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Germ cell specification in mice: Signalling, transcription regulation, and epigenetic consequences

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Summary

The specification of germ cell fate in development initiates mechanisms essential for the perpetuation of genetic information across the generations. Recent studies in mice have shown that germ cell specification requires at least three key molecular/cellular events: repression of the somatic program, re-acquisition of potential pluripotency, and an ensuing genome-wide epigenetic reprogramming. Moreover, a signalling and transcriptional principle governing these processes has been identified, raising the possibility of inducing the germ cell fate precisely from pluripotent stem cells in culture. These advances will in turn serve as a basis to explore the mechanism of germ cell specification in other mammals, including humans. The recapitulation of germ cell development in humans in culture will provide unprecedented opportunities to understand the basis of the propagation of our genome, both under normal and diseased conditions.

Introduction

The germ cell lineage is the only lineage that ensures the perpetuation and diversification of the genetic information across the generations in most multicellular organisms. The specification of germ cell fate, which establishes a group of cells as primordial germ cells (PGCs), the origin of both oocytes and spermatozoa, initiates essential events required for this process, and therefore is fundamental to development, reproduction, and heredity. There appear to exist at least two pathways to the specification of germ cell fate: In one pathway, which is called 'preformation', embryonic cells that inherit maternal determinants from the egg go on to form the germ cell lineage, whereas in the other, which is called 'epigenesis', pluripotent cells formed early in development are induced by signals from adjacent tissues to form the germ cell lineage. In mice, and presumably in all mammals, germ cell fate is induced by 'epigenesis' (Saitou 2009b) (Figure 1). Interestingly, however, in both pathways, the repression of the somatic program manifests as a key event, although the molecules underpinning this event are markedly divergent among organisms (Extavour & Akam 2003, Seydoux & Braun 2006). Furthermore, it has become increasingly evident that specified PGCs depend on some of the conserved molecules for their proliferation/survival and further development, indicating the presence of common requirements for this lineage across essentially all the species (Extavour & Akam 2003, Seydoux & Braun 2006).

In this article, I will provide a brief summary of recent advances in our knowledge of the mechanism of germ cell specification in mice, with particular emphasis on signalling and transcription regulation, and their epigenetic consequences. Evidence shows that, consistent with the function of the germ cell lineage as the transducer of genetic information, PGC specification is an integration of at least three key events: repression of

the somatic program, re-acquisition of potential pluripotency, and an ensuing genome-wide epigenetic reprogramming (Saitou 2009a). More detailed knowledge of the mechanism of PGC specification will provide a critical foundation for induction of the germ cell lineage from pluripotent stem cells in vitro not only in mice but also in other mammals, including humans. These advances will in turn lead to a more detailed mechanistic understanding of germ cell specification (e.g., biochemical analysis of the mechanisms involved in PGC specification), as well as advances in reproductive and regenerative medicine.

Transcriptional regulation for PGC specification

PGCs in mice have been shown to originate from some of the most proximal epiblast cells, to become identifiable during early gastrulation by their characteristic alkaline phosphatase (AP) activity, and to form a distinct AP-positive cluster with approximately 40 cells at the base of the incipient allantois in the extraembryonic mesoderm (ExM) at around embryonic day (E) 7.25 [early/mid-bud (E/MB) stage] (Ginsburg *et al.* 1990, Lawson & Hage 1994). Subsequently, and concomitant with an increase in their number, they start to migrate one by one toward the developing hindgut endoderm and move through it. They then exit the endoderm to appear in the mesentery, and at around E10.5 colonize the embryonic gonads, where they proliferate further and initiate a differentiation either into oocytes or spermatozoa depending on their and somatic sexes (Bowles & Koopman 2007).

The precise origin of the germ cell lineage and the mechanism of PGC specification have long been elusive; this has mainly been due to the lack of specific markers that delineate the earliest processes of germ cell specification. For example, it has been known that Oct4, a key gene for pluripotency (Niwa 2007), serves as a specific marker for the germ cell lineage, but this is only true after ~E7.75 (Yeom *et al.* 1996), much later than PGC specification (PGC specification occurs earlier than E7.0). A series of studies based on single-cell gene expression analysis, including single-cell cDNA microarray analysis, have identified key regulators and global transcription events associated with PGC specification (Saitou *et al.* 2002, Ohinata *et al.* 2005, Yabuta *et al.* 2006, Kurimoto *et al.* 2008).

B-lymphocyte induced maturation protein 1 (Blimp1, also known as Prdm1), a PR (PRDI-BF1 and RIZ) domain-containing transcriptional regulator, has long been known as a "master regulator" of plasma cell differentiation from B-cells (Calame *et al.* 2003), and has more recently been shown to mark the origin of the germ cell lineage in the epiblast (Ohinata *et al.* 2005). *Blimp1* begins to be expressed in a subset of the most proximal posterior epiblast cells at around E6.25 [pre/no-streak (P/0S) stage]. The *Blimp1*-expressing cells increase in number, move posteriorly, and develop into PGCs with AP activity, stella expression, and Hox gene repression at around E7.25 (Figure 1

and 2).

Blimp1-positive cells initially express the Hox genes as well as many genes known to be involved in embryonic development, especially pattern specification and mesoderm induction (including genes such as *Hoxa1*, *Hoxa2*, *Hoxa3*, *Hoxb1*, *Hoxb2*, *Hoxd1*, *Hoxd9*, *Sna1*, *Tbx3*, *Tbx6*, *Mesp1*, *Sp5*, *Mixl1*, *Sall3*, *Cdx1*, *Isl1*, *Ets1*, *Etv2*, *Nfkbia*, *Foxf1*, *Plxna2*, and *Smad7*), but subsequently down-regulate them from around E6.75-E7.0 [late-streak/no-bud (LS/0B) stages] (Yabuta *et al.* 2006, Kurimoto *et al.* 2008). Conversely, *Blimp1*-positive cells initially down-regulate the expression of genes associated with pluripotency, including Sox2, *Nanog* and *Zic3*, but regain them from around the LS/0B stage (Yabuta *et al.* 2006, Kurimoto *et al.* 2008). Therefore, the PGC precursors appear to be initially propelled toward a somatic mesodermal fate, but then regain their potentially pluripotent nature.

At the E/MB stages, compared to their somatic neighbours, which would share a common origin, PGCs up- and down-regulate ~500 and ~330 genes ("germ cell specification" and "somatic program" genes), respectively (Kurimoto *et al.* 2008). Accordingly, the specification genes include those for 'germ cell development' in the Gene Ontogeny (GO) functional annotation (*Blimp1*, *stella*, *fragilis*, *Dnd1*, *Kit*). They do not include, however, a particularly large number of genes in the other functional categories, suggesting that the PGC-specific genes consist of a unique set of genes. The specification genes include transcriptional regulators such as *Blimp1*, *Prdm14*, *Sox3*, *Tcfap2c*, *Elf3*, *Elk1*, *Isl2*, *Mycn*, *Klf2*, *Fiat*, *Sp8*, *Smad3*, *Sox2*, *Nanog*, *Zic3*, *Tcfe3*, *Epc1*, *Six4*, and *Eya3*, some of which should have critical functions in PGC specification (see below). In addition to the genes associated with pattern specification and mesoderm development, the somatic genes include many cell-cycle-associated genes, such as S-phase promoting factors (e.g., *Ccne1*, *Ccnd1*, *Cdc25a*, *Cdc6*, *Pold2*, *E2F3*, and *Myc*) and regulators of DNA methylation (e.g., *de novo* DNA methyltransferases (*Dnmt3a*, *Dnmt3b*) and *Uhrf1*, the essential factor for the recruitment of *Dnmt1* to replication foci) (Bostick *et al.* 2007, Sharif *et al.* 2007). The precise gene expression signature for the established PGCs provides a basis for the analysis of the function of each gene up- or down-regulated in PGCs.

