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Review

Primordial germ cells in the mouse

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Historical perspective

Germ cells may be defined as those cells, all of whose surviving descendants will become sperm or eggs. In all sexually reproducing animals and plants, these cells play a uniquely important role, namely the transmission (after meiotic recombination) of genetic information from one generation to the next. The eventual production of sperm and eggs (spermatogenesis and oogenesis) has been intensively studied by reproductive physiologists in various species of both Vertebrates and Invertebrates for many years.

In contrast, the origin of the germ cell lineage, including the emergence and fate of the first, primordial germ cells (often termed PGCs), has received less attention, perhaps because it is of less practical importance, but also because it is technically more difficult. However, zoologists realized more than a century ago that, in certain organisms, cells were set aside very early in development to segregate the germ cell lineage from the other, mortal, somatic lineages. For example, Boveri carried out a classic series of observations between 1887 and 1910 on the nematode *Ascaris*, showing that the germ cell lineage was segregated during the first few cleavage divisions, retaining its chromatin complement intact, while all the somatic lineages suffered "chromatin diminution" in which large terminal regions of the chromosomes were discarded. These and subsequent studies on *Ascaris* are summarized by Nieukoop and Sutasurya (1981).

Similarly, when embryologists examined developing frog eggs, they observed aggregates of mitochondria, protein, and RNA in the cytoplasm of the vegetal pole, even as early as the unfertilised egg. These aggregates appeared to segregate into the germ cell lineage ("germ plasm", see Mahowald and Hennen, 1971). Confirmed by later studies in *Xenopus*, the findings were matched in *Drosophila* by the finding that the pole cells, the origin of all subsequent *Drosophila* germ cells, contained specific cytoplasm ("pole plasm") rich in germ cell determinants (Illmensee and Mahowald, 1974). They were the very first cells to be formed, at the posterior end of the fertilized egg. Similarly, in *Caenorhabditis elegans*, the other favourite invertebrate model organism, there are polar granules (P granules) in the unfertilised egg which are asymmetrically distributed to daughter cells at each of the first four cleavage divisions, to be concentrated finally in the P4 cells that are the ancestors of the entire *C.elegans* germ-cell lineage (Strome and Wood, 1982). The germ cell lineage in zebrafish and also in chick has been followed from the two-cell stage onwards, using germline-specific expression of vertebrate homologues of the *vasa* gene, known to be a germline determinant in *Drosophila* (Knaut et al., 2000; Tsunekawa et al., 2000).

The belief therefore grew up that germ cells, evidently a uniquely important and special cell type, were segregated early in development, and inherited from the mother's egg a special sort of cytoplasm (germ plasm, pole plasm, polar granules) rich in germ-cell determinants.

Origin of the mouse germ line

But mice were a problem. Try as they might, developmental biologists were unable to identify any cells in the cleavage embryo (Kelly, 1977) nor even in the late blastocyst (Gardner, 1977) that gave rise only to germ cells. Nor was it possible to identify, by electron microscopy or any other means, cytoplasmic aggregations in the egg or early embryo that could be construed as equivalent to germ plasm (see Eddy and Hahnel, 1983). Once the embryo implanted (4–5 days *postcoitum*), it became even less amenable to experimental investigation.

Later in development, however, primordial germ cells

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were relatively easy to identify. Thanks to their high level of tissue nonspecific alkaline phosphatase (TNAP) activity, primordial germ cells (PGCs) were identified in mouse embryos 8.5 days postcoitum (dpc) as long ago as 1954 by Chiquoine. TNAP appears not to be needed for germ cell survival (MacGregor et al., 1995), but it has proved invaluable as a marker. Thus, Mintz and Russell (1957) were able to follow the migration pathway of mouse PGCs from the base of the allantois at 8.5 dpc to their entry into the genital ridges, the site of the future gonads. Ozdzenski (1967), using the same TNAP marker, identified PGCs at the base of the allantois a few hours earlier. However, with the histological techniques available at the time, no TNAP-positive PGCs could be identified earlier than 8.0 dpc, so it was not clear where the germ cell lineage had originated.

