The mammalian germline as a pluripotency cycle

Harry G. Leitch* and Austin Smith*

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 de-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., 2003). Those cells not allocated to the germline will undergo somatic differentiation and ultimately perish. Entry to the germ cell lineage allows the possibility of establishing a new organism in the next generation, sustaining a germline cycle (see Glossary, Box 1) (Fig. 2B).

The molecular mechanisms for germ cell induction vary between species, but the basic strategy of inducing the germline early in development from pluripotent progenitors is conserved (Extavour and Akam, 2003). In mammals, the pluripotency network is dismantled in the soma and can only be reinstated by artificial reprogramming strategies (Yamanaka and Blau, 2010). By contrast, many pluripotency genes are expressed in PGCs, although it is not clear how they function in these cells, which do not normally exhibit pluripotent behaviour. In this Hypothesis, we re-examine

Wellcome Trust-Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QR, UK.

*Authors for correspondence (hgl21@cam.ac.uk; austin.smith@cscr.cam.ac.uk)

the connection between the germline and pluripotency (see Glossary, Box 1). We discuss the extent to which pluripotency may be maintained in a latent form throughout the germline cycle and consider possible reasons for this. We propose a unifying view of naive pluripotency (see Glossary, Box 1), mammalian PGC specification and the germline cycle.

PGC induction in mice

The mouse is the major model for studying mammalian germ cell induction. In other mammals, including humans, the early development of PGCs appears to follow a similar course (Buehr, 1997; Leitch et al., 2013c), but little is known about the conservation of the underlying mechanisms.

PGC induction commences prior to formation of the primitive streak and initiation of somatic lineage commitment (Lawson et al., 1991; Ohinata et al., 2005). Up until embryonic day (E) 6.25, the whole post-implantation mouse epiblast can be induced to form PGCs *in vitro* (Ohinata et al., 2009). This capacity subsequently diminishes (Hayashi et al., 2011; Ohinata et al., 2009). Exactly how long PGC recruitment continues in the embryo is not certain, although it has not been demonstrated after E7.0 (Ohinata et al., 2009). It is possible that potency to form PGCs is lost prior to germ layer formation.

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

A Preformation

Drosophila oocyte *Drosophila* embryo

B Induction

Mouse embryo
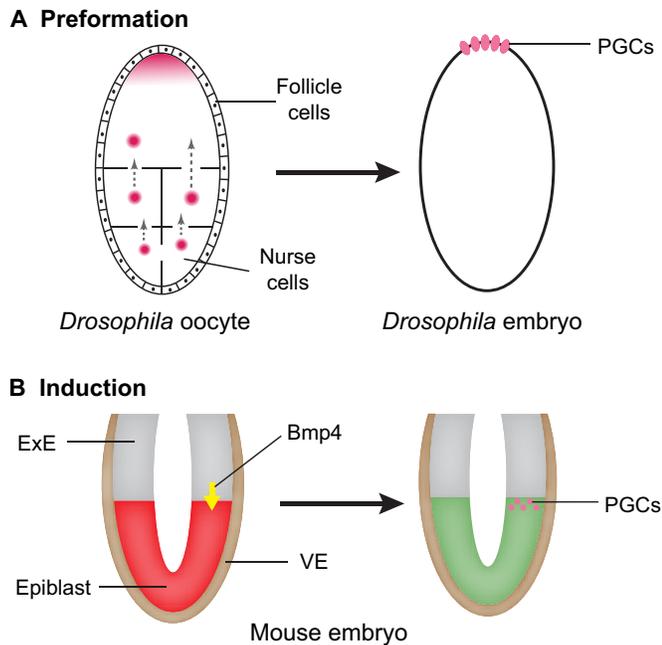
Fig. 1. Preformation and induction models of PGC specification. Schematic representation of the two modes of germ cell specification. (**A**) Preformation, as occurs in *Drosophila*. The nurse cells synthesise RNAs and proteins (pink) that are localised to the posterior pole of the oocyte. This germ 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. Papaioannou 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
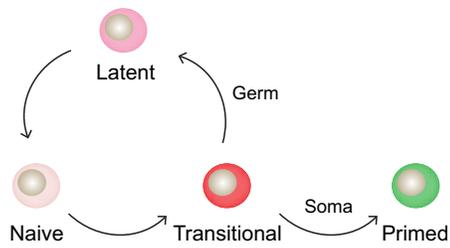
B *In vivo*
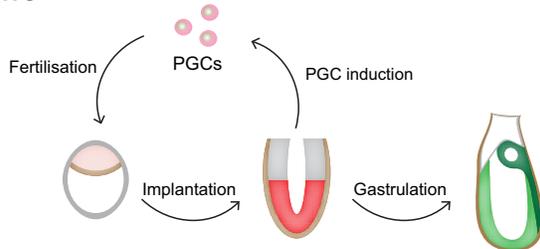
C *In vitro*
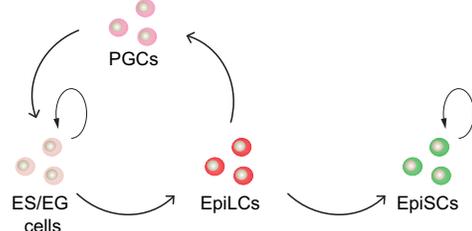
Fig. 2. The pluripotency cycle. (**A**) Schematic summary of different states of pluripotency. (**B**) Pluripotent states *in vivo*. Naive pluripotency is established in the pre-implantation epiblast then progresses through a transitional phase from which PGCs are segregated prior to priming for somatic 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 recalcitrant 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 Fgf5, 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.