Key regulators for PGC specification

Most notably, Blimp1 has been found to be one of the most critical regulators for PGC specification (Ohinata *et al.* 2005, Vincent *et al.* 2005, Kurimoto *et al.* 2008) (Figure 2). In the absence of Blimp1 activity, AP-positive PGC-like cells are formed, but they are smaller in number, form aberrant aggregates, and do not show migration toward the hindgut endoderm. Single-cell cDNA microarray analysis has shown that the *Blimp1*-deficient PGC-like cells fail to repress almost all the somatic genes (Kurimoto *et al.* 2008). In contrast, while they exhibit severe impairment in the up-regulation of highly PGC-specific genes, they more or less up-regulate approximately half of the specification

genes (~250) (Kurimoto *et al.* 2008). However, a rigorous quantitative comparison of single-cell gene expression levels and frequencies of the specification genes between the wild-type and *Blimp1*-deficient cells shows that Blimp1 does exert positive and quantitative effects on the up-regulation and concerted expression of many of the highly PGC-specific genes (Kurimoto *et al.* 2008). Consistently, the effect of *Blimp1* on PGC specification is dose-dependent, with fewer PGCs specified in its heterozygous mutants (Ohinata *et al.* 2005, Vincent *et al.* 2005, Robertson *et al.* 2007). These observations indicate that, while Blimp1 is essential for repressing all the somatic genes, it may also be important for creating an epigenetic state for the expression of highly PGC-specific genes.

Prdm14, another PR-domain containing protein, is also a critical regulator for PGC specification (Yamaji *et al.* 2008). *Prdm14* is evolutionally conserved at least in vertebrates. It has been reported that in humans, *Prdm14* is up-regulated in some cancers (Hu *et al.* 2005, Nishikawa *et al.* 2007) and also in ES cells (Assou *et al.* 2007, Tsuneyoshi *et al.* 2008). In mice, Prdm14 starts to be expressed in the morula and continues to be expressed in the inner mass cells of the blastocysts, but this expression is transient and disappears quickly by ~E5.0. Subsequently, its expression re-initiates specifically in PGC precursors at around E6.5 (P/ES stage) and continues exclusively in PGCs until about ~E13.5-E14.5 both in males and females. In contrast to *Blimp1*, *Prdm14* does not appear to be expressed in any cell types other than PGCs. In the absence of Prdm14, AP-positive PGC-like cells are formed, and they repress the Hox genes, but fail to up-regulate Sox2 (Yamaji *et al.* 2008). They also fail to undergo proper genome-wide epigenetic reprogramming (see below), partly due to a failure to repress Glp, and eventually disappear perhaps due to the failure to proliferate. A global transcriptome analysis of *Prdm14*-deficient PGC-like cells shows that they acquire characteristics somewhat similar to *Blimp1*-positive, *Hoxb1*-negative LS/0B stage cells but do not fully mature into PGCs at the E/MB stage (M.S., unpublished observation). Importantly, the initial expression of *Prdm14* in PGCs is Blimp1-independent, but its subsequent maintenance and/or up-regulation is strictly dependent on Blimp1. Thus, Blimp1 and Prdm14 are the two major transcriptional regulators orchestrating the birth of the germ cell lineage in mice (Figure 2).

Transcription factor Tcfap2c (also known as AP2γ), a member of the five closely related transcription factors bearing a characteristic basic helix-span-helix domain (Eckert *et al.* 2005), also plays a key role for PGC specification (Weber *et al.* 2010). Tcfap2c seems to be expressed maternally and is detected in all the cells at least up to the blastocyst stage but subsequently becomes confined to the extraembryonic ectoderm and the PGCs (Auman *et al.* 2002, Werling & Schorle 2002). Epiblast-specific deletion of *Tcfap2c* shows that, in the absence of *Tcfap2c*, AP-positive PGC-like cells appear to be formed normally, but their numbers become drastically reduced by E8.0 and they do not show

characteristic migration toward the hindgut endoderm (Weber *et al.*), a phenotype similar to that in *Blimp1*-deficient embryos. Since *Blimp1* expression precedes *Tcfap2c* and *Blimp1* deletion leads to the loss of *Tcfap2c* up-regulation, Tcfap2c would be a critical downstream target of Blimp1 for PGC specification (Figure 2).

It has recently been suggested that the RNA-binding protein Lin28 may play a role as an upstream regulator for Blimp1 (West *et al.* 2009). Lin28 is an RNA-binding protein bearing a cold-shock domain and two retroviral-type zinc finger domains of the CCHC type, and has been shown to inhibit the processing and maturation of *let-7* miRNA family members (for review see (Bussing *et al.* 2008)). Notably, *LIN28* is one of four genes (the others being *OCT4*, *SOX2*, and *NANOG*) that together suffice to reprogram human fibroblast cells into induced pluripotent stem (iPS) cells (Yu *et al.* 2007), although the role of LIN28 in this process has yet to be determined. On the other hand, in pathologic Reed-Sternberg cells, let-7a is highly expressed and suppresses BLIMP1 by binding to a target site in the BLIMP1 3' UTR (Nie *et al.* 2008). Using an in vitro ES-cell differentiation strategy, a study by West et al. showed that *Lin28* is an essential regulator of PGC specification through inhibition of *let-7* maturation and consequent induction of *Blimp1* (West *et al.* 2009) (Figure 2). The precise expression of Lin28 in the epiblast and in the initial phase of PGC specification and whether Lin28 is indeed critical in PGC specification in vivo remain to be determined.

There are a number of genes that are known to play critical roles in the early phase of PGC development, presumably after the PGC fate is established (for review see (Saitou 2009b)). It has long been known that the receptor tyrosine kinase Kit and its ligand stem cell factor (SCF) regulate the migration, proliferation and/or survival of PGCs (Mintz & Russell 1957, McCoshen & McCallion 1975, Buehr *et al.* 1993, Gu *et al.* 2009), presumably through the AKT/mTOR/Bax signalling pathway (De Miguel *et al.* 2002, Stallock *et al.* 2003, Runyan *et al.* 2006). Similarly, mutations of RNA-binding proteins such as Nanos3, Tial1, and Dnd have been known to lead to the loss of germ cells due to the failure of survival and/or proliferation of PGCs (Beck *et al.* 1998, Tsuda *et al.* 2003, Youngren *et al.* 2005). It is also of note that hypoxia-inducible factor-2a (Hif2a) has been shown to play a critical role for PGC specification and/or early PGC development, presumably by regulating Oct4 expression in PGCs (Covello *et al.* 2006). The precise mechanisms of the actions of these molecules in PGCs and the modes of their regulation by the key specification genes, such as Blimp1 and Prdm14, remain to be investigated. It will also be important to clarify the precise roles of key pluripotency genes such as Oct, Sox2, and Nanog for PGC specification (Okamura *et al.* 2008), although Oct4 and Nanog have been shown to be critical to the survival of PGCs at relatively later stages, i.e., ~E9.5 (Kehler *et al.* 2004, Chambers *et al.* 2007, Yamaguchi *et al.* 2009).

