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Review

Primordial germ cell specification: the importance of being 'blimped'

Susana M. Chuva de Sousa Lopes[1] and Bernard A.J. Roelen[2]

[1]Hubrecht Institute, Developmental Biology and Stem Cell Research, CT Utrecht, The Netherlands and

[2]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, CM Utrecht, The Netherlands

Summary. In mouse embryos, the expression of Blimp1 has recently revealed a population of allocated primordial germ cell precursors 24 hours earlier than previously thought. Those 'blimped' precursors have been shown to give rise, by mitotic division, to germ cells only and no other cell lineages. Here, we try to understand the events that lead to Blimp1 expression in the primordial germ cell precursors and speculate on what can be the role of Blimp1 during primordial germ cell specification and gastrulation in the mouse. Finally, we discuss the possible involvement of Blimp1 in the two know modes of germ line segregation (epigenesis and preformation).

Key words: Blimp1, Primordial germ cell, Embryo, Mouse, Mammals

What are primordial germ cells?

The allocation of cells to the germ cell lineage, a key decision separating the germ and the somatic lineages in sexually reproducing animals, is still far from being well understood. Depending on the species, the formation of primordial germ cells (PGCs) is assured either by inheritance of maternal cytoplasmic determinants, as in the fruitfly and zebrafish, or by inductive cell interactions, as for instance, in the newt and mouse (and presumably all mammals). In the mouse (*Mus musculus*), PGCs are set aside early during embryonic development, during gastrulation, at a peripheral place in the embryo (Ozdzenski, 1967; Ginsburg et al., 1990). These cells subsequently embark on a migratory journey to the genital ridge(s) where they settle and will eventually enter meiosis. All epiblast cells are potentially able to become PGCs if they happen to be at the right time in the right place during embryonic development (Tam and Zhou, 1996). The right place is the proximal-posterior part of the epiblast, close to the border with the extraembryonic ectoderm. The right time would be around embryonic day (E)6.25-7.5 (Fig. 1).

In the mouse, PGCs were first described by Chiquoine using the Gomori histochemical technique for alkaline phosphomonoesterase activity (Chiquoine, 1954). Later, a small cluster of PGCs as early as E7.0-E7.5 in the base of the allantoic bud was identified (Ozdzenski, 1967; Ginsburg et al., 1990). PGCs show high levels of tissue non-specific alkaline phosphatase (Hahnel et al., 1990), a product of the gene *Akp2*, in the cytoplasmic membrane and the Golgi apparatus (Clark and Eddy, 1975). This simple staining technique is still the golden standard to identify PGCs today in the mouse (Fig. 2), even though new molecular markers have been identified, including *Oct4* (Scholer et al., 1989; Yeom et al., 1996), *Ifitm3/Mil1/Fragillis* (Saitou et al., 2002; Tanaka and Matsui, 2002; Lange et al., 2003), *Dppa3/PGC7/Stella* (Saitou et al., 2002; Sato et al., 2002), *Nanog* (Yamaguchi et al., 2005), *Dppa4/Pita2* (Maldonado-Saldivia et al., 2007), *Dppa5/Esg1* (Western et al., 2005) and *Prdm1/Blimp1* (Chang et al., 2002; Ohinata et al., 2005; Vincent et al., 2005). *Blimp1* differs from the other mentioned genes in that it is not expressed in the pluripotent inner cell mass of blastocysts, but starts being expressed in the primitive endoderm.

Before Blimp1, there was BMP

The bone morphogenetic protein (BMP) signalling pathway has been demonstrated to be instrumental early during the formation of PGCs. BMP ligands are secreted by the extraembryonic tissues, extraembryonic ectoderm

Offprint requests to: Susana M. Chuva de Sousa Lopes, Hubrecht Institute, Developmental Biology and Stem Cell Research, Uppsalaan 8, 3584 CT Utrecht, The Netherlands. e-mail: smc80@cam.ac.uk

and proximal-posterior visceral endoderm at E5.5-E6.5 (Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001) and activate the BMP signalling pathway in the proximal epiblast via Smad1/5/8 (Hayashi et al., 2002; de Sousa Lopes et al., 2004). Several BMP ligands are involved, including BMP2, BMP4 and BMP8b, and if the BMP signalling pathway is not (or is incompletely) activated in the proximal epiblast, the cell populations clonally derived from the proximal epiblast cells will be defective. In this case not only the PGCs, but also the allantois and the visceral yolk sac are abnormal (Winnier et al., 1995; Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001). This clearly indicates that the wave of BMP signalling is not exclusively involved in PGC induction, but in the induction of all cell populations derived from the proximal epiblast. Interestingly, BMP2 and BMP4 have an additive effect on the number of formed PGCs, whereas for BMP8b such an additive effect has not been observed (Ying et al., 2000; Ying and Zhao, 2001).