Table 1. Diagnostic features of mouse pluripotent and somatic cells

Cell type	Core pluripotency factors* expressed	Naive pluripotency factors[‡] expressed	DNA methylation	Chimaera formation by blastocyst injection	X chromosome status (in females)
ES cell	Yes	Yes	Low[§]	Yes	XaXa
EG cell	Yes	Yes	Low[§]	Yes	XaXa
PGC	Yes	Yes[¶]	Low	No	XaXi → XaXa
EpiSC	Yes[#]	No	?	No	XaXi
Somatic cell	No	No	High	No	XaXi

*Oct4, Sox2, Nanog (co-expression).
[‡]Rex1, Stella, Esrrb, Klf2, Klf4 (co-expression).
[§]In the presence of 2i/LIF.
[¶]Klf4 is not expressed in PGCs.
[#]Sox2 and Nanog are present but low in EpiSCs.

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 (see Glossary, Box 1) suggests that they may retain a latent pluripotency (see Glossary, Box 1). Latency may reflect subjugation of the pluripotency network to germline determination factors in the absence *in vivo* 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 *Klf4* (Durcova-Hills et al., 2008). The extent to which the pluripotency network interacts with or is independent of distinct germline determinants, such as *Blimp1* (Ohinata 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 verus 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.

Competing interests statement
The authors declare no competing financial interests.

References

Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L. and Rossant, J. (1996). A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122**, 243-252.

Bao, S., Tang, F., Li, X., Hayashi, K., Gillich, A., Lao, K. and Surani, M. A. (2009). Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. *Nature* **461**, 1292-1295.

Bernemann, C., Greber, B., Ko, K., Sterneckert, J., Han, D. W., Araúzo-Bravo, M. J. and Schöler, H. R. (2011). Distinct developmental ground states of epiblast stem cell lines determine different pluripotency features. *Stem Cells* **29**, 1496-1503.

Bock, C. and Wutz, A. (2013). DNA methylation: a matter of culture. *Nat. Struct. Mol. Biol.* **20**, 249-251.

Borgel, J., Guibert, S., Li, Y., Chiba, H., Schübeler, D., Sasaki, H., Forné, T. and Weber, M. (2010). Targets and dynamics of promoter DNA methylation during early mouse development. *Nat. Genet.* **42**, 1093-1100.

Bradley, A., Evans, M., Kaufman, M. H. and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255-256.

Brons, I. G. M., Smithers, L. E., Trotter, M. W. B., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A. et al. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191-195.

Brook, F. A. and Gardner, R. L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc. Natl. Acad. Sci. USA* **94**, 5709-5712.

Buehr, M. (1997). The primordial germ cells of mammals: some current perspectives. *Exp. Cell Res.* **232**, 194-207.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643-655.

Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L. and Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature* **450**, 1230-1234.

Chuva de Sousa Lopes, S. M., Hayashi, K., Shovlin, T. C., Mifsud, W., Surani, M. A. and McLaren, A. (2008). X chromosome activity in mouse XX primordial germ cells. *PLoS Genet.* **4**, e30.

Durcova-Hills, G., Adams, I. R., Barton, S. C., Surani, M. A. and McLaren, A. (2006). The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. *Stem Cells* **24**, 1441-1449.