Epigenetic reprogramming following PGC specification

PGCs have been shown to undergo extensive epigenetic reprogramming, including genome-wide DNA demethylation, erasure of parental imprints, and re-activation of the inactive X-chromosome (for review see (Sasaki & Matsui 2008)). However, the mechanisms underlying these events have long been elusive. A precise understanding of the molecular mechanisms leading to PGC specification, the crucial onset of the entire epigenetic reprogramming process in the germ cell lineage, will be key to addressing this issue (Figure 2 and 3).

It has been shown that *Blimp1*-positive PGC precursors at around E6.75 [early/mid-streak (E/MS) stage] bear genome-wide epigenetic modifications [di- and tri-methylation of H3 lysine4 (H3K4me2 and me3) and acetylation of H3 lysine9 (H3K9ac) (active modifications), mono-, di-, and tri-methylation of H3 lysine9 (H3K9me1, me2, and me3), and di- and tri-methylation of H3 lysine 27 (H3K27me2 and me3) (repressive modifications)] apparently indistinguishable from their somatic neighbours (Seki *et al.* 2007). Subsequently, however, from around E7.75 onwards, PGCs that have started their migration begin to show the genome-wide reduction of the two major repressive modifications, DNA methylation and H3K9me2 (Seki *et al.* 2005, Seki *et al.* 2007). Although the mode of reduction of genome-wide DNA methylation in migrating PGCs has not yet been precisely analyzed, the global reduction of H3K9me2 in migrating PGCs seems to be a progressive, cell-by-cell process, with nearly all the PGCs exhibiting low H3K9me2 levels by E8.75 (Seki *et al.* 2007).

As discussed earlier, upon PGC specification, Blimp1 and Prdm14 are directly or indirectly involved in the repression of key machineries for both de novo and maintenance DNA methylation, and in the repression of the histone methyltransferase Glp, an essential enzyme for genome-wide H3K9me1 and me2 in embryonic development (Tachibana *et al.* 2005). On the other hand, although some of the JmjC domain-containing histone lysine demethylases (see review, (Klose *et al.* 2006, Shi 2007)) are indeed expressed in PGCs, none of them are specific to PGCs (i.e., similarly expressed in somatic neighbours). These observations support the idea that the specific repression of active enzymes in PGCs may be a key to understanding the genome-wide reduction of DNA methylation and H3K9me2. Since there is experimental evidence that supports the presence of active mechanisms for the erasure of epigenetic modifications (Hajkova *et al.* 2002, Seki *et al.* 2007), there should exist some intricate interplay between active and passive mechanisms [DNA methylation being reduced passively by cell division, especially after E9.0 (see below), and H3K9me1/2 being reduced either through a turnover of methyl groups or a replacement of the entire H3 molecule] in PGCs for the epigenetic reprogramming.

On the other hand, genome-wide H3K27me3, another repressive modification mediated by the polycomb repressive complex 2 (PRC2), becomes up-regulated in migrating PGCs

from around E8.25 onwards in a progressive, cell-by-cell manner, with nearly all the PGCs showing high H3K27me3 levels by E9.5 (Seki *et al.* 2005, Seki *et al.* 2007). Ezh2, Eed and Suz12, the three core components of the PRC2 complex that mediate the tri-methylation of H3K27 (Cao & Zhang 2004), are expressed at similar levels both in the PGCs and their somatic neighbours by at least E8.25 (Yabuta *et al.* 2006, Kurimoto *et al.* 2008). It may therefore be the case that the preceding reduction of DNA methylation and H3K9me1/2 would play a role in the up-regulation of H3K27me3.

Notably, a majority of migrating PGCs (~60%) from around E8.0 to around E9.0, a key period of epigenetic reprogramming, are shown to be in the G2 phase of the cell cycle (Seki *et al.* 2007). Blimp1 may be a key factor for the G2 arrest, since it is involved in the repression of S-phase promoting factors (e.g., *Ccne1*, *Ccnd1*, *Cdc25a*, *Cdc6*, *Pold2*, *E2F3*, and *Myc*) (see above) (Kurimoto *et al.* 2008)). Moreover, concomitant with this period, PGCs seem to transiently pause their global transcription by RNA polymerase II (RNAPII) (Seki *et al.* 2007). These observations indicate that PGC specification creates a unique cellular state for the epigenetic reprogramming: PGCs repress key active epigenetic enzymes, are arrested at the G2 phase of the cell cycle, and pause Pol II-based transcription.

Furthermore, it has been reported that migrating PGCs up-regulate the symmetrical di-methylation of histone H4 arginine3 (H4R3me2), which appears to be conferred by a protein arginine methyltransferase, Prmt5 (Ancelin *et al.* 2006). Prmt5 catalyzes ω-N^G, N'^G-symmetric di-methylation of arginine residues, in a variety of target proteins, including histone H4, H2A, and H3, and the spliceosomal proteins SmD1, SmD3, and SmB/B' (Bedford & Richard 2005). Ancelin et al. have shown that Blimp1 forms a complex with Prmt5 in cultured cell lines and co-localizes with Prmt5 in the nuclei of migrating PGCs. The co-localization of Blimp1 and Prmt5 in the PGC nuclei appears to be seen only between E8.5 and E10.5, and after E11.5, both proteins seem to translocate into the cytoplasm, coincident with the down-regulation of H4R3me2 in the PGC nuclei. The function of Prmt5 in PGC specification and the significance of the putative Blimp1/Prmt5 complex in PGCs remain to be explored.

In sum, it has now become evident that PGC specification involves a complex genetic program that leads to a genome-wide epigenetic reprogramming (Figure 2 and 3). Further clarification of the functions of the many genes involved in PGC specification, including Blimp1, Prdm14, and Tcfap2c, is therefore critical to understanding the initial set-up and the consequences of the epigenetic reprogramming in the germ cell lineage.

A signalling principle for PGC specification

It has long been known, through gene-knockout studies, that bone morphogenetic protein (Bmp) signals are essential for the generation of AP-positive PGCs from the epiblast:

Bmp4 and Bmp8b emitted from the extraembryonic ectoderm (ExE) and Bmp2 emitted apparently from the visceral endoderm (VE), as well as Alk2, a type I receptor for Bmp signalling and the signal transducers known as Smads (Smad1, 4, and 5), are critical for the generation of AP-positive PGCs (for review see (Saitou 2009b)). However, due to the absence of sensitive markers for the detection of nascent PGCs, the precise mechanism through which the Bmp signals generate the germ cell lineage has been elusive. Hence, numerous questions remain unanswered: What are the direct target cells of Bmps, which receptor complexes do the Bmps use, what are their downstream target genes, how do the different Bmps cooperate to induce PGCs, and are there any secondary signals that are required for PGC specification?