Mouse embryos with only one active copy of the *Bmp4* gene form less PGCs, but the allantois appears to be normal in size. This would indicate that the formation of PGCs is BMP4-dose dependent, whereas the allantois is not. Two logical explanations for this are that either the proximal epiblast cells have a preference for becoming allantois, and only if sufficient cells are formed, cells can become PGCs; or that when only few allantois cells are initially formed, these cells are capable of compensatory growth.

How important BMP signalling is for the formation of PGCs in mammals other than the mouse remains to be elucidated. Interestingly, embryos of another rodent, the guinea pig (*Cavia porcellus*) show a surprising morphological variation to the mouse embryo (reviewed by Eakin and Behringer, 2004). Guinea pig embryos are cup-shaped like mouse embryos, but the extraembryonic ectoderm does not contact the epiblast either before (Fig. 3A,B) or during early gastrulation (Fig. 3C). It is thus unclear whether any signalling molecule produced by the extraembryonic ectoderm is involved in the induction of PGCs or other cell populations derived from the proximal epiblast in the guinea pig, or in other less related mammals. On the other hand, the visceral endoderm is responsible for part of the BMP signalling and has been shown necessary for the formation of PGCs in the mouse (Ying and Zhao, 2001; de Sousa Lopes et al., 2004). Comparison of the expression of BMP ligands in these two rodents would be useful to elucidate the role of the BMP signalling pathway in PGC formation.

What induces Blimp1 in PGC precursors?

At E6.0, before any sign of PGC precursors, the products of *Ifitm3/Fragillis/Mil1* (Tanaka and Matsui, 2002; Lange et al., 2003), *Nodal* (Beck et al., 2002) and *Cripto/Tdgf1* (Ding et al., 1998) become asymmetrically expressed in a proximal-distal gradient in the epiblast, whereas *Fgf8* expression is restricted to the posterior part of the epiblast (Crossley and Martin, 1995). At E6.25, just before gastrulation, about 6 cells located in the posterior-proximal epiblast upregulate Blimp1 (B lymphocyte-induced maturation protein), a process referred to as ‘blimping’ (McLaren and Lawson, 2005)