Durcova-Hills, G., Tang, F., Doody, G., Tooze, R. and Surani, M. A. (2008). Reprogramming primordial germ cells into pluripotent stem cells. *PLoS ONE* **3**, e3531.

Extavour, C. G. and Akam, M. (2003). Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* **130**, 5869-5884.

Farthing, C. R., Ficz, G., Ng, R. K., Chan, C.-F., Andrews, S., Dean, W., Hemberger, M. and Reik, W. (2008). Global mapping of DNA methylation in mouse promoters reveals epigenetic reprogramming of pluripotency genes. *PLoS Genet.* **4**, e1000116.

Fog, C. K., Galli, G. G. and Lund, A. H. (2012). PRDM proteins: important players in differentiation and disease. *Bioessays* **34**, 50-60.

Gardner, R. L. and Rossant, J. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. *J. Embryol. Exp. Morphol.* **52**, 141-152.

Gardner, R. L., Lyon, M. F., Evans, E. P. and Burtenshaw, M. D. (1985). Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo. *J. Embryol. Exp. Morphol.* **88**, 349-363.

Greber, B., Wu, G., Bernemann, C., Joo, J. Y., Han, D. W., Ko, K., Tapia, N., Sabour, D., Sterneckert, J., Tesar, P. et al. (2010). Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* **6**, 215-226.

Hackett, J. A., Reddington, J. P., Nestor, C. E., Dunican, D. S., Branco, M. R., Reichmann, J., Reik, W., Surani, M. A., Adams, I. R. and Meehan, R. R. (2012). Promoter DNA methylation couples genome-defence mechanisms to epigenetic reprogramming in the mouse germline. *Development* **139**, 3623-3632.

Hajkova, P. (2011). Epigenetic reprogramming in the germline: towards the ground state of the epigenome. *Philos. Trans. R. Soc. B* **366**, 2266-2273.

Hayashi, K. and Surani, M. A. (2009a). Self-renewing epiblast stem cells exhibit continual delineation of germ cells with epigenetic reprogramming in vitro. *Development* **136**, 3549-3556.

Hayashi, K. and Surani, M. A. (2009b). Resetting the epigenome beyond pluripotency in the germline. *Cell Stem Cell* **4**, 493-498.

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, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H. and Saitou, M. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science* **338**, 971-975.

Huang, Y., Osorno, R., Tsakiridis, A. and Wilson, V. (2012). In vivo differentiation potential of epiblast stem cells revealed by chimeric embryo formation. *Cell Rep.* **2**, 1571-1578.

Imamura, M., Miura, K., Iwabuchi, K., Ichisaka, T., Nakagawa, M., Lee, J., Kanatsu-Shinohara, M., Shinohara, T. and Yamanaka, S. (2006). Transcriptional repression and DNA hypermethylation of a small set of ES cell marker genes in male germline stem cells. *BMC Dev. Biol.* **6**, 34.

Johnson, A. D., Crother, B., White, M. E., Patient, R., Bachvarova, R. F., Drum, M. and Masi, T. (2003). Regulative germ cell specification in axolotl embryos: a primitive trait conserved in the mammalian lineage. *Philos. Trans. R. Soc. B* **358**, 1371-1379.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S. et al. (2004). Generation of pluripotent stem cells from neonatal mouse testis. *Cell* **119**, 1001-1012.

Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomelí, H., Nagy, A., McLaughlin, K. J., Schöler, H. R. et al. (2004). Oct4 is required for primordial germ cell survival. *EMBO Rep.* **5**, 1078-1083.

Kimura, T. and Nakano, T. (2011). Induction of pluripotency in primordial germ cells. *Histol. Histopathol.* **26**, 643-650.

Ko, K., Tapia, N., Wu, G., Kim, J. B., Bravo, M. J. A., Sasse, P., Glaser, T., Ruau, D., Han, D. W., Greber, B. et al. (2009). Induction of pluripotency in adult unipotent germline stem cells. *Cell Stem Cell* **5**, 87-96.

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.

Labosky, P. A., Barlow, D. P. and Hogan, B. L. (1994). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* **120**, 3197-3204.

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.

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.

Leeb, M. and Wutz, A. (2011). Derivation of haploid embryonic stem cells from mouse embryos. *Nature* **479**, 131-134.