The identification of Blimp1 and Prdm14 as genes that specifically mark the very onset of PGC specification in the epiblast and the generation of robust reporters for their expression have made it possible to investigate the precise roles of signalling molecules in PGC specification. By analyzing the expression of *Blimp1* and *Prdm14* using their expression reporters in various signalling mutants and in isolated epiblasts cultured under serum-free, defined conditions, a recent study succeeded in constructing an integrated model regarding the roles of signalling activities that confer the germ cell fate to the epiblast cells (Ohinata *et al.* 2009).

In all the mutants for *Bmp4*, *Bmp8b*, or *Smad1*, the expression of *Blimp1* and *Prdm14* in the epiblasts is severely impaired, indicating that Bmp signals are necessary for the earliest step in PGC specification. On the other hand, in mutants for *Smad2* or *FoxH1*, in which nearly all the epiblast cells adopt a posterior fate due to impaired formation of anterior VE (AVE), a signalling center emitting the anteriorization/anti-posteriorization signals (e.g., Lefty1 against Nodal, Dkk1 against Wnt, Cerberus-like against Bmp, etc.) (Arnold & Robertson 2009), *Blimp1* expression expands into all the epiblast cells contacting the ExE, as well as those locating much more distally. This indicates that in the absence of the anteriorizing signals, all the pre-streak stage epiblast cells may adopt the germ cell fate as long as they receive Bmp signals from the ExE. Consistently, in vitro epiblast cultures show that in response to Bmp4, essentially all the isolated epiblast cells from E5.5 to E6.0 are competent to express *Blimp1* and *Prdm14*, and take on the AP-positive germ cell fate: Through the complex of Alk3 (or Alk6) and one of the type II receptors (most likely BmprII) via Smad1 and Smad5, Bmp4 induces both *Blimp1* and *Prdm14* in the epiblast in a dose-dependent manner. In addition, Bmp2 also induces *Blimp1* and *Prdm14* in the epiblast, although less efficiently than Bmp4. Notably, this study shows that Bmp8b does not directly induce *Blimp1* and *Prdm14* in the epiblast but rather controls (A)VE development to an appropriate level: In *Bmp8b* mutants, AVE becomes enlarged and apparently emits an extra amount of anteriorizing signals which prevent the Bmp4 signalling. These findings show that the three Bmp molecules Bmp4, Bmp8b, and Bmp2 cooperate during early gastrulation to endow the most proximal

posterior epiblast cells with a sufficient level of Bmp-Smad signalling for their adoption of the germ cell fate (Figure 4).

A previous report has shown that Bmp4 signalling through the VE is essential for PGC specification (de Sousa Lopes *et al.* 2004). In contrast, the study by Ohinata et al. has shown that Bmp4 signals directly to the epiblast cells. The culture used in the previous study included serum and feeder cells, some un-identified factor(s) from which could have complicated the experimental outcome. Furthermore, for detecting PGCs, the previous study depended almost solely on AP staining, which is not a strict criterion to identify PGCs in culture. These points may explain the discrepancy of the conclusions between the two studies.

It is also of note that only the epiblast cells from E5.5 to E6.25 are competent to express *Blimp1* and *Prdm14* and subsequently take on the AP-positive germ cell fate in response to Bmp4 (Ohinata *et al.* 2009): Epiblast cells at E5.25 show neither *Blimp1* nor AP activity, whereas those later than E6.5 exhibit *Blimp1* but fail to show AP activity and stella in response to BMP4, indicating that the epiblast cells alter their responsiveness to BMP4 in a developmental stage-dependent manner. Importantly, this study shows that Wnt3, which begins to be expressed in the epiblast at around E5.5 (Liu *et al.* 1999, Kemp *et al.* 2005), is a key factor conferring to the epiblasts the Bmp4 responsiveness, although the molecular underpinning of the "competence" and how Wnt3 might provide it need to be clarified. On the other hand, the fact that epiblast cells at E6.5 do express *Blimp1* in response to Bmp4 but do not exhibit AP activity or stella, indicates that Blimp1 alone is insufficient to confer the germ cell fate. In good agreement with these findings, Blimp1 has been shown to be expressed in multiple cell lineages during development and adulthood, and in many of these cases it plays a critical role for the lineage specification or maintenance (Horsley *et al.* 2006, Kallies *et al.* 2006, Martins *et al.* 2006, Magnusdottir *et al.* 2007, Robertson *et al.* 2007). The epigenetic landscape of the cells in which Blimp1 is induced would therefore be a key to how Blimp1 affects the fate of the cells. Accordingly, the genes controlled by Blimp1 in PGC specification and in other contexts such as epidermal or plasma-cell differentiation are very different (Kurimoto *et al.* 2008).

One of the most significant demonstrations of this study is that under a defined condition essentially with Bmp4, a majority of the competent epiblast cells go on to develop into pre-meiotic PGC-like cells after ~84-132 hours of culture (Ohinata *et al.* 2009). The induced PGC-like cells not only show proper gene expression, but also exhibit characteristic epigenetic reprogramming, including the reduction of genome-wide H3K9me2, up-regulation of H3K27me3, and erasure of the genomic imprints, an attribute exclusive to PGCs, during the 132-hour culture. These findings indicate that PGC specification may be an instructive event that allows a major part of the subsequent

development of PGCs to progress in a cell-autonomous manner. Finally, the induced PGC-like cells contribute to functional spermatogenesis when directly transplanted into neonatal germ-cell-less testes of *W/W*^{v} mice or by the gonad reconstruction assay, providing an unequivocal and stringent demonstration of the germ cell fate induction.

PGC specification from pluripotent stem cells in culture

There have been several reports regarding the derivation of gametes from embryonic stem (ES) cells in culture (Hubner *et al.* 2003, Toyooka *et al.* 2003, Geijsen *et al.* 2004, Nayernia *et al.* 2006). However, the generation of fully functional gametes that support normal embryonic development and adult physiology has not yet been achieved. The methodologies in these reports use serum and do not involve directed differentiation toward the germ cell lineage, but rather select rare cells expressing some germ-cell markers after prolonged culture for random differentiation. Accordingly, complex processes required for germ cell development in vivo have not been monitored properly.

In recent years, there has been considerable progress in our understanding of the molecular basis of many aspects of germ cell development, including PGC specification, as reviewed here, male versus female germ cell differentiation, meiosis, spermatogonial stem cell biology, spermatogenesis, and oocyte development (for review see (Handel & Schimenti 2010, Bowles & Koopman 2007, Kanatsu-Shinohara *et al.* 2008, Edson *et al.* 2009)). Based on the information we have obtained, for the successful generation of gametes from pluripotent stem cells in culture, it would be essential and perhaps most efficient to recapitulate what happens in vivo from the very beginning (i.e., PGC specification from the epiblast) as precisely as possible and in a step-by-step fashion.