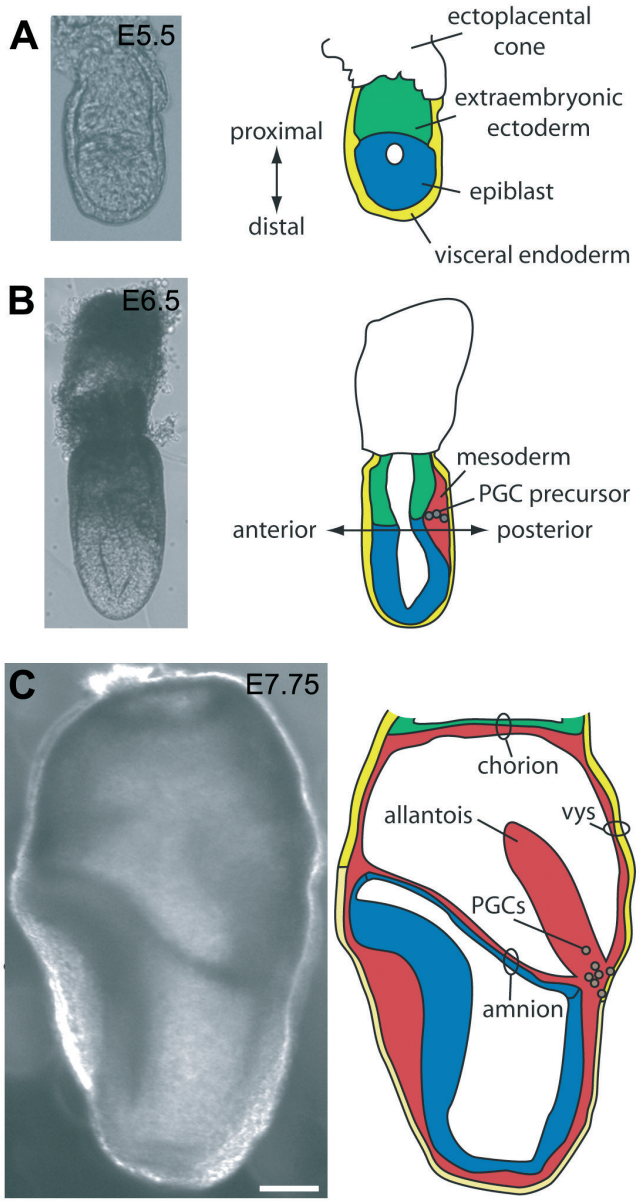

Fig. 1. Early postimplantation development of mouse embryos. Photomicrographs of **(A)** E5.5, **(B)** E6.5 and **(C)** E7.75 mouse embryos are on the left, schematic drawings of these embryos with explanatory text are on the right. Blue: epiblast/ectoderm; brown: PGCs; gray: PGC precursors; green: extraembryonic ectoderm; red: mesoderm; yellow: visceral endoderm; light yellow: definitive endoderm. Abbreviations: PGCs, primordial germ cells; vys, visceral yolk sac. Scale bar in A is 40 μm, in B and C 100 μm.

and become the first cells allocated to the germ cell lineage (Ohinata et al., 2005; Vincent et al., 2005). Although these 'blimped' PGC precursors are lineage restricted, they are not yet specified to the germ cell lineage (Chuva de Sousa Lopes et al., 2007). Moreover, the expression of *Blimp1* is not specific to the PGC precursors at E6.25, but it is shared by the visceral endoderm (Ohinata et al., 2005; Vincent et al., 2005).

What regulates the increase in Blimp1 expression in the 6 cells of the posterior-proximal epiblast that will become PGC precursors is still a matter of debate. The expression of *Fgf8* in the posterior epiblast is probably not involved in the induction of 'blimped' PGC precursors, because *Fgf8* mutant embryos exhibit a normal number of PGCs (Sun et al., 1999). However, the expression domains of *Ifitm3*, *Nodal* and *Cripto* are further restricted to the proximal-posterior epiblast at E6.25 and may play an important role in the inductive process. In agreement, the whole E5.5-E5.75 epiblast (both the proximal and the distal region) is able to respond to exogenous BMP4 and generate PGCs (Fig. 4), whereas at E6.0-6.25 when the domains of expression of Ifitm3, Nodal and Cripto become restricted to the proximal part of the embryo, only the proximal, but not the distal epiblast, retains this capacity (Okamura et al., 2005).

Genetic deletion of *Ifitm3* will provide further information on its role during PGC development. Ifitm3, a transmembrane protein, may play a role in the cell-cell adhesion and communication between PGC precursors (Matsui and Okamura, 2005). Nodal, its accessory co-receptor Cripto and Smad2 belong to the same signalling pathway (TGFß, signalling pathway). Genetic deletion of *Smad2* results in embryos with a multitude of PGC clusters formed around the developing embryo (Tremblay et al., 2001). The deficiency of *Nodal* leads to an anteriorized embryo (Camus et al., 2006). Nodal is generated and secreted with a prodomain attached, which is cleaved by Spc1 and Spc4, two enzymes generated mainly by the extraembryonic ectoderm (Beck et al., 2002). The exclusive expression of uncleaved Nodal precursor in the mouse embryo, as a result of the genetic ablation of the Nodal prodomain cleavage site, does give rise to a posteriorized embryo (Ben-Haim et al., 2006). In agreement, E6.0 embryos cultured without the extraembryonic ectoderm (the source of Spc1 and Spc4) express Blimp1 throughout the epiblast (Chuva de Sousa Lopes et al., 2007) probably as a result of the presence of uncleaved Nodal. Together, these data suggest that not only the BMP signalling pathway via Smad1/5, but also the TGFß, signalling pathway via Smad2 is involved in the allocation of 'blimped' PGC precursors.

Is being 'blimped' the first sign of gastrulation?

Other genes that, similarly to *Blimp1*, start being expressed in the posterior-proximal epiblast just before gastrulation commences are *Eomesodermin* (Russ et al., 2000), *Evx1* (Dush and Martin, 1992), *Brachyury* and *Wnt3* (Rivera-Perez and Magnuson, 2005). The expression domains of these genes are however broader than that of *Blimp1*. The deletion of *Blimp1* does not block gastrulation, but in contrast gastrulation seems to occur normally (Ohinata et al., 2005; Vincent et al., 2005). In these *Blimp1*-deficient embryos a population of PGC progenitors may even be formed. Could the induction of the first 6 'blimped' PGC precursors be the first sign of the formation of the primitive streak, and therefore the first sign of gastrulation? The expression of *Blimp1* may not be necessary for gastrulation to occur, but it does co-localise with the proximal-posterior region of the epiblast. It is exactly this part of the embryo that undergoes the deformation that precedes formation of

