The mammalian germline as a pluripotency cycle

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Summary
Naive pluripotency refers to the capacity of single cells in regulative embryos to engender all somatic and germline cell types. Only germ cells – conventionally considered to be unipotent – can naturally re-acquire pluripotency, by cycling through fertilisation. Furthermore, primordial germ cells express, and appear to be functionally dependent upon, transcription factors that characterise the pluripotent state. We hypothesise that germ cells require pluripotency factors to control a developmentally restricted epigenome. Consequently, they harbour latent potential, as manifested in teratocarcinogenesis or direct conversion into pluripotent stem cells in vitro. Thus, we suggest that there exists an unbroken cycle of pluripotency, naive in the early epiblast and latent in the germline, that is sustained by a shared transcription factor network.

Key words: DNA methylation, Embryonic germ cells, Embryonic stem cells, Germline, Pluripotency, Primordial germ cells

Introduction
The germline of animals can be formed in two distinct ways (Fig. 1). In many organisms, preformation of germ plasm segregates the germ and soma from the very start of development. In this mode, the germline is perpetually kept apart from somatic cells across the generations. Alternatively, the germline may be induced within a population of pluripotent cells, as in mammals. Ectopic expression of germline genes in the soma is potentially tumorigenic (Simpson et al., 2005) whereas failure to specify a germline is a reproductive dead end. Therefore, germ cell specification via induction requires precise orchestration to ensure timely restriction from the soma. Evidence suggests that induction may be the ancestral form of primordial germ cell (PGC) specification and that preformation has emerged multiple times during evolution to escape the developmental constraints imposed by the necessity for reliable germline induction (Johnson et al., 2012). Upregulation of pluripotency genes includes re-expression of pre-implantation epiblast cells can produce all lineages of the body, including the germline (Gardner and Rossant, 1979; Gardner et al., 2005). Mouse blastocyst microinjection demonstrated that pre-implantation epiblast cells are said to exhibit naive pluripotency (see Glossary, Box 1). The major inductive signal for PGCs is Bmp4 emanating from the extra-embryonic ectoderm (Lawson et al., 1999; Ohinata et al., 2009). Bone morphogenetic protein (BMP) stimulation leads to the upregulation of Blimp1 (Prdm1) and Prdm14, the two crucial determinants of the germ cell lineage, shortly followed by re-expression of naive pluripotency markers (Saitou and Yamaji, 2012). Upregulation of pluripotency genes includes re-expression of Stella (also known as Dppa3 and Pgc7), which has been used as the definitive marker of specified PGCs (McLaren and Lawson, 2005; Saitou et al., 2002).

Pluripotency in vivo and in vitro
Mouse blastocyst microinjection demonstrated that pre-implantation epiblast cells can produce all lineages of the body, including the germline (Gardner and Rossant, 1979; Gardner et al., 1985). They can also give rise to embryonic stem (ES) cells (see Glossary, Box 1) in vitro (Brook and Gardner, 1997; Nichols et al., 2009). Mouse ES cells in turn can re-integrate into the blastocyst and colonise both soma and germline (Bradley et al., 1984). Thus, pre-implantation epiblast and ES cells are said to exhibit naive pluripotency (Nichols and Smith, 2012; Nichols and Smith, 2009). Following implantation, epiblast cells lose the capability to colonise the blastocyst (Rossant et al., 1978). However, cell transplantation studies have shown that, up to E6.5, the distal epiblast (usually destined to form ectoderm) can give rise to proximal epiblast derivatives, such as extra-embryonic mesoderm and PGCs, and vice versa (Tam and Zhou, 1996). Thus, despite the onset of regional specification, early post-implantation epiblast cells remain pluripotent. This population does not readily give rise to ES cells, but instead can yield post-implantation epiblast stem cells (EpiSCs) (see Glossary, Box 1) (Brons et al., 2007; Tesar et
Fig. 1. Preformation and induction models of PGC specification.
Schematic representation of the two modes of germ cell specification. (A) Preformation, as occurs in *Drosophila*. The nurse cells synthesise RNAs and proteins (pink) that are localised to the posterior pole of the oocyte. This germ plasm will be segregated into PGCs. (B) Induction, as occurs in the mouse. Signals from the extra-embryonic ectoderm (grey) induce the formation of PGCs (dark pink) from within pluripotent epiblast (red). Non-induced cells (green) go on to form the soma. ExE, extra-embryonic ectoderm; VE, visceral endoderm (brown). Adapted from Extavour and Akam (Extavour and Akam, 2003).

