 2006)), or adopt the apoptotic pathway instead of changing their potential totipotent fate (for example W mutations, Nanos3 knock out mice (Tsuda *et al.*, 2003)). In the adult gonad, reprogramming of the somatic sexual identity has been achieved through deletion of lineage – specific transcription factors (Matson *et al.*, 2011; Uhlenhaut *et al.*, 2009) but an equivalent alteration of female or male adult germ cell fate has not been described. Germline deletion of key germ cell determinants like Blimp1 and Prdm14 avoids the establishment of a viable founding population of germ cells. Characteristically, the repression of Hoxb1 does not occur in Blimp1 null cells and Prmd14 null PGC-like cells fail to re-express pluripotency genes (Ohinata *et al.*, 2005; Yamaji *et al.*, 2008). The exact fate of Blimp1 -/- and Prdm14 -/- PGC-like cells is not fully clear. It will be interesting to investigate the effect of the conditional deletion of these genes in a time subsequent to PGC specification, but before their natural exclusion from germ cell nuclei. It could be that the transcriptional network established at the time of specification is sufficiently robust to maintain germ cell fate using redundant or unrelated transcription factors. It could also be that when DNA and histone modifications are established there is no further need of instructive factors. Since is easier to induce reprogramming into related lineages than into unrelated ones, it could be that the 'signature' established at the time of germ cell specification prevents reprograming of the germ line to any not pluripotent state. It will be interesting to investigate how Blimp1 and Prmd14 are integrated into a gene regulatory network to establish the germ cell fate and how in silico and *in vivo* conditional perturbation of this network could modulate germline identity. We also know that DNA methylation within a CpG context is the key mark that can be inherited through cell division and contributes to a stable lineage commitment. So to understand how the transcriptional network that induces germ cell fate relates to the reprogramming events that characterize PGCs is a key subject of investigation.

In human PGCs, the gene regulatory network that is associated with seminomas is founded in transcription factors that are key to the regulation of pluripotency, like OCT4 and NANOG, and also in transcriptional regulators of early germ cells as BLIMP1 and TCFAP2AC (Hoei-Hansen *et al.*, 2004; Weber *et al.*, 2010). An over-dose of NANOG (and STELLA) seems to contribute to the deviation of normal development (Gilbert *et al.*, 2011). Interestingly, in mouse embryos and in mouse ES cells, a tight dosage of Nanog expression is critical to secure the most naïve pluripotent state (Miyanari and Torres-Padilla, 2012). It will be interesting to determine if the chromosomal alterations observed in human GCT could lead to an extra-dosage of NANOG and investigate if

the allelic regulation of its expression could be at the basis of the malignant transformation.

It is becoming a worldwide challenge to find the most suitable *in vitro* conditions that would allow a detailed investigation of the cellular and molecular mechanisms that govern germ cell specification and development in humans (Hayashi *et al.*, 2012; Panula *et al.*, 2011). For ethical reasons, direct experimental manipulation of the human embryo is highly regulated. Specification of human PGCs occurs within the first month of gestation and clinically detectable spontaneous abortions that may be available for research occur when PGCs have already reached the gonadal ridges. This makes the human epiblast elusive to direct analysis. It would thus be valuable to explore and design new methodologies that would mimic this developmental window in order to study how the selection of PGC precursors occurs in the human epiblast. We expect that some of the key genes involved in PGC specification in the mouse are also involved in the equivalent human process. But we expect also many surprises due to the highly divergent structure of the human embryo with respect to the mouse and to the different biochemical requirements for the maintenance of mouse ES cells and human ES cells. The characterization of PGC specification in the human epiblast could contribute to a major ultimate goal for human health. Insights gained from this investigation could directly impact to the potential autologous generation of germ cells from patients with fertility disruptions, probably via an iPS mediated strategy.

Alternatively, human PGC development could be modelled by using human ES cells or cell lines derived from GCTs. Since the generation of viable offspring from these cells is not a testable option, several criteria should be met in order to be able to unequivocally identify the *in vitro* obtained human germ cell-like cells as authentic germ cells. Ultimately, it would be extremely exciting if the whole germ cell development would be recapitulated in vitro. The first step towards this goal would be to obtain specified PGCs that, besides a characteristic gene expression pattern, properly undergo genome-wide demethylation and chromatin remodelling. These cells should then be proofed as competent to enter and undergo meiosis. Finally, maternal and paternal imprints should be differentially established and maintained while in culture.

It is probably a fundamental and an ambitious aim that has long fascinated developmental biologists of all times. Maybe, the technical advances reached so far to *in vitro* recapitulate mouse germ cell development and also other aspects of regenerative medicine, have now set an attractive scenario to experimentally tackle it.