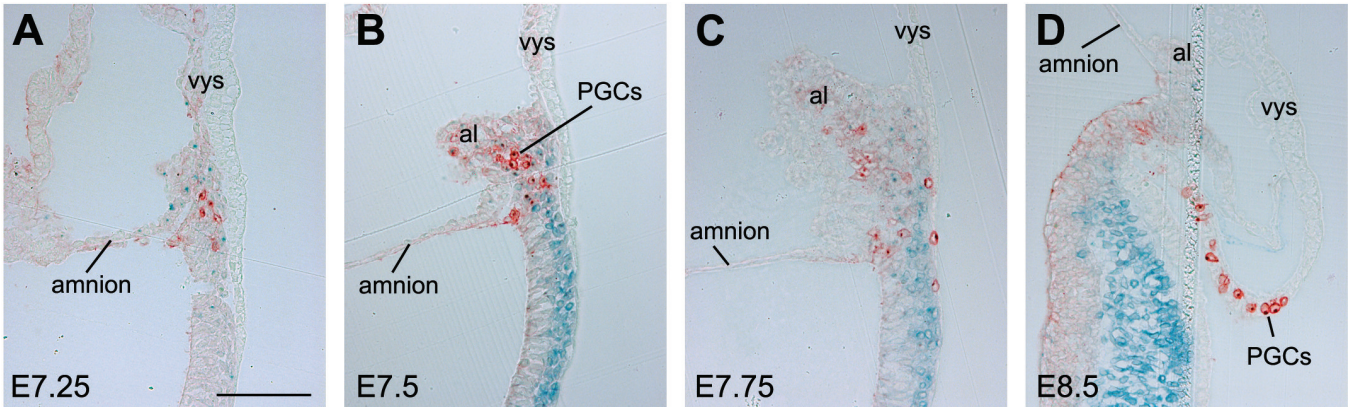

Fig. 2. Formation of primordial germ cells in the mouse embryo. Photomicrographs of sagittal sections of mouse embryos of **(A)** E7.25, **(B)** E7.5, **(C)** E7.75 and **(D)** E8.5 showing their posterior part. The embryos have been stained for alkaline phosphatase activity (red). Note the characteristic "dot" staining the Golgi apparatus and the cell membrane of the PGCs. PGCs are formed at the base of the allantois and move to the endoderm, later the hindgut wall. Abbreviations: PGCs, primordial germ cells; vys, visceral yolk sac; al, allantois. Scale bar is 100 µm.