al., 2007), which exhibit features characterised as primed pluripotency (see Glossary, Box 1) (Nichols and Smith, 2009). Notably, EpiSCs have lost expression of several pluripotency factors and have upregulated germ layer specification genes. They form multi-differentiated teratomas but rarely contribute to chimaeras by blastocyst injection. However, they are capable of colonising the gastrulation-stage epiblast (Huang et al., 2012). EpiSCs can be derived up until at least E7.5, suggesting that they may represent a late stage of epiblast development (Osorno et al., 2012).

Although the window for ES cell derivation does not extend beyond the peri-implantation epiblast, the capacity to regain naive pluripotency is not lost in all cells of the post-implantation embryo. Specifically, once formed, PGCs can convert in culture into naive pluripotent stem cells, called embryonic germ (EG) cells (see Glossary, Box 1) (Matsui et al., 1992; Resnick et al., 1992). PGCs themselves do not contribute to chimaeras when injected into blastocysts (V. Papatoannou and R. Gardner, personal communication) (Durcova-Hills et al., 2006) and there is no evidence that they give rise to any somatic cell types during normal development. By contrast, EG cells, like ES cells, contribute widely to chimaeras, including colonisation of the germline, following blastocyst injection (Labosky et al., 1994; Stewart et al., 1994). The derivation of EG cells has therefore been widely considered as a reprogramming phenomenon (Durcova-Hills et al., 2008; Kimura and Nakano, 2011).

Recent models of germline specification propose that PGCs are themselves reprogrammed from epiblast cells that have embarked on a path of somatic differentiation (Hayashi and Surani, 2009b; Kurimoto et al., 2008; Yabuta et al., 2006). The implication of these models is that the link between pluripotency and the germline is not continuous and, therefore, that the boundary between soma and germ is breached.

Key features of ES cells, EG cells, EpiSCs and PGCs are summarised in Table 1.

**Post-implantation progressions**
Implantation is rapidly followed by organisation of the epiblast into an epithelium, accompanied by the downregulation of naive pluripotency markers, such as Rex1 (Zfp42) and Nanog (Chambers et al., 2003; Nichols and Smith, 2012; Pelton et al., 2002). Changes in the pluripotency transcription factor network are further exemplified by a switch in enhancer usage at the *Oct4* (*Pou5f1*)
A Pluripotent states

![Diagram of the pluripotency cycle]

B In vivo

![Diagram of the pluripotency cycle]

C In vitro

![Diagram of the pluripotency cycle]

Fig. 2. The pluripotency cycle. (A) Schematic summary of different states of pluripotency. (B) Pluripotent states in vivo. Naive pluripotency is established in the pre-implantation epiblast then progresses through a transitional phase from which PGCs are segregated prior to priming for somatic differentiation. PGCs harbour latent pluripotency, which can be revealed during EG cell conversion or teratocarcinogenesis. In vivo they give rise to the gametes and following fertilisation naive pluripotency is re-established in the blastocyst. (C) Pluripotent states in vitro. Naive pluripotency is captured in self-renewing ES cells, which can give rise to a transitory population of epiblast-like cells (EpiLCs) in culture (Hayashi et al., 2011). EpiLCs exhibit similar features to the transitional post-implantation epiblast, including the capacity to give rise to PGCs. These PGCs can form functional gametes in vivo (Hayashi et al., 2011; Hayashi et al., 2012) or EG cells in vitro (Mansour et al., 2012). If not induced to form PGCs, differentiating ES cells can give rise to EpiSCs which self-renew in culture and are pluripotent, but recalltiant to PGC generation (Hayashi et al., 2011).

gene (Yeom et al., 1996). Dissolution of this naive network is likely to be a requirement for ensuing lineage specification (Nichols and Smith, 2012). Indeed, downregulation of naive pluripotency markers is accompanied by the appearance of ‘primed’ markers such as Fgfs, Otx2 and Nodal (Ang et al., 1996; Mesnard et al., 2006). Significantly, however, the central pluripotency factors, Oct4 and Sox2, remain expressed.