Leeb, M., Walker, R., Mansfield, B., Nichols, J., Smith, A. and Wutz, A. (2012). Germline potential of parthenogenetic haploid mouse embryonic stem cells. *Development* **139**, 3301-3305.

Leitch, H. G., McEwen, K. R., Turp, A., Encheva, V., Carroll, T., Grabole, N., Mansfield, W., Nashun, B., Knezovich, J. G., Smith, A. et al. (2013a). Naive pluripotency is associated with global DNA hypomethylation. *Nat. Struct. Mol. Biol.* **20**, 311-316.

Leitch, H. G., Nichols, J., Humphreys, P., Mulas, C., Martello, G., Lee, C., Jones, K., Surani, M. A. and Smith, A. (2013b) Rebuilding pluripotency from primordial germ cells. *Stem Cell Reports* doi: 10.1016/j.stemcr.2013.03.004 (in press).

Leitch, H. G., Tang, W. W. C. and Surani, M. (2013c). Primordial germ-cell development and epigenetic reprogramming in mammals. *Curr. Top. Dev. Biol.* **104**, 149-187.

Lengner, C. J., Gimelbrant, A. A., Erwin, J. A., Cheng, A. W., Guenther, M. G., Welstead, G. G., Alagappan, R., Frampton, G. M., Xu, P., Muffat, J. et al.

(2010). Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. *Cell* **141**, 872-883.

Li, J. Y., Pu, M. T., Hirasawa, R., Li, B. Z., Huang, Y. N., Zeng, R., Jing, N. H., Chen, T., Li, E., Sasaki, H. et al. (2007). Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog. *Mol. Cell. Biol.* **27**, 8748-8759.

Magnúsdóttir, E., Gillich, A., Grabole, N. and Surani, M. A. (2012). Combinatorial control of cell fate and reprogramming in the mammalian germline. *Curr. Opin. Genet. Dev.* **22**, 466-474.

Mak, W., Nesterova, T. B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A. P. and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. *Science* **303**, 666-669.

Mansour, A. A., Gafni, O., Weinberger, L., Zviran, A., Ayyash, M., Rais, Y., Krupalnik, V., Zerbib, M., Amann-Zalcenstein, D., Maza, I. et al. (2012). The H3K27 demethylase Utx regulates somatic and germ cell epigenetic reprogramming. *Nature* **488**, 409-413.

Marks, H., Kalkan, T., Menafra, R., Denissov, S., Jones, K., Hofemeister, H., Nichols, J., Kranz, A., Stewart, A. F., Smith, A. et al. (2012). The transcriptional and epigenomic foundations of ground state pluripotency. *Cell* **149**, 590-604.

Matsui, Y., Zsebo, K. and Hogan, B. L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841-847.

McLaren, A. and Lawson, K. A. (2005). How is the mouse germ-cell lineage established? *Differentiation* **73**, 435-437.

Mesnard, D., Guzman-Ayala, M. and Constam, D. B. (2006). Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. *Development* **133**, 2497-2505.

Mikkelsen, T. S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B. E., Jaenisch, R., Lander, E. S. and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. *Nature* **454**, 49-55.

Monk, M. (1990). Changes in DNA methylation during mouse embryonic development in relation to X-chromosome activity and imprinting. *Philos. Trans. R. Soc. B* **326**, 299-312.

Monk, M., Boubelik, M. and Lehnert, S. (1987). Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99**, 371-382.

Nichols, J. and Smith, A. (2009). Naive and primed pluripotent states. *Cell Stem Cell* **4**, 487-492.

Nichols, J. and Smith, A. (2012). Pluripotency in the embryo and in culture. *Cold Spring Harb. Perspect. Biol.* **4**, a008128.

Nichols, J., Silva, J., Roode, M. and Smith, A. (2009). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* **136**, 3215-3222.

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.

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.

Osorno, R., Tsakiridis, A., Wong, F., Cambray, N., Economou, C., Wilkie, R., Blin, G., Scotting, P. J., Chambers, I. and Wilson, V. (2012). The developmental dismantling of pluripotency is reversed by ectopic Oct4 expression. *Development* **139**, 2288-2298.

Pearson, J. C., Lemons, D. and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. *Nat. Rev. Genet.* **6**, 893-904.

Pelton, T. A., Sharma, S., Schulz, T. C., Rathjen, J. and Rathjen, P. D. (2002). Transient pluripotent cell populations during primitive ectoderm formation: correlation of in vivo and in vitro pluripotent cell development. *J. Cell Sci.* **115**, 329-339.