Because many of the PGCs at 8.5 dpc were embedded in the endoderm as it began to invaginate to form the hind gut, there grew a general belief that the germ cell lineage in mammals originated in extraembryonic endoderm. This belief persisted in textbooks of embryology for many years. However, several lines of evidence suggested that, at least in the mouse, PGCs were derived from the epiblast (embryonic ectoderm), not from the endoderm (for summary, see McLaren, 1983). The transplantation experiments of Gardner and Rossant (1979) established this epiblast origin beyond doubt.

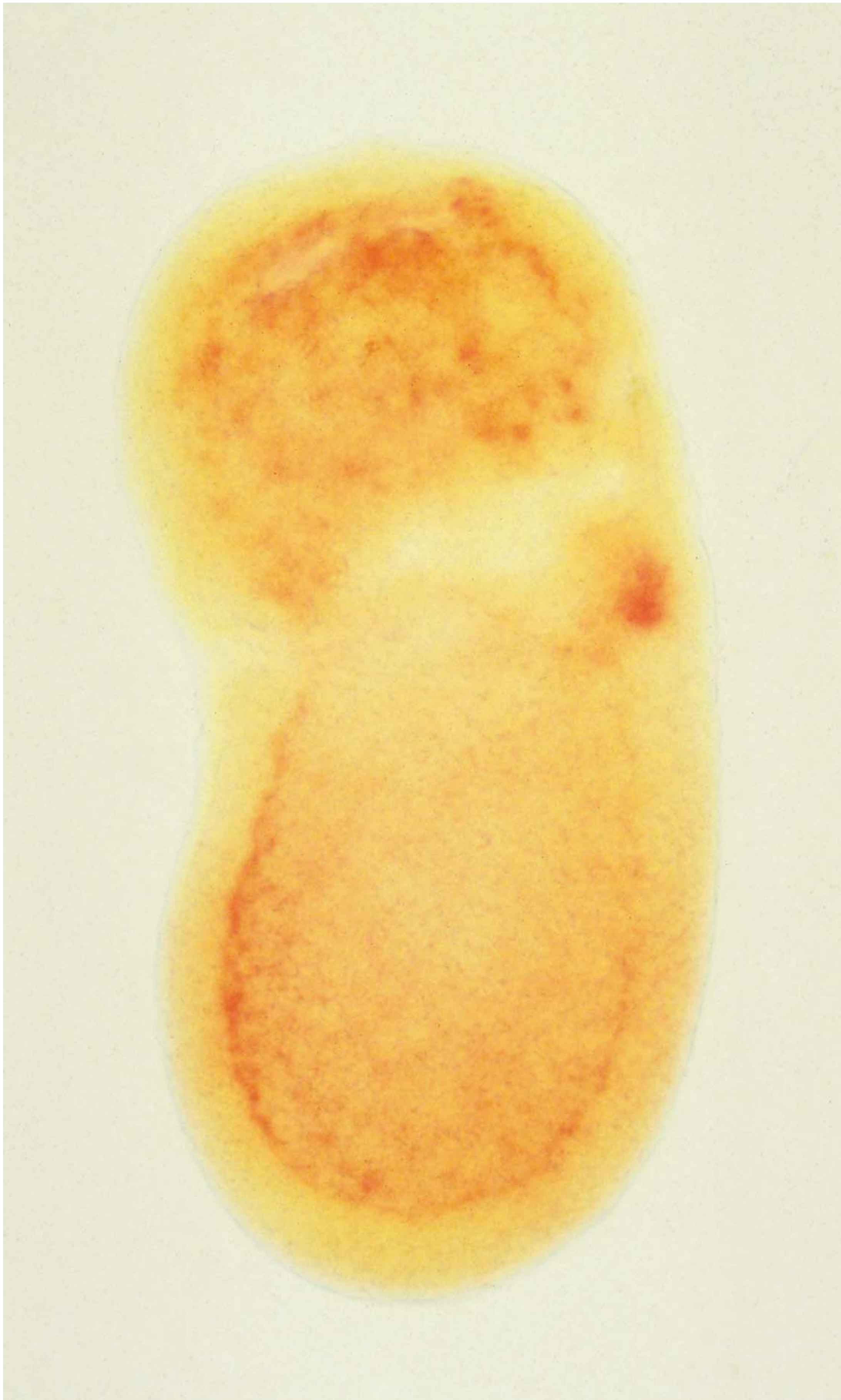
In an early approach to fate-mapping, Snow (1981) cut out segments of the mouse egg cylinder at 7 dpc and cultured each segment and its donor embryo for 24-36 h to see to what extent the fate in vitro would mirror its in vivo expectation. The correlation turned out to be very close. In particular, fragment 7 (extraembryonic, at the posterior end of the primitive streak, at the base of what would subsequently become the allantois) turned out to contain most of the PGCs, while the donor embryo contained very few PGCs. Encouraged by the knowledge that PGC ancestors were located in the same position at 7–7.5 dpc as they were at 8–8.5 dpc, a sensitive whole-mount technique was devised to identify alkaline phosphatase, allowing the visualisation of a cluster of PGCs as early as 7.25 dpc (Ginsburg et al., 1990) (Fig. 1).