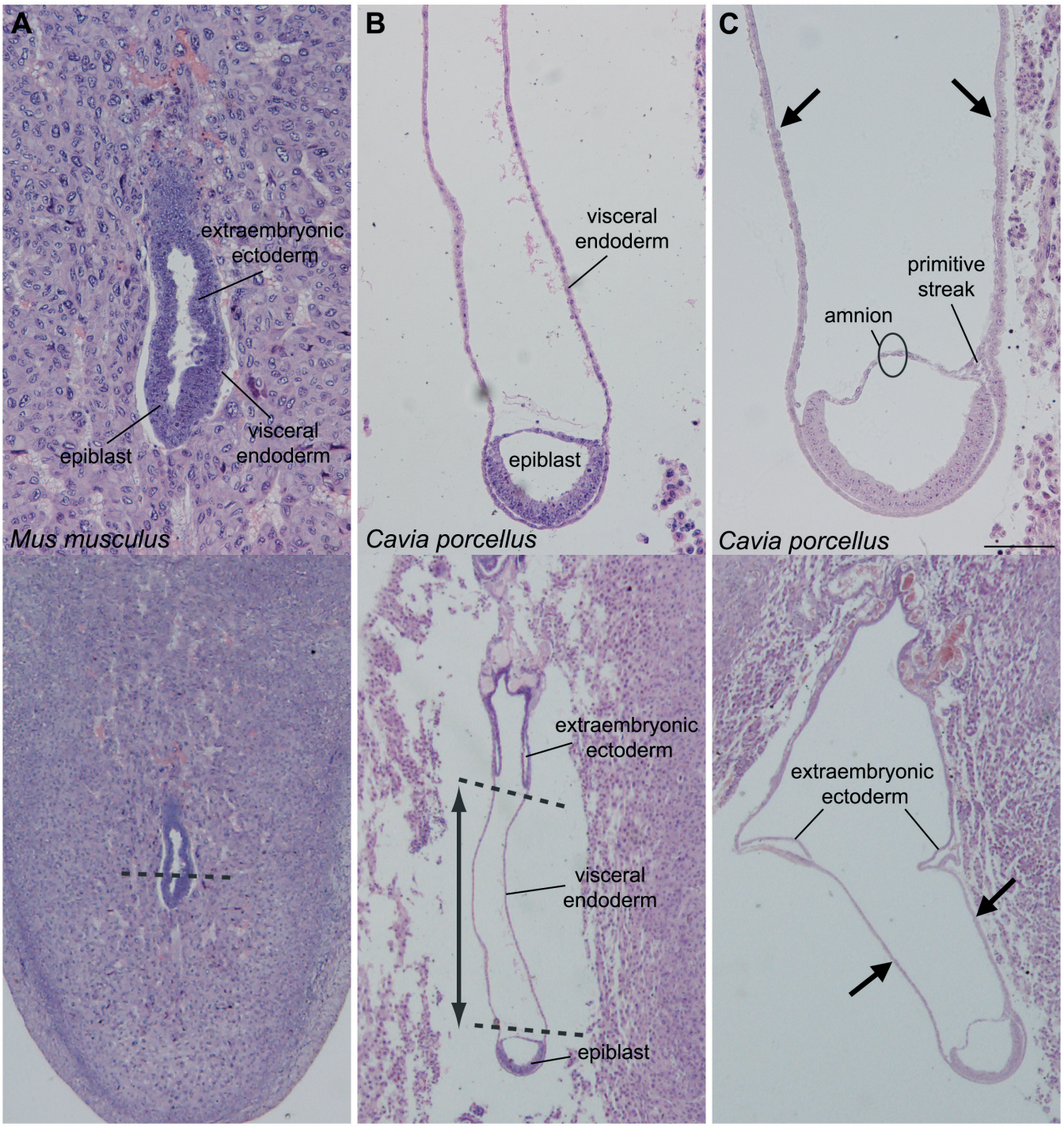

Fig. 3. Comparison between mouse and guinea pig embryos. Photomicrographs of sagittal sections of **(A)** a mouse embryo and **(B)** a guinea pig embryo before gastrulation; and **(C)** a guinea pig embryo at the begin of gastrulation. All embryos are in deciduum. Histological slides were from the Hill collection, previously at the Hubrecht Institute in Utrecht, now in the Museum für Naturkunde in Berlin. Note that in the mouse **(A)** embryo the extraembryonic ectoderm contacts the epiblast (dotted line), whereas in the guinea pig these two tissues are far apart (dotted lines separated by double arrow in **B**, lower panel). The epiblast in the guinea pig embryo does cavitate **(B)**. The mesoderm migrates as a single layer of cells from the primitive streak **(C)** to form the amnion and line the visceral endoderm (black arrows). Scale bar is 100 μm in the top panels.

the primitive streak. The formation of multiple primitive streaks has been described in mouse embryos double-mutant for *Cerberus-like* and *Lefty1* (Perea-Gomez et al., 2002), two known Nodal antagonists expressed by the anterior visceral endoderm. It would indeed be interesting to analyse the expression of Blimp1 in those double mutants. Finally, time-lapse analysis of the 'blimping' process in the mouse embryo would also contribute greatly to our understanding of gastrulation

From PGC precursor to PGC

It is unclear how the number of 'blimped' PGC precursors, forming a tight cluster at the base of the allantois, increases from 6 to approximately 40 cells between E6.25-E7.25 (Ohinata et al., 2005). The number of 'blimped' PGC precursors seems to increase linearly between E6.25-E7.5 (Ohinata et al., 2005), in contrast to the exponential increase in PGC numbers observed between E8.5-E13.5 (Tam and Snow, 1981). Moreover, it has been demonstrated that between E6.25-E7.25, epiblast cells are still being recruited to the germ cell lineage (Lawson and Hage, 1994; Tam and Zhou, 1996; Chuva de Sousa Lopes et al., 2007). Therefore, 'blimped' PGC precursors, similarly to specified PGCs, may acquire a doubling time of 16 hours, as opposed to the 5-7 hour doubling time observed in the rest of the epiblast cells (Snow, 1977; Tam and Snow, 1981; Lawson and Hage, 1994).