The signalling requirements that are responsible for the expression of key transcriptional regulators for PGC specification from the epiblast have been revealed, which has led to the generation of functional PGC-like cells under a serum-free, defined condition (Ohinata *et al.* 2009). Several methodologies to induce ES cells into epiblast-like states under a serum-free, defined condition have been reported (for review see (Murry & Keller 2008)). It would therefore be possible to induce PGC-like cells from pluripotent stem cells, including ES cells and induced pluripotent stem (iPS) cells (Takahashi & Yamanaka 2006, Okita *et al.* 2007, Takahashi *et al.* 2007, Wernig *et al.* 2007, Yu *et al.* 2007, Park *et al.* 2008), by first differentiating them toward an epiblast-like state.

Recent studies have reported the derivation in culture of pluripotent stem cells from the epiblast, i.e., EpiSCs, which bear properties similar to those of the original epiblast cells, including gene expression and epigenetic states (Brons *et al.* 2007, Tesar *et al.* 2007). EpiSCs can be derived from the epiblast cells at E5.5 to E6.5 in the presence of Activin and basic fibroblast growth factor (FGF). They can also be induced from ES cells by culturing them with Activin and bFGF (Guo *et al.* 2009). These studies have raised the

important possibility of inducing PGC-like cells from EpiSCs or EpiSC-like cells cultured under certain modified conditions. Indeed, it has been shown that EpiSCs are a somewhat heterogeneous cell population that includes *Blimp1*- and stella-positive cells, although these cells are present in very small numbers (the stella-positive cells constitute 0.5% of the total EpiSC population), and that EpiSCs have many important properties that are similar to those of PGCs, including gene expression, epigenetic states, and differentiation potential (Hayashi & Surani 2009). The emergence of stella-positive PGC-like cells in the EpiSC culture is blocked by Dorsomorphin, an inhibitor of Bmp signalling, and enhanced by Bmp4 (up to ~1.5% of cells becoming stella-positive, a similar ratio for the emergence of stella-positive cells in randomly differentiated embryoid bodies) (West *et al.* 2009), indicating that a signalling pathway operating in vivo is indeed responsible for the emergence of PGC-like cells in culture (Hayashi & Surani 2009). The function of the PGC-like cells has not been definitively demonstrated by transplantation assays. Further modification of the culture conditions would lead to a more efficient generation of PGC-like cells in culture.

Perhaps one of the most important challenges following a precise recapitulation of PGC specification would be to explore the conditions necessary to support a long-term proliferation of PGCs in vitro. The long-term proliferation of PGCs in turn would provide a foundation for exploring the conditions needed to support further germ cell development in vitro. There have been several attempts to establish such long-term proliferation, but PGCs in culture either go on to undergo apoptosis after several rounds of cell divisions (Dolci *et al.* 1991, Matsui *et al.* 1991) (Pesce *et al.* 1993) or de-differentiate into pluripotent embryonic germ (EG) cells (Matsui *et al.* 1992, Labosky *et al.* 1994, De Miguel *et al.* 2002). PGCs in vivo go on to enter the cell-cycle arrest at the G1 phase in the male genital ridges or the meiotic prophase in the female genital ridges, both after around E13.5 (McLaren 2003). However, PGCs proliferate actively, especially when they migrate out from the developing hindgut endoderm (see above) (Seki *et al.* 2007) and in the genital ridges in earlier stages (~E10.5-E11.5). Understanding of the mechanisms for PGC survival and proliferation would therefore be critical for establishing the conditions for long-term proliferation of PGCs in culture.

Perspectives: Implications for PGC development in other mammals, including humans

In the last decade of intensive research, a major part of the framework for the signalling and transcription in PGC specification in mice has been established. In particular, the identification of Blimp1 and Prdm14 as critical transcriptional regulators for PGC specification has been a key breakthrough. The finding that essentially all the epiblast cells from E5.5 to E6.0 are competent to express Blimp1 and Prdm14 and go on to form PGCs in response to Bmp4, constitutes an important basis from which to explore conditions for inducing PGC-like cells directionally from pluripotent stem cells.

However, many basic questions still remain to be addressed, including the precise mechanism by which Bmp4-Smad signalling activates *Blimp1* and *Prdm14* expression, the molecular nature of the competence of the epiblast cells for the germ cell fate and how Wnt3 signalling would be involved in it, the biochemical mechanisms of the action of Blimp1 and Prdm14, and the precise mechanism and functional significance of the epigenetic reprogramming in PGCs after their specification. Investigations into some of these questions will require a precise in vitro PGC induction system.

There still exists only very limited information regarding the mechanism of germ cell specification in other mammals, including humans. Although the characteristics of pre-implantation development are similar between mice and humans, those of the early post-implantation development, including gastrulation, are somewhat different (Sadler 2010). In mice, the most proximal epiblast cells maintain direct contact with the extraembryonic ectoderm cells from ~E5.0 to E6.5 and receive a Bmp4 signal from these cells to adopt the germ cell fate. In humans, before gastrulation, at around day 8 of development, some of the pluripotent epiblast cells form amnioblasts, and there appears a cavity, called the amniotic cavity, between the amnioblast and the epiblast (Sadler 2010) (Figure 5). Accordingly, the amnioblast cells directly contact the cytotrophoblast cells and, during the second week of development, most of the epiblast cells become separated from the trophoblast layer by the amnion and the amniotic cavity (Sadler 2010) (Figure 5). There has been no information in terms of which part of the epiblast acquires the germ cell fate or which tissue emits the key signal for this fate. It is at the end of the third week that PGCs are detected by their high AP activity in the wall of the yolk sac at the base of the allantois and future umbilical cord (Sadler 2010). Clearly, it is very difficult to fully investigate the mechanism of germ cell specification in human embryos due to the difficulty of obtaining the appropriate materials, and for ethical reasons. Therefore, to gain further insight into the precise mechanism of PGC specification in humans, it will be important to investigate the mechanism of PGC specification in mammals whose early post-implantation development is similar to that of humans, such as the rabbit (Viebahn *et al.* 1995). The accumulation of knowledge regarding the PGC specification in mice should provide a basis for such investigations.

On the other hand, studies involving human ES and iPS cells have already shown that it is possible to induce human PGC-like cells from human ES and iPS cells (Clark *et al.* 2004, Kee *et al.* 2006, Tilgner *et al.* 2008, Bucay *et al.* 2009, Kee *et al.* 2009, Park *et al.* 2009). Many of these studies involve random differentiation of ES cells by EB formation and subsequent detection of PGC-like cells by PGC markers identified in mice. It has also been shown that addition of BMP4 in culture substantially increases the ratio of the appearance of PGC-like cells (Kee *et al.* 2006, Kee *et al.* 2009). Although the demonstration of the function of induced human PGC-like cells will necessarily be made difficult by ethical considerations, an ES-cell-based PGC formation, if properly

performed, would provide invaluable opportunities to improve our understanding of germ cell development in humans. Continued detailed investigations into the mechanism of germ cell development in mice, as well as studies involving other mammals and human ES and iPS cells, should provide a critical foundation for a precise mechanistic understanding of the propagation of our genome both under normal and diseased conditions.