Global epigenetic changes also occur post-implantation. DNA methylation is at its nadir during blastocyst development but has increased significantly by E6.5 (Borgel et al., 2010; Monk et al., 1987; Smith et al., 2012). However, the regulatory elements of naive pluripotency genes escape DNA methylation in the post-implantation epiblast, only becoming fully methylated later in development (Bao et al., 2009; Li et al., 2007; Osorno et al., 2012). Conversely, CpG islands associated with late germline genes, such as Dazl and Sycp3, are specifically targeted for methylation and gene silencing (Borgel et al., 2010; Hackett et al., 2012). Preventing inappropriate expression of these genes in somatic tissues may be important to avoid tumorigenesis (Simpson et al., 2005). Subsequently, PGC development is uniquely associated with extensive loss of DNA methylation (Hajkova, 2011; Seisenberger et al., 2012). This allows later germline factors to be expressed, constituting an elegant control system. DNA demethylation is also essential to erase imprints and remove epimutations.

Although the early post-implantation epiblast has been reported to give rise to ES cell-like pluripotent stem cells in high-density culture and the presence of leukaemia inhibitory factor (LIF) (see Glossary, Box 1) (Bao et al., 2009), the efficiency and dynamics of this process contrast markedly when compared with derivation from PGCs. Recently, we have shown that more than 20% of single PGCs at E7.5 or E8.5 can generate EG cell lines (Leitch et al., 2013b). This is orders of magnitude more efficient than derivation from post-implantation epiblast, and is more akin to generation of ES cells from naive epiblast cells, which has an efficiency of up to 50% (Nichols et al., 2009) (J. Nichols, personal communication). Furthermore, ES cell derivation from the post-implantation epiblast entails a culture process of 2–4 weeks that is not amenable to tracing of single cells (Bao et al., 2009). It is possible that occasional specification of PGCs in protracted high-density cultures leads to formation of EG cells. Alternatively, sporadic reversion to a naive state may occur. It is difficult to discriminate between these two possibilities because EG cells cannot be definitively distinguished from ES cells other than by their origin, as only some lines exhibit erasure of imprints (Leitch et al., 2013a).

Rapid developmental progressions subsequent to implantation, and the associated downregulation of the key transcription factors that safeguard naive pluripotency, may restrict responsiveness to the stimuli that facilitate generation of ES cells. Chromatin modifications and genome-wide accumulation of DNA methylation might also contribute to resistance to direct ES cell derivation after implantation. Indeed, naive ES cells are characterised by global DNA hypomethylation (Leitch et al., 2013a; Yamaji et al., 2013). Although ES cells cultured in serum exhibit relatively high levels of DNA methylation, there should be selection in culture against de novo methylation of genes important for self-renewal. It might be interesting, therefore, to investigate whether ES cells may be obtained from post-implantation epiblasts that are mutant for de novo DNA methyltransferases (Dnmt3a/b).

Are primed pluripotent cells restricted to a somatic fate?