Around E7.5, a second inductive event must take place so the PGC precursors become specified or committed to the germ cell lineage, in the sense that no other cells can enter the germ line. This event is responsible for dramatic changes in the PGC precursors. In particular, they change their transcription expression profile by upregulating several markers of pluripotency (Dppa3, Nanog, Sox2, Akp2) and downregulating markers of differentiation (Brachyury, Fgf8, Snail) (Yabuta et al., 2006). E-cadherin has been described to play an important role maintaining the tight clump of PGC precursors, a necessary event for PGC specification to occur (Okamura et al., 2003). After specification, PGCs acquire a migratory phenotype and move to the wall of the hindgut (Fig 1).

The role of Blimp1 in PGC specification

The human ortholog of *Blimp1* was first identified as

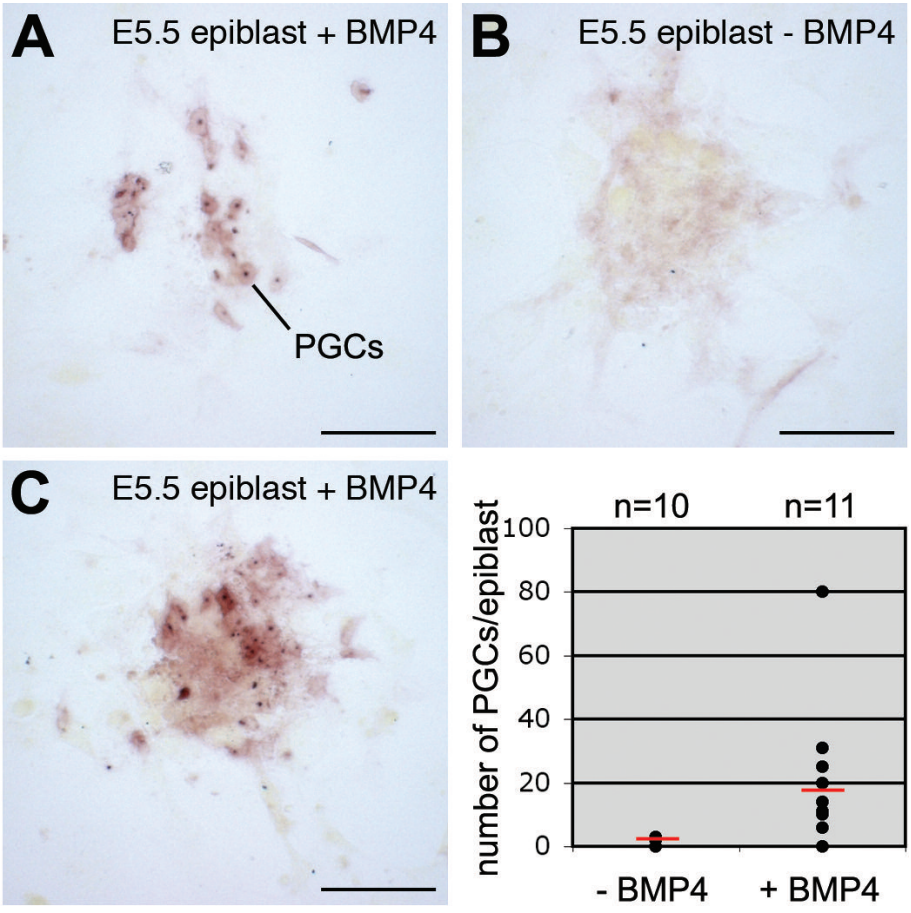

Fig. 4. BMP4 induces PGC formation in E5.5 epiblast cells. E5.5 epiblast explants were cultured for 4 days on a layer of mouse embryonic fibroblasts in the presence **(A, C)** and absence **(B)** of 100 ng/ml BMP4. Thereafter, explants were stained for alkaline phosphatase activity and the number of PGCs counted (lower right panel). The median is depicted in red, n is the number of individual epiblast explants analysed. Abbreviations: PGCs, primordial germ cells. Scale bar is 100 μm.

a repressor of interferon-ß expression (Keller and Maniatis, 1991). Characterization of the protein revealed a proline-rich region, five Krüppel-type zinc finger motifs and a SET-PR domain, suggestive of lysine-specific histone methyltransferase activity. Blimp1 was independently identified as being involved in the differentiation of lymphocytes (Turner et al., 1994). Through the zinc finger domains, Blimp1 is capable of recruiting the histone H3 lysine methyltransferase G9a to the interferon-ß, promoter, resulting in transcriptional silencing (Gyory et al., 2004).