Rathjen, J., Lake, J. A., Bettess, M. D., Washington, J. M., Chapman, G. and Rathjen, P. D. (1999). Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. *J. Cell Sci.* **112**, 601-612.

Resnick, J. L., Bixler, L. S., Cheng, L. and Donovan, P. J. (1992). Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550-551.

Rossant, J., Gardner, R. L. and Alexandre, H. L. (1978). Investigation of the potency of cells from the postimplantation mouse embryo by blastocyst injection: a preliminary report. *J. Embryol. Exp. Morphol.* **48**, 239-247.

Saitou, M. and Yamaji, M. (2012). Primordial germ cells in mice. *Cold Spring Harb. Perspect. Biol.* **4**, a008375.

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.

Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W. and Reik, W. (2012). The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Mol. Cell* **48**, 849-862.

Silva, J., Nichols, J., Theunissen, T. W., Guo, G., van Oosten, A. L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I. and Smith, A. (2009). Nanog is the gateway to the pluripotent ground state. *Cell* **138**, 722-737.

Simpson, A. J. G., Caballero, O. L., Jungbluth, A., Chen, Y.-T. and Old, L. J. (2005). Cancer/testis antigens, gametogenesis and cancer. *Nat. Rev. Cancer* **5**, 615-625.

Smallwood, S. A., Tomizawa, S.-I., Krueger, F., Ruf, N., Carli, N., Segonds-Pichon, A., Sato, S., Hata, K., Andrews, S. R. and Kelsey, G. (2011). Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat. Genet.* **43**, 811-814.

Smith, A. (2010). Pluripotent stem cells: private obsession and public expectation. *EMBO Mol. Med.* **2**, 113-116.

Smith, Z. D., Chan, M. M., Mikkelsen, T. S., Gu, H., Gnirke, A., Regev, A. and Meissner, A. (2012). A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature* **484**, 339-344.

Stewart, C. L., Gadi, I. and Bhatt, H. (1994). Stem cells from primordial germ cells can reenter the germ line. *Dev. Biol.* **161**, 626-628.

Sugimoto, M. and Abe, K. (2007). X chromosome reactivation initiates in nascent primordial germ cells in mice. *PLoS Genet.* **3**, e116.

Takagi, N. and Abe, K. (1990). Detrimental effects of two active X chromosomes on early mouse development. *Development* **109**, 189-201.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.

Tam, P. P. and Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev. Biol.* **178**, 124-132.

Tang, S. J., Hoodless, P. A., Lu, Z., Breitman, M. L., McInnes, R. R., Wrana, J. L. and Buchwald, M. (1998). The Tlx-2 homeobox gene is a downstream target of BMP signalling and is required for mouse mesoderm development. *Development* **125**, 1877-1887.

Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L. and McKay, R. D. G. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196-199.

Weber, S., Eckert, D., Nettersheim, D., Gillis, A. J. M., Schäfer, S., Kuckenberg, P., Ehlermann, J., Werling, U., Biermann, K., Looijenga, L. H. J. et al. (2010). Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. *Biol. Reprod.* **82**, 214-223.

Yabuta, Y., Kurimoto, K., Ohinata, Y., Seki, Y. and Saitou, M. (2006). Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. *Biol. Reprod.* **75**, 705-716.

Yamaguchi, S., Kurimoto, K., Yabuta, Y., Sasaki, H., Nakatsuji, N., Saitou, M. and Tada, T. (2009). Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells. *Development* **136**, 4011-4020.

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. Genet.* **40**, 1016-1022.

Yamaji, M., Ueda, J., Hayashi, K., Ohta, H., Yabuta, Y., Kurimoto, K., Nakato, R., Yamada, Y., Shirahige, K. and Saitou, M. (2013). PRDM14 ensures naive pluripotency through dual regulation of signaling and epigenetic pathways in mouse embryonic stem cells. *Cell Stem Cell* **12**, 368-382.

Yamanaka, S. and Blau, H. M. (2010). Nuclear reprogramming to a pluripotent state by three approaches. *Nature* **465**, 704-712.

Yeom, Y. I., Fuhrmann, G., Ovitt, C. E., Brehm, A., Ohbo, K., Gross, M., Hübner, K. and Schöler, H. R. (1996). Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* **122**, 881-894.

Ying, Q. L., Nichols, J., Chambers, I. and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281-292.