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References

ANCELIN, K., LANGE, U.C., HAJKOVA, P., SCHNEIDER, R., BANNISTER, A.J., KOUZARIDES, T., AND SURANI, M.A. (2006). Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat. Cell Biol.8*: 623-630.

AOYAMA, M., SUN-WADA, G.H., YAMAMOTO, A., YAMAMOTO, M., HAMADA, H., AND WADA, Y. (2012). Spatial Restriction of Bone Morphogenetic Protein Signaling in Mouse Gastrula through the mVam2-Dependent Endocytic Pathway. *Dev. Cell.*22: 1163-1175.

BARRIOS, F., FILIPPONI, D., CAMPOLO, F., GORI, M., BRAMUCCI, F., PELLEGRINI, M., OTTOLENGHI, S., ROSSI, P., JANNINI, E.A., AND DOLCI, S. (2012). SOHLH1 and SOHLH2 control Kit expression during postnatal male germ cell development. *J. Cell Sci.* 125: 1455-1464.

BARRIOS, F., FILIPPONI, D., PELLEGRINI, M., PARONETTO, M.P., DI SIENA, S., GEREMIA, R., ROSSI, P., DE FELICI, M., JANNINI, E.A., AND DOLCI, S. (2010). Opposing effects of retinoic acid and FGF9 on Nanos2 expression and meiotic entry of mouse germ cells. *J. Cell Sci.* 123: 871-880.

BOWLES, J., FENG, C.W., SPILLER, C., DAVIDSON, T.L., JACKSON, A., AND KOOPMAN, P. (2010). FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Dev. Cell.* 19: 440-449.

CHEN, H.F., KUO, H.C., CHIEN, C.L., SHUN, C.T., YAO, Y.L., IP, P.L., CHUANG, C.Y., WANG, C.C., YANG, Y.S., AND HO, H.N. (2007). Derivation, characterization and differentiation of human embryonic stem cells: comparing serum-containing versus serum-free media and evidence of germ cell differentiation. *Hum. Reprod.* 22: 567-577.

CLARK, A.T., BODNAR, M.S., FOX, M., RODRIQUEZ, R.T., ABEYTA, M.J., FIRPO, M.T., AND PERA, R.A. (2004). Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum. Mol. Gen.* 13: 727-739.

DE FELICI, M., BOITANI, C., AND COSSU, G. (1985). Synthesis of glycoconjugates in mouse primordial germ cells. *Dev. Biol.* 109: 375-380.

DE JONG, J., STOOP, H., GILLIS, A.J., VAN GURP, R.J., VAN DE GEIJN, G.J., BOER, M., HERSMUS, R., SAUNDERS, P.T., ANDERSON, R.A., OOSTERHUIS, J.W.., *et al.* (2008). Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications. *J. Pathol.* 215: 21-30.

DE SOUSA LOPES, S.M., HAYASHI, K., AND SURANI, M.A. (2007). Proximal visceral endoderm and extraembryonic ectoderm regulate the formation of primordial germ cell precursors. *BMC Dev. Biol.* 7: 140.

DE SOUSA LOPES, S.M., ROELEN, B.A., MONTEIRO, R.M., EMMENS, R., LIN, H.Y., LI, E., LAWSON, K.A., AND MUMMERY, C.L. (2004). BMP signaling mediated by ALK2 in the visceral endoderm is necessary for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* 18: 1838-1849.

DI VIZIO, D., CITO, L., BOCCIA, A., CHIEFFI, P., INSABATO, L., PETTINATO, G., MOTTI, M.L., SCHEPIS, F., D'AMICO, W., FABIANI, F., *et al.* (2005). Loss of the tumor suppressor gene PTEN marks the transition from intratubular germ cell neoplasias (ITGCN) to invasive germ cell tumors. *Oncogene* 24: 1882-1894.

DINAPOLI, L., BATCHVAROV, J., AND CAPEL, B. (2006). FGF9 promotes survival of germ cells in the fetal testis. *Development* 133: 1519-1527.

DRAPER, J.S., SMITH, K., GOKHALE, P., MOORE, H.D., MALTBY, E., JOHNSON, J., MEISNER, L., ZWAKA, T.P., THOMSON, J.A., AND ANDREWS, P.W. (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat. Biotech.* 22: 53-54.