In PGCs, Blimp1 has been shown to interact with the arginine-specific histone methyltransferase Prmt5 (Ancelin et al., 2006). This complex is present in the nucleus of the PGCs from E8.5-E11.5 and is responsible for the high levels of symmetrical dimethylation of arginine 3 on histone H2A and/or H4 tails (H2A/H4R3me2s) observed. At E11.5, the Blimp1-Prmt5 complex translocates from the nucleus to the cytoplasm, which results in the derepression of *Dhx38* (Ancelin et al., 2006). In agreement, PGCs exhibit high chromatin remodelling activity during migration (Seki et al., 2005).

The expression of Blimp1 is probably not necessary for the formation of PGC precursors at E6.25, as mouse embryos deficient for *Blimp1* still express a tight cluster of *Ifitm3*-expressing cells with high levels of alkaline phosphates activity at E7.5 (Ohinata et al., 2005). We favour the hypothesis that Blimp1 does play an important role during PGC specification and, moreover, that it needs a partner to do so. This would explain why 'blimped' PGC precursors do not specify immediately after the upregulation of Blimp1 expression, but rather 'wait' 24 hours from the occurrence of the first

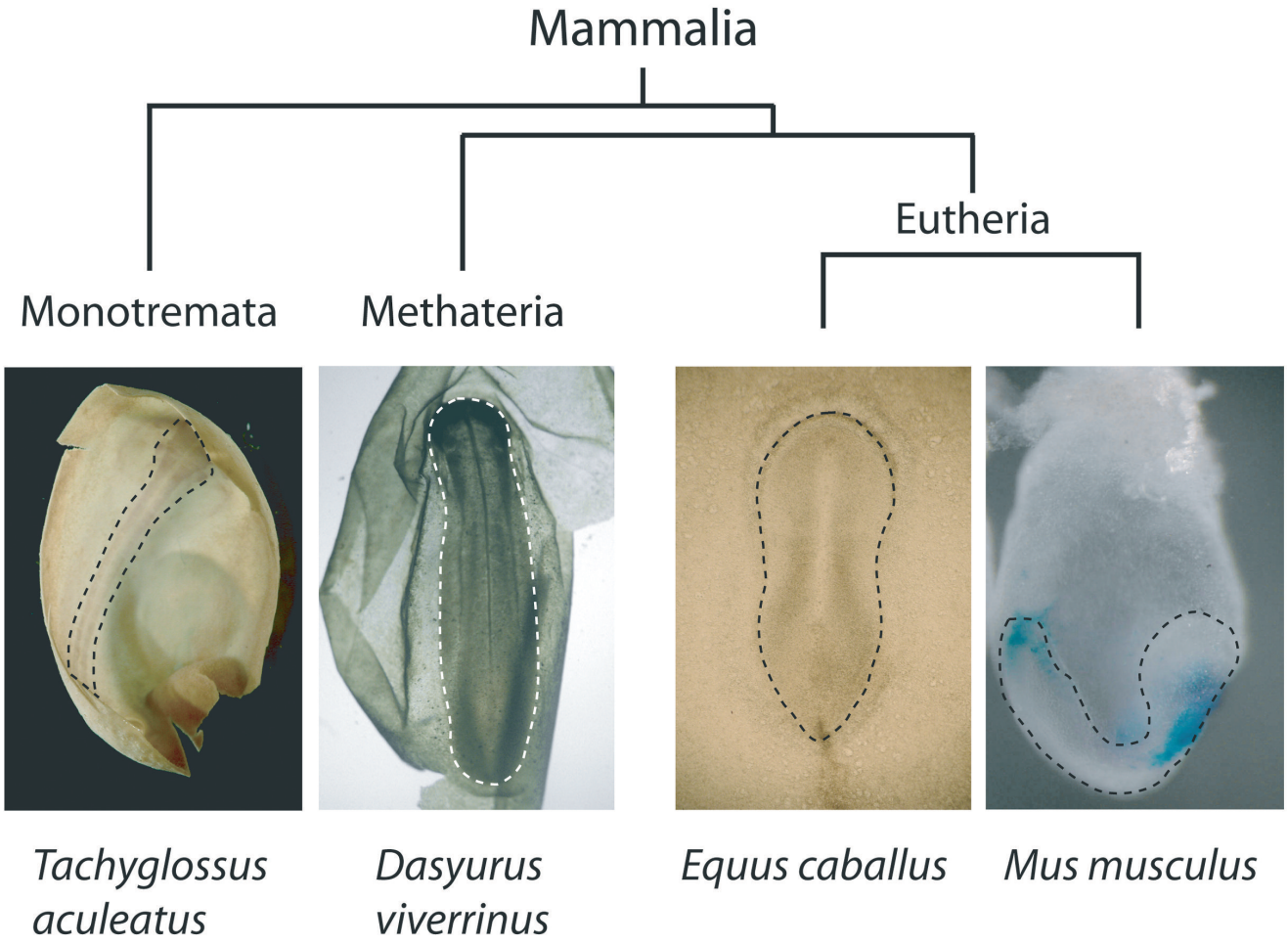

Fig. 5. Mammalian embryos. Shown are, from left to right, embryos of a short-beaked echidna, eastern quoll, horse and house mouse. Anterior is on the top, posterior on the bottom, except for the mouse embryo where anterior is right and posterior is left. Note the 'flat' morphology of the embryos, in contrast to the cup-shaped morphology of the mouse embryo, and the large area of extraembryonic structures. The embryonic regions are indicated by dotted lines. Embryos of the short-beaked echidna and eastern quoll belong to the Hubrecht collection, previously at the Hubrecht Institute in Utrecht, now in the Museum für Naturkunde in Berlin.

'blimped' PGC precursors to acquire PGC characteristics, and in particular to repress genes of the somatic program. Blimp1 has also been associated with transcriptional repression of cell cycle regulators, including *Myc*, which in PGCs is downregulated upon specification (Yabuta et al., 2006). The hypothetical partner for Blimp1 should be upregulated around E7.25 in the PGC precursors and is probably an enzyme with an epigenetic function, because Blimp1 has been observed to bind and form active complexes with the methyltransferases G9a and Prmt5.

Does Blimp1 play a role in preformed PGCs?