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Figure Legends

Figure 1. Schematic representation of the emergence of primordial germ cells (PGCs) in the mouse embryo

At the time of implantation (~E4.5), the blastocyst is composed of three cell types, trophoectoderm (TE, purple), primitive endoderm (PE, yellow), and epiblast (blue). The TE cells in direct contact with the epiblast proliferate and form a simple epithelial sheet of extraembryonic ectoderm (ExE), which surrounds a small central cavity (the proamniotic cavity). Epiblast cells also undergo cavitation and form a cup-shaped epithelial sheet by E5.5. The epiblast cells have competence to differentiate into all somatic cells as well as germ cells. The initial embryonic patterning including anterior-posterior polarity formation, gastrulation, and germ cell specification is mediated by signallings from the ExE and PE-derived visceral endoderm (VE) that cover the epiblast (for relevant signals for PGC specification, see text and Figure 4). DVE: distal visceral endoderm; AVE: anterior visceral endoderm; ExM: extraembryonic mesoderm; EM: embryonic mesoderm; DE: definitive endoderm; PGCs: primordial germ cells.

Figure 2. A model of PGC specification orchestrated by the two PR domain-containing proteins, Blimp1 and Prdm14

(A) Expression of *Blimp1* (left) and *Prdm14* (right) in the LS stage embryo visualized by the *Blimp1*-mVenus and *Prdm14*-mVenus reporters, respectively. Blimp1 is expressed in the nascent PGC precursors emerging from the most proximal part of the posterior epiblast as well as in the visceral endoderm. Prdm14 is exclusively expressed in the germ cell lineage and pluripotent cell lines.

(B) A summary of genetic pathways for PGC specification. See text for details. Black arrows and black lines with terminal bars indicate genetic pathways for activation and for repression, respectively, as demonstrated by in vivo experiments. Dotted arrows and dotted lines with terminal bars indicate genetic pathways for activation and for repression, respectively, as proposed based on in vitro experiments.

Figure 3. A summary of the genome-wide epigenetic reprogramming revealed by immunohistochemistry in migrating PGCs

The established PGCs that start migration undergo a genome-wide epigenetic reprogramming, which includes genome-wide reduction of DNA methylation and H3K9me2, and genome-wide up-regulation of H3K27me3 and H4R3me2. PGCs repress active enzymes for repressive epigenetic modifications (DNA methylation and H3K9me1/2) prior to the reprogramming, become arrested at the G2 phase of the cell cycle and turn off RNA polymerase II-based transcription during the reprogramming. See text for details.

Figure 4. Signalling activities leading to PGC specification in the mouse embryo

At around E5.5, epiblast cells acquire the competence to respond to Bmp4 by the activities of Nodal and Wnt3. On the other hand, Nodal signalling mediated by Smad2/FoxH1 specifies DVE cells that start to provide signals against posteriorization. Signals from ExE including Bmp8b apparently prevent proximal VE from differentiating to DVE, thus restricting the anti-posteriorization activities. At around E6.0-6.25, DVE moves anteriorly (red arrow) to form AVE and a subset of the competent epiblast cells that receive the highest level of Bmp4 signals from the ExE are specified as Blimp1- and Prdm14-positive PGC precursors. The germ cell competence may be sustained until around the LS stage in the proximal part of posterior epiblast cells. The abbreviations are as shown in Figure 1.

Figure 5. Schematic representation of the early post-implantation development of human embryos

The implanted human embryo at around 8 days of development consists of a syncytiotrophoblast (not shown), cytotrophoblast (CT, purple), epiblast (Epi, blue) and hypoblast (HB, yellow). In contrast to the mouse embryo, the epiblast cells at this stage have already formed an amnioblast (AB) and an amniotic cavity appears between the epiblast and AB. At around 12 days of development, just prior to the onset of gastrulation, the epiblast cells are no longer in direct contact with the CTs. Epi: epiblast; Hypo: hypoblast; AB: amnioblast; CT: cytotrophoblast; En: extraembryonic endoderm; SpM: extraembryonic splanchnopleuric mesoderm; SoM: extraembryonic somatopleuric mesoderm.

Figure 1

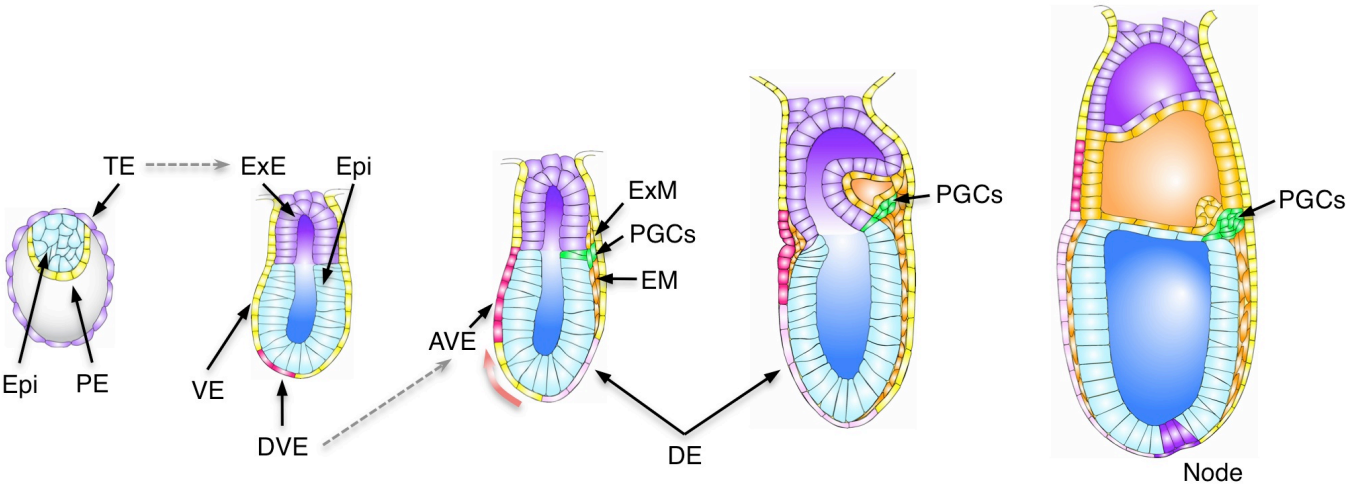
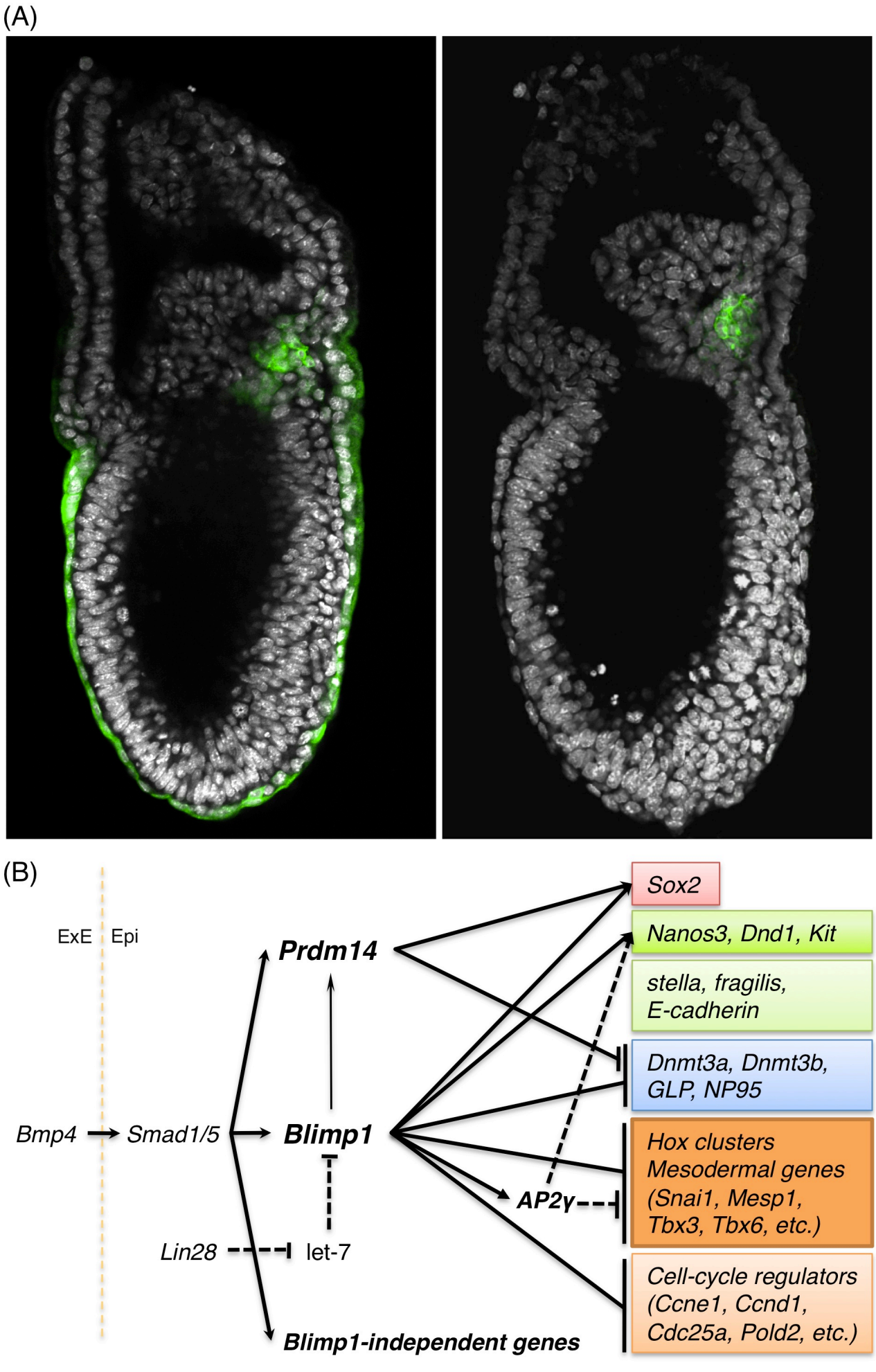