ECKERT, D., BIERMANN, K., NETTERSHEIM, D., GILLIS, A.J., STEGER, K., JACK, H.M., MULLER, A.M., LOOIJENGA, L.H., AND SCHORLE, H. (2008). Expression of BLIMP1/PRMT5 and concurrent histone H2A/H4 arginine 3 dimethylation in fetal germ cells, CIS/IGCNU and germ cell tumors. *BMC Dev. Biol.* 8: 106

EGUIZABAL, C., MONTSERRAT, N., VASSENA, R., BARRAGAN, M., GARRETA, E., GARCIA-QUEVEDO, L., VIDAL, F., GIORGETTI, A., VEIGA, A., AND IZPISUA BELMONTE, J.C. (2011). Complete meiosis from human induced pluripotent stem cells. *Stem cells* 29: 1186-1195.

FILIPPONI, D., HOBBS, R.M., OTTOLENGHI, S., ROSSI, P., JANNINI, E.A., PANDOLFI, P.P., AND DOLCI, S. (2007). Repression of kit expression by Plzf in germ cells. *Mol. Cell. Biol.* 27: 6770-6781.

GEENS, M., SERMON, K.D., VAN DE VELDE, H., AND TOURNAYE, H. (2011). Sertoli cell-conditioned medium induces germ cell differentiation in human embryonic stem cells. *J. Ass. Reprod. Gen.* 28: 471-480.

GEIJSEN, N., HOROSCHAK, M., KIM, K., GRIBNAU, J., EGGAN, K., AND DALEY, G.Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427: 148-154.

GILBERT, D., RAPLEY, E., AND SHIPLEY, J. (2011). Testicular germ cell tumours: predisposition genes and the male germ cell niche. *Nat. Rev. Cancer* 11: 278-288.

GINSBURG, M., SNOW, M.H., AND MCLAREN, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* 110: 521-528.

GODDARD, N.C., MCINTYRE, A., SUMMERSGILL, B., GILBERT, D., KITAZAWA, S., AND SHIPLEY, J. (2007). KIT and RAS signalling pathways in testicular germ cell tumours: new data and a review of the literature. *Int. J. Androl.* 30: 337-348; discussion 349.

HACKETT, J.A., ZYLICZ, J.J., AND SURANI, M.A. (2012). Parallel mechanisms of

epigenetic reprogramming in the germline. *Trends Genet.* 28: 164-174.

HANOVER, J.A., KRAUSE, M.W., AND LOVE, D.C. (2012). Bittersweet memories: linking metabolism to epigenetics through O-GlcNAcylation. *Nat. Rev. Mol. Cell Biol.* 13: 312-321.

HAYASHI, K., KOBAYASHI, T., UMINO, T., GOITSUKA, R., MATSUI, Y., AND KITAMURA, D. (2002). SMAD1 signaling is critical for initial commitment of germ cell lineage from mouse epiblast. *Mech. Dev.* 118: 99-109.

HAYASHI, K., OHTA, H., KURIMOTO, K., ARAMAKI, S., AND SAITOU, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell* 146: 519-532.

HAYASHI, Y., SAITOU, M., AND YAMANAKA, S. (2012). Germline development from human pluripotent stem cells toward disease modeling of infertility. *Fert. Ster.* 97: 1250-1259.

HOEI-HANSEN, C.E., NIELSEN, J.E., ALMSTRUP, K., HANSEN, M.A., SKAKKEBAEK, N.E., RAJPERT-DEMEYTS, E., AND LEFFERS, H. (2004). Identification of genes differentially expressed in testes containing carcinoma in situ. *Mol. Hum. Reprod.* 10: 423-431.

HOLMBERG, J., AND PERLMANN, T. (2012). Maintaining differentiated cellular identity. *Nat. Rev. Genet.* 13: 429-439.

KAWAKAMI, T., OKAMOTO, K., SUGIHARA, H., HATTORI, T., REEVE, A.E., OGAWA, O., AND OKADA, Y. (2003). The roles of supernumerical X chromosomes and XIST expression in testicular germ cell tumors. *J. Urol.* 169: 1546-1552.

KEE, K., ANGELES, V.T., FLORES, M., NGUYEN, H.N., AND REIJO PERA, R.A. (2009). Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. *Nature* 462: 222-225.

KEE, K., GONSALVES, J.M., CLARK, A.T., AND PERA, R.A. (2006). Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells. *Stem Cells Dev.* 15: 831-837.

KIMURA, T., SUZUKI, A., FUJITA, Y., YOMOGIDA, K., LOMELI, H., ASADA, N., IKEUCHI, M., NAGY, A., MAK, T.W., AND NAKANO, T. (2003). Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production. *Development* 130: 1691-1700.