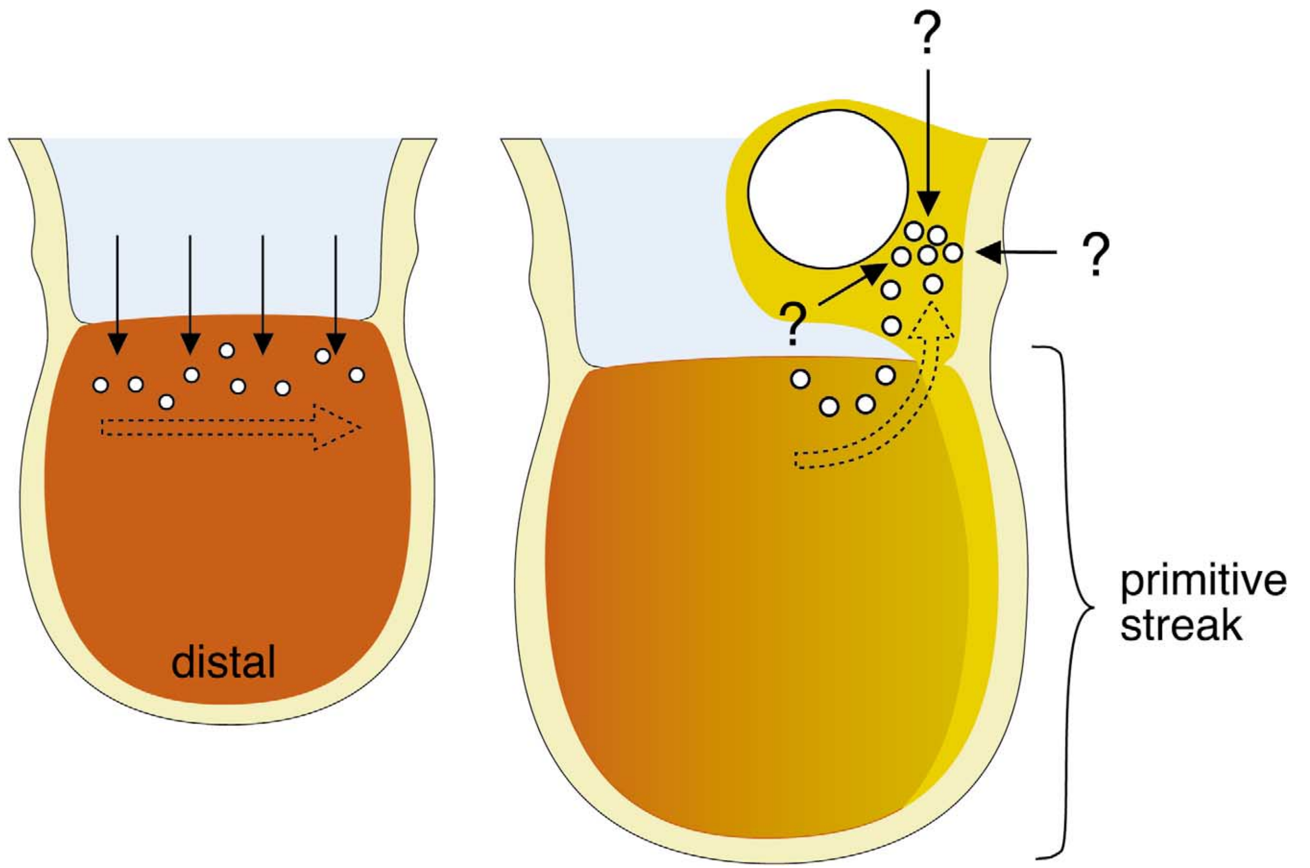
In 1994, Lawson and Hage, by injecting single epiblast cells at 6.0 and 6.5 dpc with a lineage marker and following the fate of their clonal descendants, were able to show that the ancestors of the PGCs were derived from proximal epiblast cells, adjacent to the extraembryonic ectoderm. During the course of gastrulation the cells in this location moved through the posterior primitive streak into the extraembryonic region. Each of the injected epiblast cells gave rise to a marked clone, which was identified and analysed after about 40 h in culture. None of these clones contained only PGCs, proving that, even at 6.5 dpc, the PGC lineage was not determined (lineage-restricted). For those clones that included PGCs, the PGCs constituted less than 15% of the total clone, and less than 10% of the total number of PGCs that were present at the end of the culture period. Clonal analysis established that germ cell fate was determined in a group of about 45 progenitor cells, at about 7.2 dpc, placing the lineage-restriction event at the location of the alkaline-phosphatase-positive cluster visualised earlier by Ginsburg et al. (1990). Once lineage-restricted, the PGCs slowed down their doubling time from the 6.6–6.8 h of the surrounding migrating extraembryonic mesoderm cells to about 16 h (similar to the doubling time of PGCs from 8.5 to 13.5 dpc as estimated by Tam and Snow, 1981).