Germ cells in the mouse are thought to be induced, in contrast to germ cells in for instance, the zebrafish (*Danio rerio*), the chicken (*Gallus gallus*), the African clawed frog (*Xenopus leavis*), the nematode worm (*Caenorhabditis elegans*) or the fruitfly (*Drosophila melanogaster*), in which germ cells are formed by inheritance of maternal cytoplasmic components (preformation). It is not immediately clear why these two separate mechanisms evolved, but in the mouse, the embryo has first to develop extraembryonic cell lineages, the trophectoderm, the primitive endoderm and to some extent the extraembryonic mesoderm, that insure a proper implantation (and future placentation) of the embryo before it focuses on its own development. Nevertheless, after the formation of the extraembryonic lineages, the germ line is the first "embryonic" cell lineage to be restricted, just before the embryo gastrulates to form the three definitive germ layers (ectoderm, mesoderm, endoderm). This shows that also in the mouse, like the zebrafish, the frog or the fruitfly, specifying the germ line has high developmental priority. The early segregation of the germ line which occurs at a single moment during development is probably necessary to guarantee that the germ lineage maintain a naïve epigenetic signature.

Blimp1 has shown to be important both in *Xenopus* and zebrafish embryonic development (de Souza et al., 1999; Roy and Ng, 2004; Wilm and Solnica-Krezel, 2005). However, a role during germ cell development or expression of Blimp1 in their germ cells has not been observed. It is feasible that in organisms where PGC identity is inherited by acquisition of maternal determinants, the repression of the somatic program in the germ cells is achieved using different molecules. Therefore there may be no role for Blimp1 in the preformation mode of germ cell formation.

Finally, although the data available from placental (eutheria) mammals suggests an epigenetic mode of germ cell formation, a preformation mode of germ cell formation in egg-laying (monotremata) and marsupial (metatheria) mammals should not be ruled out (Extavour and Akam, 2003). In this respect, it is noteworthy that some phyla exhibit both the preformation and the epigenesis mode of germ cell formation side by side (Extavour and Akam, 2003). In addition, among placental mammals, the mouse embryo may be regarded as atypical, as embryos of most studied mammals exhibit a 'flat' morphology (including human embryos), in contrast to the cup-shaped embryos of rodents (Fig. 5). The cup-shaped mouse embryo has a relatively smaller distance between the anterior and posterior part, and the movement of cells from anterior-posterior and distal-proximal is therefore greatly facilitated. The compact character of the mouse embryo and intimate relationship with the extraembryonic tissues also privileges the formation of small specific inductive regions. In 'flat' embryos, it can be expected that the mechanisms responsible for this are slightly different. The time and place of germ cell origin in egg-laying and marsupial mammals, as well as in most placental mammals, and the existence of 'blimped' PGC precursors remains to be determined.

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