TE
ExE
Epi
Epi
PE
VE
DVE
AVE
ExM
PGCs
EM
DE
PGCs
PGCs
Node
E4.5
E5.5
E6.25
E6.75
E7.25

Figure 2


(A)
(B)
ExE
Epi
Prdm14
Bmp4
Smad1/5
Blimp1
Lin28
let-7
AP2γ
Blimp1-independent genes
Sox2
Nanos3, Dnd1, Kit
stella, fragilis, E-cadherin
Dnmt3a, Dnmt3b, GLP, NP95
Hox clusters Mesodermal genes (Snai1, Mesp1, Tbx3, Tbx6, etc.)
Cell-cycle regulators (Ccne1, Ccnd1, Cdc25a, Pold2, etc.)

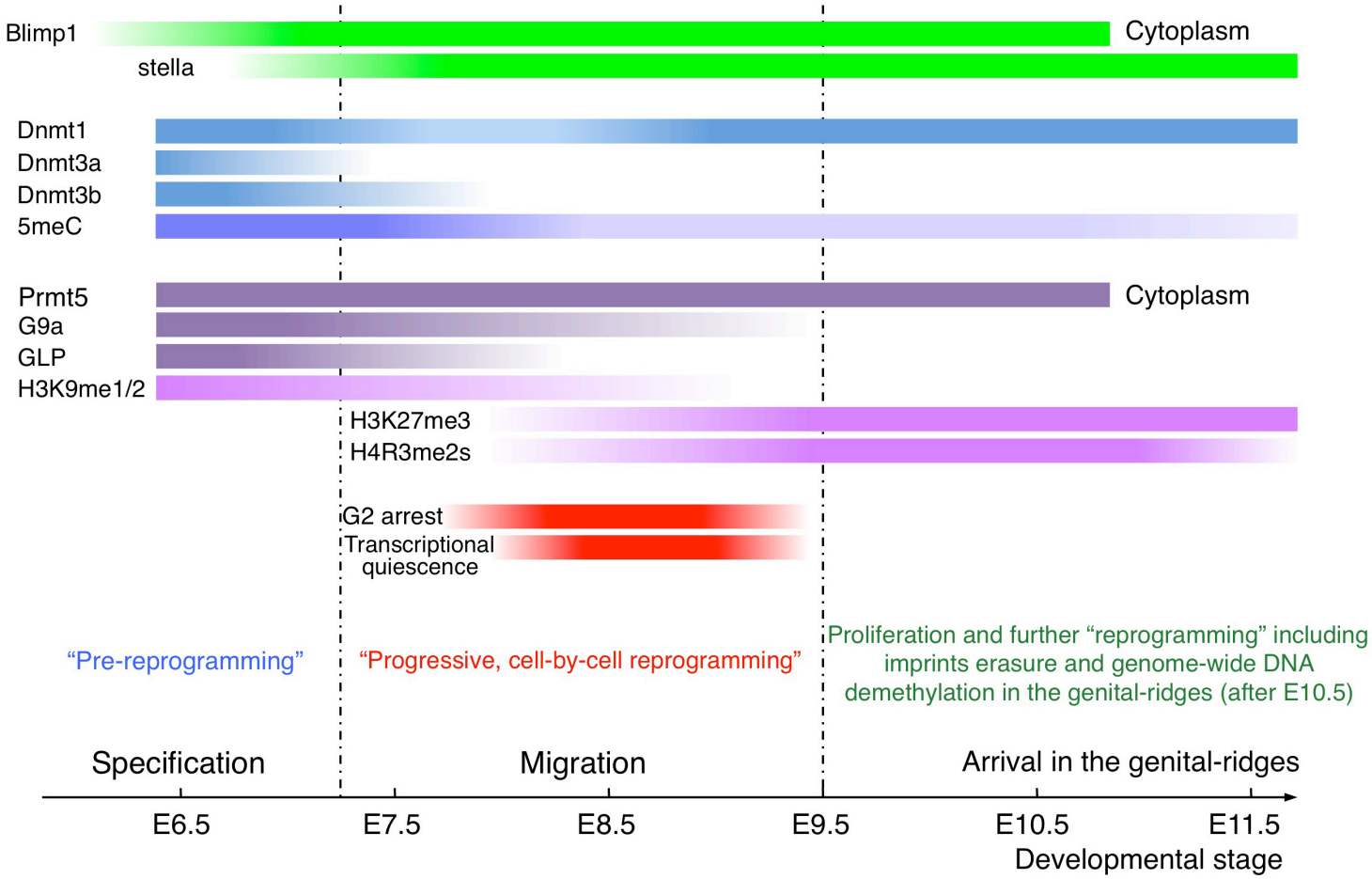
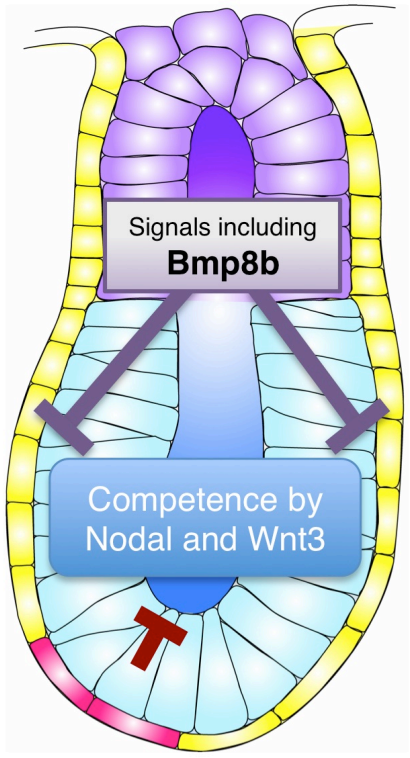
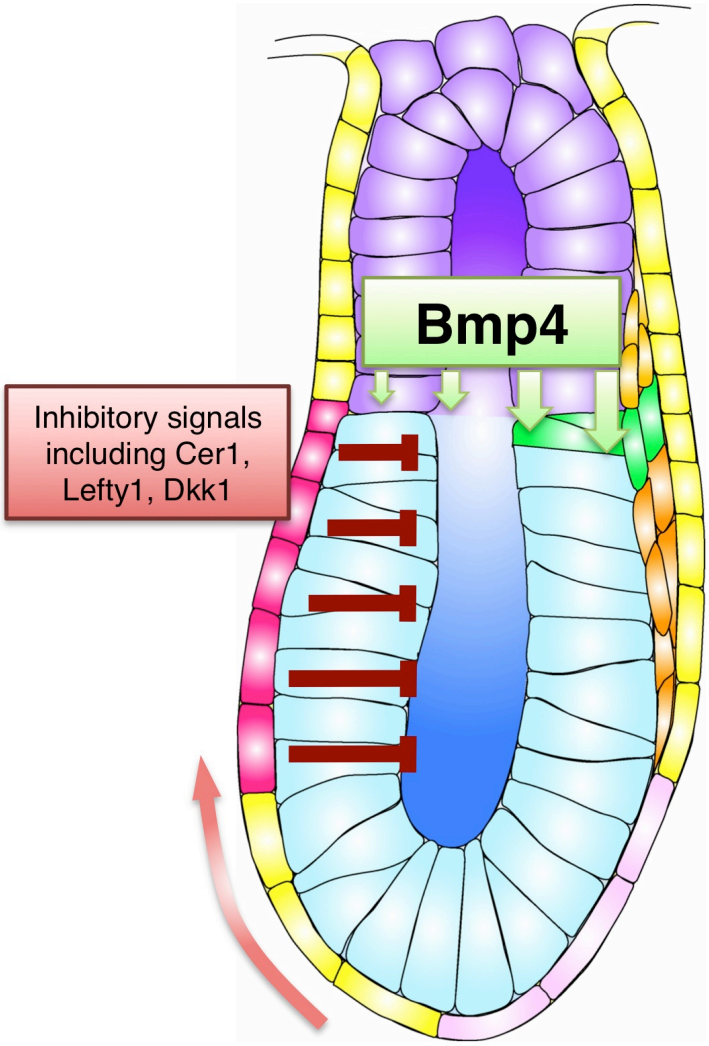