Evidently, the founding of the mouse germ-cell lineage is accompanied by a change in cell-cycle regulation, as well as by a marked increase in alkaline phosphatase expression. *Oct-4*, a Pou transcription factor (Schöler et al., 1990), is still widely expressed in the epiblast at this time, and only becomes restricted to the germ-cell lineage at about 8.0 dpc. Little was known until recently about gene expression in PGC founders and their immediate ancestors. However, mutants defective for the signalling molecule BMP4 proved to be devoid of both PGCs and allantois (Lawson et al, 1999). *Bmp8b* mutants had a similar though less marked effect on germ cell number. In vitro studies have established that *Bmp4* and *Bmp8b* signalling pathways act synergistically (Ying et al., 2001). A chimera experiment involving normal ES cells established that BMP4 was required in the extraembryonic ectoderm, rather than in the epiblast cells themselves. Evidently, a BMP-mediated signal was required to predispose the adjacent proximal epiblast cells to give rise to PGCs among their descendants. Other subsequent signal(s) may well be required to complete the PGC determination event in the extraembryonic "cluster" location (Fig. 2, from McLaren, 1999). Using elegant transplantation techniques, Tam and Zhou (1996) established that the proximal epiblast cells were in no way predetermined for a PGC fate: even cells from the distal tip of the epiblast could give rise to PGCs if transplanted at the appropriate time to a proximal location. Distal epiblast cells explanted in vitro, if juxtaposed to extraembryonic ectoderm, could also give rise to cells resembling PGCs (Yoshimizu et al., 2001).