KRETZSCHMAR, M., DOODY, J., AND MASSAGUE, J. (1997). Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* 389: 618-622.

KURIMOTO, K., YABUTA, Y., OHINATA, Y., SHIGETA, M., YAMANAKA, K., AND SAITOU, M. (2008). Complex genome-wide transcription dynamics orchestrated by Blimp1 for the specification of the germ cell lineage in mice. *Genes Dev.* 22: 1617-1635.

LAWSON, K.A., DUNN, N.R., ROELEN, B.A., ZEINSTRA, L.M., DAVIS, A.M., WRIGHT, C.V., KORVING, J.P., AND HOGAN, B.L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* 13: 424-436.

LAWSON, K.A., AND HAGE, W.J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. Ciba Foundation symposium 182: 68-84; discussion 84-91.

LAWSON, K.A., MENESES, J.J., AND PEDERSEN, R.A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* 113: 891-911.

LIU, P., WAKAMIYA, M., SHEA, M.J., ALBRECHT, U., BEHRINGER, R.R., AND BRADLEY, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Gen.* 22: 361-365.

MATSON, C.K., MURPHY, M.W., SARVER, A.L., GRISWOLD, M.D., BARDWELL, V.J., AND ZARKOWER, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* 476: 101-104.

MATSON, C.K., AND ZARKOWER, D. (2012). Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. *Nat. Rev. Gen.* 13: 163-174.

MCLAREN, A. (1999). SIGNALLING FOR GERM CELLS. *Genes Dev.* 13: 373-376.

MEDRANO, J.V., RAMATHAL, C., NGUYEN, H.N., SIMON, C., AND REIJO PERA, R.A. (2012). Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro. *Stem cells* 30: 441-451.

MIYANARI, Y., AND TORRES-PADILLA, M.E. (2012). Control of ground-state pluripotency by allelic regulation of Nanog. *Nature* 483: 470-473.

MOREMEN, K.W., TIEMEYER, M., AND NAIRN, A.V. (2012). Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 13: 448-462.

OHINATA, Y., OHTA, H., SHIGETA, M., YAMANAKA, K., WAKAYAMA, T., AND SAITOU, M. (2009). A signaling principle for the specification of the germ cell lineage in mice. *Cell* 137: 571-584.

OHINATA, Y., PAYER, B., O'CARROLL, D., ANCELIN, K., ONO, Y., SANO, M., BARTON, S.C., OBUKHANYCH, T., NUSSENZWEIG, M., TARAKHOVSKY, A., *et al.* (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 436: 207-213.

OKAMURA, D., HAYASHI, K., AND MATSUI, Y. (2005). Mouse epiblasts change responsiveness to BMP4 signal required for PGC formation through functions of extraembryonic ectoderm. *Mol. Reprod. Dev.* 70: 20-29.

OOSTERHUIS, J.W., AND LOOIJENGA, L.H. (2005). Testicular germ-cell tumours in a broader perspective. *Nat. Rev. Cancer* 5: 210-222.

PANULA, S., MEDRANO, J.V., KEE, K., BERGSTROM, R., NGUYEN, H.N., BYERS, B., WILSON, K.D., WU, J.C., SIMON, C., HOVATTA, O., *et al.* (2011). Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells. *Hum. Mol. Gen.* 20: 752-762.

PARK, T.S., GALIC, Z., CONWAY, A.E., LINDGREN, A., VAN HANDEL, B.J., MAGNUSSON, M., RICHTER, L., TEITELL, M.A., MIKKOLA, H.K., LOWRY, W.E., *et al.* (2009). Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells. *Stem cells* 27: 783-795.

PERRETT, R.M., TURNPENNY, L., ECKERT, J.J., O'SHEA, M., SONNE, S.B., CAMERON, I.T., WILSON, D.I., RAJPERT-DE MEYTS, E., AND HANLEY, N.A. (2008). The early human germ cell lineage does not express SOX2 during *in vivo* development or upon *in vitro* culture. *Biol. Reprod.* 78: 852-858.

RICHARDS, M., FONG, C.Y., AND BONGSO, A. (2010). Comparative evaluation of different *in vitro* systems that stimulate germ cell differentiation in human embryonic stem cells. *Fert. Ster.* 93: 986-994.

SAGA, Y. (2008). Sexual development of mouse germ cells: Nanos2 promotes the male germ cell fate by suppressing the female pathway. *Dev. Growth Diff.* 50 *Suppl* 1, S141-147.

SAITOU, M., BARTON, S.C., AND SURANI, M.A. (2002). A molecular programme for the specification of germ cell fate in mice. *Nature* 418: 293-300.