We have previously described EpiSCs as being in a primed pluripotent state (Nichols and Smith, 2009). EpiSC cultures are heterogeneous (Bernemann et al., 2011; Greber et al., 2010), but the balance of evidence suggests they generally resemble gastrulation-stage epiblast, in which case they might not be competent for germline specification. Certainly, EpiSCs do not respond to the in vitro stimuli that can be used to induce PGCs efficiently from pre-gastrulation epiblast or differentiating ES cells (Hayashi et al., 2011). There is evidence that a small subpopulation of EpiSCs can express some PGC markers, but the ability of such cells to make functional gametes has not been tested (Hayashi and Surani, 2009a). It has also been suggested that EpiSCs might be able to contribute to PGCs when reintroduced to the post-implantation embryo (Huang et al., 2012). However, this is based on alkaline phosphatase staining and further evidence of PGC identity is lacking.
We hypothesise that prior to gastrulation the post-implantation epiblast is in a state of transitional pluripotency (see Glossary, Box 1) (Fig. 2) (Nichols and Smith, 2012; Smith, 2010). During this phase, naive pluripotency dissolves as key transcription factors, including Nanog, Esrrb and Klf4, are downregulated while germ cell competence is progressively enabled (Hayashi et al., 2011). As discussed above, EpiSCs probably approximate to a later stage of epiblast development in which somatic specification is promoted through lineage priming (see Glossary, Box 1) but PGC-forming capacity has been largely or completely lost (Fig. 2). Consistent with this, the global gene expression pattern of EpiSCs differs markedly from that of the pre-gastrulation epiblast and the intermediate stage of ES cell differentiation towards PGCs [called epiblast-like cells (EpiLCs)] (Hayashi et al., 2011).

It would be of great interest to capture the proposed transitional epiblast state as self-renewing cultures. Such cells should be distinct from ES cells but have the capacity to produce both germline and soma. They may also be revertible to the naive ES cell state through environmental manipulation. The in vitro conversion of ES cells into a population called EPL (‘early primitive ectoderm-like’ or ‘early epiblast-like’) cells (Rathjen et al., 1999) may hint that transitional cells could be propagated in culture.

**Reprogramming or segregation?**

Although PGCs are induced prior to gastrulation, the reprogramming model of PGC specification posits that PGCs are recovered from epiblast cells that have embarked upon somatic differentiation (Hayashi and Surani, 2009b). This is based on the detection of transcripts for mesodermal and Hox genes in the epiblast precursors of PGCs (Saitou et al., 2002; Yabuta et al., 2006). However, in a comprehensive single-cell microarray study, the expression profile of the earliest PGC precursors analysed (at E6.5) clusters closely with that of pre-gastrulation epiblast cells (Kurimoto et al., 2008). These cells are *Blimp1*-positive but do not express key markers of somatic lineages. Transcripts for genes such as brachyury (*T*) do appear subsequently in PGC precursors (Kurimoto et al., 2008). However, *T* transcripts can also be readily detected in undifferentiated ES cells (Marks et al., 2012). Moreover, *T* is directly induced by Wnt signalling and is more appropriately considered a positional marker at this early stage rather than a marker of mesodermal differentiation (Tang et al., 1998). Transcriptional upregulation of other mesoderm-associated genes (Kurimoto et al., 2008) may also be a consequence of the location of PGC precursors and their exposure to Wnt and BMP stimuli from the extra-embryonic ectoderm, rather than a reliable indication that they have initiated somatic differentiation. Whether any of these factors are expressed appreciably at the protein level has not been determined. Transient expression of Hox genes in PGCs also does not necessarily denote somatic specification, as they reflect axial identity within the embryo rather than lineage (Pearson et al., 2005).

The most demonstrable ‘differentiated’ feature of early PGCs is the presence in females of an inactive X chromosome (Chuva de Sousa Lopes et al., 2008; Sugimoto and Abe, 2007). In mice, X chromosome reactivation correlates with emergence of naive pluripotency (Mak et al., 2004; Silva et al., 2009). However, it does not follow that X-chromosome inactivation defines loss of pluripotency. Indeed, random X-chromosome inactivation begins soon after implantation in all epiblast cells, not just in PGCs. Thus, the onset of X inactivation marks transitional epiblast. This probably reflects the requirement for dosage compensation to navigate gastrulation (Takagi and Abe, 1990). Although X-chromosome activation status broadly correlates with DNA methylation changes and cell potency during mouse development (Monk, 1990), there is no evidence for a functional association with naive pluripotency and, indeed, this correlation appears to be less apparent in other mammals (Lengner et al., 2010).

Based on the preceding observations, it is questionable whether nascent PGCs initiate functional differentiation towards mesoderm, as has been suggested (Hayashi and Surani, 2009b; Kurimoto et al., 2008; Yabuta et al., 2006). An alternative view is that PGCs might be segregated directly from transitional epiblast. In this scenario, rather than being ‘reprogrammed’, PGCs may be induced from epiblast cells without experiencing productive somatic gene expression. Thus, preservation from somatic differentiation might be a feature of all stages of the germline cycle.