Saitou et al. (2002) derived single-cell cDNAs from the Fragment 7 region (Snow, 1981), and separated them into putative PGC and putative somatic cell samples. Differential screening of a PGC-specific cDNA library led to the isolation of two genes of particular interest. *Fragilis* is a member of an interferon-inducible gene family, other members of which show homotypic adhesion and regulation of cell-cycle control (properties possibly characteristic of the

Fig. 1. Whole mount of mouse embryo in midgastrulation, stained for alkaline phosphatase (TNAP). The strongly expressing cluster of cells that represents the origin of the mouse germ cell lineage lies on the right-hand side, at the upper end of the primitive streak, in the extraembryonic region (note the enlarging exocoelomic cavity and the patches of alkaline-phosphatase-positive cells in the ectoplacental cone). The embryonic region lies below and a layer of visceral endoderm surrounds the whole structure.





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primordial germ cell cluster). The expression of *Fragilis* is first seen in the proximal epiblast at about 6.0 dpc. *Smad1* is also expressed in the epiblast at this time, and is responsible for signal transduction. *Smad1* mutant embryos have fewer PGCs. *Fragilis* expression strengthens and moves posteriorly, so that by 7.2 dpc it occupies a relatively large area (including about 150 cells) at the proximal end of the primitive streak and base of the future allantois, i.e., in the region of the PGC cluster (Fig. 3).

The other gene identified by Saitou et al. (2002) is a novel gene, *Stella* (equivalent to *Pgc7*, identified by Sato et al., 2002). *Stella* is first expressed at about 7.2 dpc, in the PGC cluster region, in the centre of *Fragilis* expression. It appears to be germ-cell-specific, since it continues to be expressed in PGCs as they migrate along the hind gut and into the genital ridges. *Stella* is also expressed in preimplantation embryos and in ES cells (Sato et al., 2002).

Expression of mesoderm-specific genes (*Brachyury, Fgf8*) is maintained for a time in nascent germ cells, and other germline-specific genes (*Tnap, Oct4*) are upregulated. However, there is marked (though transient) downregulation of region-specific homeobox genes (*HoxB1, Hoxa1, Lim1, Evx1*), also *Smad1* (Lange, Saitou, and Surani, personal communication), all of which are expressed by the surrounding somatic cells. This is reminiscent of the repression of transcription seen in the early germline of both *C. elegans* (Seydoux et al., 1996) and *Drosophila* (Van Doren et al., 1998), suggesting that transcriptional downregulation of somatic genes may be a conserved characteristic of germ cell determination. Chang and Calame (2002) report that *Blimp-1* (B lymphocyte-induced maturation protein-1), a transcriptional repressor, is expressed in the mouse germline up until about 13 dpc.

Are mammals unique?

Thus, the germ cell lineage in mice appears to be induced by external signals, midway through gastrulation, rather than originating from preformed cytoplasmic determinants present in early embryogenesis (as in frogs, also *Drosophila* and *C. elegans*). Should not the mechanism of such a key developmental process have been conserved during evolution?

The difference between the mechanism for establishing the germ cell lineage in frogs and mice becomes less starting when one finds that it is remarkably similar to the difference between the mechanism for establishing the germ cell lineage in frogs and newts (both are Amphibia). In Anura (frogs, toads), PGCs have an early, endodermal origin, with an uninterrupted "germ line" characterised by germ plasm localised to specific cells. In Urodeles (newts, salamanders), PGCs were reported to arise from presumptive lateral plate mesoderm in midgastrulation, with no indications of germ plasm (Humphrey, 1929; Nieuwkoop, 1947). The later experiments of Nieuwkoop (1969) and Satasurja and Nieuwkoop (1974) on the Urodele *Ambystoma* established that the PGCs, along with other mesodermal tissues, originated in ectoderm, under the inductive influence of the ventral yolk mass, during the course of gastrulation—an origin remarkably similar to that now established for the mouse.