SKAKKEBAEK, N.E. (1972). Possible carcinoma-in-situ of the testis. *Lancet* 2: 516-517.

SPILLER, C., WILHELM, D., AND KOOPMAN, P. (2009a). Cell cycle analysis of fetal germ cells during sex differentiation in mice. *Biol. Cell* 101: 587-598.

SPILLER, C.M., WILHELM, D., AND KOOPMAN, P. (2009b). Retinoblastoma 1 Protein Modulates XY Germ Cell Entry into G1/G0 Arrest During Fetal Development in Mice. *Biol. Reprod.* 82: 433-443.

STEVENS, L.C. (1967). Origin of testicular teratomas from primordial germ cells in mice. *J. National Cancer Institute* 38: 549-552.

SUZUKI, A., AND SAGA, Y. (2008). Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes Dev.* 22: 430-435.

TANG, F., BARBACIORU, C., BAO, S., LEE, C., NORDMAN, E., WANG, X., LAO, K., AND SURANI, M.A. (2010). Tracing the derivation of embryonic stem cells from the inner cell mass by single-cell RNA-Seq analysis. *Cell stem cell* 6: 468-478.

TENG, D.H., HU, R., LIN, H., DAVIS, T., ILIEV, D., FRYE, C., SWEDLUND, B., HANSEN, K.L., VINSON, V.L., GUMPPER, K.L., *et al.* (1997). MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. *Cancer Res.* 57: 5221-5225.

TILGNER, K., ATKINSON, S.P., GOLEBIEWSKA, A., STOJKOVIC, M., LAKO, M., AND ARMSTRONG, L. (2008). Isolation of primordial germ cells from differentiating human embryonic stem cells. *Stem cells* 26: 3075-3085.

TSUDA, M., SASAOKA, Y., KISO, M., ABE, K., HARAGUCHI, S., KOBAYASHI, S., AND SAGA, Y. (2003). Conserved role of nanos proteins in germ cell development. *Science* 301: 1239-1241.

UHLENHAUT, N.H., JAKOB, S., ANLAG, K., EISENBERGER, T., SEKIDO, R., KRESS, J., TREIER, A.C., KLUGMANN, C., KLASEN, C., HOLTER, N.I., *et al.* (2009). Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 139: 1130-1142.

WEBER, S., ECKERT, D., NETTERSHEIM, D., GILLIS, A.J., SCHAFER, S., KUCKENBERG, P., EHLERMANN, J., WERLING, U., BIERMANN, K., LOOIJENGA, L.H., *et al.,* (2010). Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. *Biol. Rep.* 82: 214-223.

WERMANN, H., STOOP, H., GILLIS, A.J., HONECKER, F., VAN GURP, R.J., AMMERPOHL, O., RICHTER, J., OOSTERHUIS, J.W., BOKEMEYER, C., AND LOOIJENGA, L.H. (2010). Global DNA methylation in fetal human germ cells and germ cell tumours: association with differentiation and cisplatin resistance. *J. Path.* 221: 433-442.

WEST, F.D., MACHACEK, D.W., BOYD, N.L., PANDIYAN, K., ROBBINS, K.R., AND STICE, S.L. (2008). Enrichment and differentiation of human germ-like cells mediated by feeder cells and basic fibroblast growth factor signaling. *Stem cells* 26: 2768-2776.

WEST, F.D., MUMAW, J.L., GALLEGOS-CARDENAS, A., YOUNG, A., AND STICE, S.L. (2011). Human haploid cells differentiated from meiotic competent clonal germ cell lines that originated from embryonic stem cells. *Stem cells Dev.* 20: 1079-1088.

WESTERN, P. (2009a). Foetal germ cells: striking the balance between pluripotency and differentiation. *Int. J. Dev. Biol.* 53: 393-409.

YAMAJI, M., SEKI, Y., KURIMOTO, K., YABUTA, Y., YUASA, M., SHIGETA, M., YAMANAKA, K., OHINATA, Y., AND SAITOU, M. (2008). Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat. Gen.* 40: 1016-1022.

YOSHIMIZU, T., OBINATA, M., AND MATSUI, Y. (2001). Stage-specific tissue and cell interactions play key roles in mouse germ cell specification. *Development* 128: 481-490.

YOUNG, J.C., JAIPRAKASH, A., MITHRAPRABHU, S., ITMAN, C., KITAZAWA, R., LOOIJENGA, L.H., AND LOVELAND, K.L. (2011). TCam-2 seminoma cell line exhibits characteristic foetal germ cell responses to TGF-beta ligands and retinoic acid. *Int. J. Androl.* 34: e204-217.