**Preserving pluripotency**

PGC specification is associated with maintained expression of some pluripotency factors, such as *Oct4*, and re-expression of others, including *Nanog* and *Klf2* (Kurimoto et al., 2008). This re-expression correlates with the capacity to give rise to EG cells (Leitch et al., 2013b). The high efficiency with which PGCs isolated from E7.5 embryos can form EG cells using LIF and 2i (Leitch et al., 2013b) suggests that they may retain a latent pluripotency (see Glossary, Box 1). Latency may reflect subjugation of the pluripotency network to germline determination in the absence of LIF or other Stat3-activating stimuli that drive conversion into EG cells in vitro (Leitch et al., 2013b).

As mentioned above, *Bmp4* is the major inductive signal for PGCs. One key target of BMP signalling, *Prdm14*, is known to regulate the expression of some pluripotency genes, such as Sox2, in PGCs (Yamaji et al., 2008). However, expression of other pluripotency genes is *Prdm14* independent (Yamaji et al., 2008).
and it is possible that their expression is stimulated or maintained in part by Bmp4. Blimp1 and Prdm14 also appear to insulate PGCs against somatic differentiation (Magnúsdóttir et al., 2012; Saitou and Yamaji, 2012). Bmp4 has been shown to prevent ES cell differentiation via induction of the Id family of antagonists of basic helix-loop-helix (bHLH) transcription factors (Ying et al., 2003). Thus, it is conceivable that BMP signalling during PGC induction acts not only to promote PGC fate, but also to reinforce resistance to somatic differentiation and preserve latent pluripotency.

**Function of pluripotency genes in the germline**

Although upregulation of pluripotency genes is one of the earliest events during PGC specification, their roles remain unclear. Oct4 and Nanog appear to be necessary for germ cell survival, but their precise functions have not been delineated (Chambers et al., 2007; Kehler et al., 2004; Yamaguchi et al., 2009). Re-expression of the naive pluripotency genes correlates with a reversal in Oct4 enhancer usage (Yeom et al., 1996). The pluripotency network might thus be reinstated to a configuration similar to that in the pre-implantation epiblast and ES cells. One notable difference in PGCs, however, is the lack of expression of the Stat3 target Kitf4 (Ducovcova-Hills et al., 2008). The extent to which the pluripotency network interacts with or is independent of distinct germline determinants, such as Blimp1 (Ohnata et al., 2005) and Tcfap2c (Tfap2c) (Weber et al., 2010), is not known. It will be instructive to determine whether gene targets of the pluripotency network are well-conserved between naive epiblast/ES cells and PGCs, or if there is either selective or extensive rewiring.

Prdm14 is a common component in both pluripotency and germline programmes. The Prdm14 null phenotype indicates that the essential role of Prdm14 is confined to the germline (Yamaji et al., 2008). However, Prdm14 has recently been shown to play a role in ES cells (Yamaji et al., 2013). Loss of Prdm14 renders ES cells prone to differentiation, and knockout cells can only be derived and propagated when differentiation is suppressed using 2i. Furthermore, Prdm14 is highly upregulated in the presence of 2i and drives genome-wide hypomethylation by suppressing the DNA methylation machinery (Leitch et al., 2013a). Although loss of Prdm14 does not seem to impede pre-implantation development, there might be functional redundancy with other Prdm proteins at this stage (Fog et al., 2012). It is also possible that subtle defects in Prdm14 mutants may be masked by the regulative (see Glossary, Box 1) capacity of the early mouse embryo. In PGCs, Prdm14 seems to be crucial for reducing the DNA methylation accrued in the post-implantation epiblast (Yamaji et al., 2008). Thus, a key role of Prdm14 in facilitating maintenance of a naive epigenome appears to be shared between the germline and ES cells. Prdm14 may therefore be considered either as a germline gene with a dual function in pluripotency or as part of the pluripotency network that also drives epigenetic re-setting in PGCs.