Blimp1
Cytoplasm
stella
Dnmt1
Dnmt3a
Dnmt3b
5meC
Prmt5
Cytoplasm
G9a
GLP
H3K9me1/2
H3K27me3
H4R3me2s
G2 arrest
Transcriptional quiescence
"Pre-reprogramming"
"Progressive, cell-by-cell reprogramming"
Proliferation and further "reprogramming" including imprints erasure and genome-wide DNA demethylation in the genital-ridges (after E10.5)
Specification
Migration
Arrival in the genital-ridges
E6.5
E7.5
E8.5
E9.5
E10.5
E11.5
Developmental stage

Figure 4



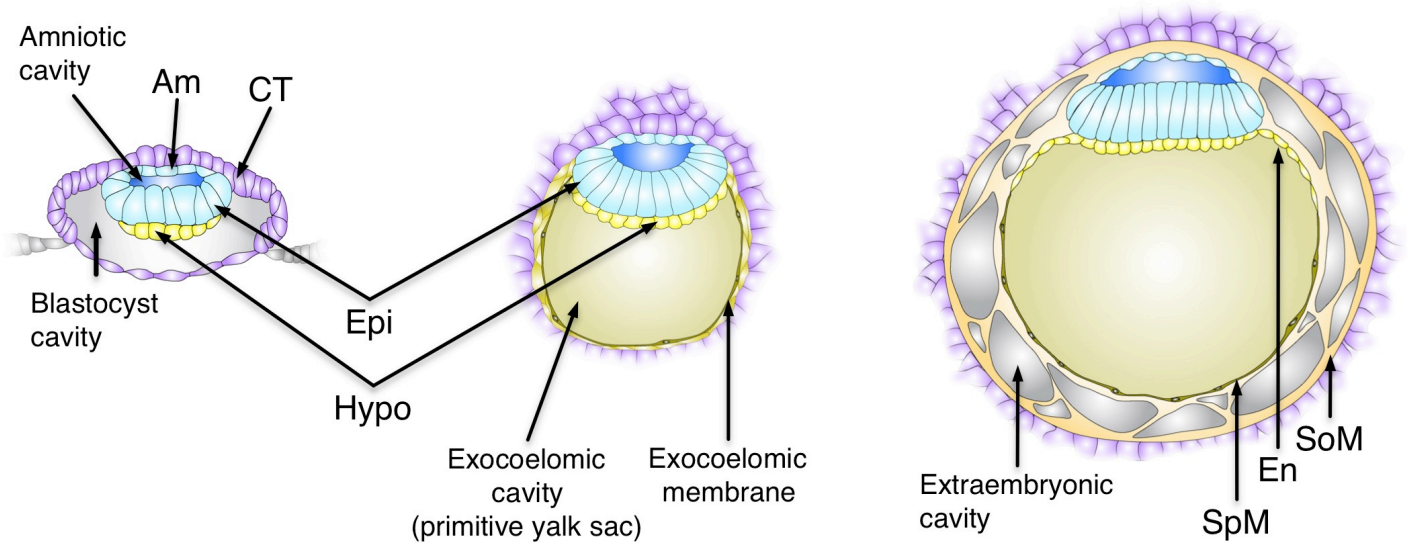
Smad2/FoxH1-mediated D/AVE formation

E5.5 ~ 5.75



E6.25

Figure 5


Amniotic cavity
Am
CT
Blastocyst cavity
Epi
Hypo
Exocoelomic cavity (primitive yalk sac)
Exocoelomic membrane
Extraembryonic cavity
SoM
En
SpM
8 days
9 days
12 days