The requirement for DNA demethylation for expression of later germline determinants highlights a fundamental difference between commitment to the germline versus somatic lineages. As somatic differentiation progresses, global DNA methylation increases further and is particularly targeted to pluripotency genes (Imamura et al., 2006; Osorno et al., 2012; Smith et al., 2012). Thus, DNA methylation is gained and pluripotency is extinguished. By contrast, germline development is associated with maintenance of the pluripotency network and loss of DNA methylation. This reflects the distinct ultimate fates of germline versus soma: whereas somatic differentiation necessarily closes down alternate lineage choices, silencing of other gene expression programmes in the germline should not be irreversible. Germline cells thus require a unique epigenomic configuration (Hajkova, 2011; Hayashi and Surani, 2009b). We speculate that the pluripotency transcription factor network may ensure stability of gene expression control during the major epigenetic erasure and reprogramming events that occur in PGCs. The dominance of the pluripotency transcription factors is exemplified by molecular reprogramming to create induced pluripotent stem cells (Takahashi and Yamanaka, 2006). The same machinery sustains continued self-renewal of ES cells in a wide-range of culture environments and epigenetic contexts (Bock and Wutz, 2013). It is particularly noteworthy that in the presence of 2i and LIF, ES cells show redistribution of repressive histone modifications (Marks et al., 2012) and low levels of DNA methylation (Leitch et al., 2013a), yet are stable and fully pluripotent.

**Regenerating pluripotency**

Following imprint erasure, germ cells commence sex-specific gametogenesis, culminating in production of two of the most highly specialised differentiated cell types – sperm and egg. Expression of pluripotency genes is not maintained throughout gametogenesis. However, spermatogonial stem cells, which do express some pluripotency factors, can sporadically convert to ES cell-like cells in vitro (Kanatsu-Shinohara et al., 2004). These so-called multipotent germline stem cells can form teratomas and in some cases have been shown to contribute to chimaeras and the germline (Kanatsu-Shinohara et al., 2004; Ko et al., 2009). Artificial activation of the egg allows formation of parthenogenetic blastocysts from which haploid ES cells can be derived (Leeb and Wutz, 2011). Haploid parthenogenetic ES cells are fully competent to contribute to chimaeras and, after diploidisation, to colonise the germline (Leeb et al., 2012). The efficiency with which the gametes give rise to pluripotent cells just a few divisions after fertilisation indicates that the sperm nucleus is also in a permissive state to regenerate pluripotency. Intriguingly, pluripotency genes are not targeted for DNA methylation in either sperm or egg (Imamura et al., 2006; Smallwood et al., 2011). This applies not only to the core pluripotency genes Oct4 and Sox2 but also to most of the naive pluripotency gene regulatory network. A notable exception is the Nanog promoter, which, although unmethylated in the oocyte, is heavily methylated in sperm (Farthing et al., 2008; Imamura et al., 2006). By contrast, although some pluripotency genes play specific roles in other cell types, the pluripotency network as a whole is downregulated and extinguished by DNA methylation in somatic cells (Imamura et al., 2006; Mikkelsen et al., 2008). Significantly, overall DNA methylation levels in sperm are comparable to those in somatic cells (Smith et al., 2012), indicating that pluripotency genes are specifically spared from methylation in the germline. Thus, although the expression of pluripotency genes varies between the different stages of the germline cycle, they are protected from DNA methylation throughout. This suggests that access to pluripotency is maintained even in the highly specialised gametes, and that these cells uniquely achieve specialisation without restriction.

**Conclusion**

The concept of naive pluripotency refers to a cellular state in which all differentiation options are accessible, even if not realised. Germline induction involves co-option of key elements of the pluripotent transcription factor network. We speculate that this may be required to maintain control of gene expression, including preventing functional expression of differentiation determinants,
and thereby to protect PGC identity during profound epigenetic reprogramming events. Thus, the induced germline and naive pluripotency are inextricably linked in both ontogeny and molecular regulation. As a consequence, competence for pluripotency is maintained throughout the germline cycle. We thus propose that the mammalian germline cycle might equally be considered a pluripotency cycle.

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Competing interests statement

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