Johnson et al. (2003b) have proposed that the mouse/axolotl regulative mode of germ cell specification is the more primitive, from which various types of predetermined germ cell development, involving localized germ cell determinants (e.g., germ plasm), have evolved independently in various animal lineages, supposedly to escape a developmental constraint imposed by ancestral embryological processes. Using the presence of a mitochondrial cloud during early development as an indicator of germ plasm, they report that not only axolotls (Fig. 4) but also lungfish (thought to be the closest living relative of the tetrapod ancestor) resemble mice in lacking any mitochondrial cloud in the oocytes, unlike zebrafish, frogs, *Xenopus*, and chick. Urodeles develop nuage-like material, but later in oogenesis, while mitochondrial clouds (which have been reported in several mammals, including human and rat) may not necessarily be associated with germ plasm. Genes that are highly conserved and expressed in the germ cell lineage of many animals include *DAZ-like* and *vasa.* RNA encoded by the *Xenopus DAZ-like* homolog is a crucial component of germ plasm (Houston and King, 2000), associated with the

Fig. 2. Diagram modified from McLaren (1999). (Left) At 6.0 dpc, a signal (solid arrows) coming from the extraembryonic ectoderm (blue) predisposes cells in the proximal layer of the epiblast (brown) towards a germ-line fate. This whole layer of PGC precursors moves (dashed arrow) towards the primitive streak and up into the extraembryonic region. (Right) At 7.0 dpc, the newly formed extraembryonic mesoderm (gold) is moving across to form the exocoelomic cavity (white). Some of the PGC precursors stop migrating and constitute the cluster of cells representing the origin of the germ cell lineage. This may involve a second (unidentified) signal or signals (solid arrows). Yellow, visceral endoderm.

Fig. 3. Diagram reproduced from Saitou et al. (2002), illustrating the *fragilis* expression pattern during gastrulation. (a) Lateral view of an early bud stage embryo with expression of *fragilis*. Anterior (A) is to the left and posterior (P) to the right. Strong expression was observed specifically at the base of the incipient allantois, the location of nascent PGCs. (b) *fragilis* expression from mid bud (farthest left) to early head fold (farthest right) stages, as viewed from the lateral side. *fragilis* expression is strong in the centre (arrowheads)—the site of the founder PGC cluster. *fragilis* expression fades at the early head fold stage (extreme right) when PGCs commence migration. Arrows indicate developing allantois where *fragilis* expression is weak or absent. (c) Lateral views of pre-streak-stage embryos (6.25 dpc) with expression of *fragilis.* Intense signal was observed in proximal epiblast cells adjacent to the extraembryonic ectoderm (arrowheads). (d) *fragilis* expression from pre-streak (6.0 dpc) (farthest left) to early bud (farthest right) stages. The initial domain of fragilis expression in the most proximal epiblast followed by its movement to the posterior region during gastrulation is shown. This expression pattern matches with the observations from clonal analysis for the origin, migration and segregation of the germ cell lineage.

mitochondrial cloud, and showing localised expression at all stages of oogenesis and embryogenesis. The axolotl homolog is also expressed in the oocyte, but the RNA is not localised, consistent with previous suggestions that axolotl embryos do not contain germ plasm. Axolotl *DAZ-like* only shows germ-cell-specific expression as the cells approach the gonad, after the establishment of the germ cell lineage (Johnson et al., 2001). *Vasa* homologs are expressed in frog germ plasm, *Drosophila* pole plasm, and in the earliest stages of embryogenesis in Zebrafish and chick, but in mice and in Axolotls, zygotic *vasa* is not expressed until the PGCs begin to colonize the gonad. Even where *vasa* homologs are expressed in early development, they may not play an essential role at this stage. Further evidence relating to the evolution of germ plasm and germ cell determinants has recently been obtained by phylogenetic analysis of the sequences encoding the *DAZ-like, vasa*, and *Oct-4* genes (Johnson et al., 2003a).

Migration

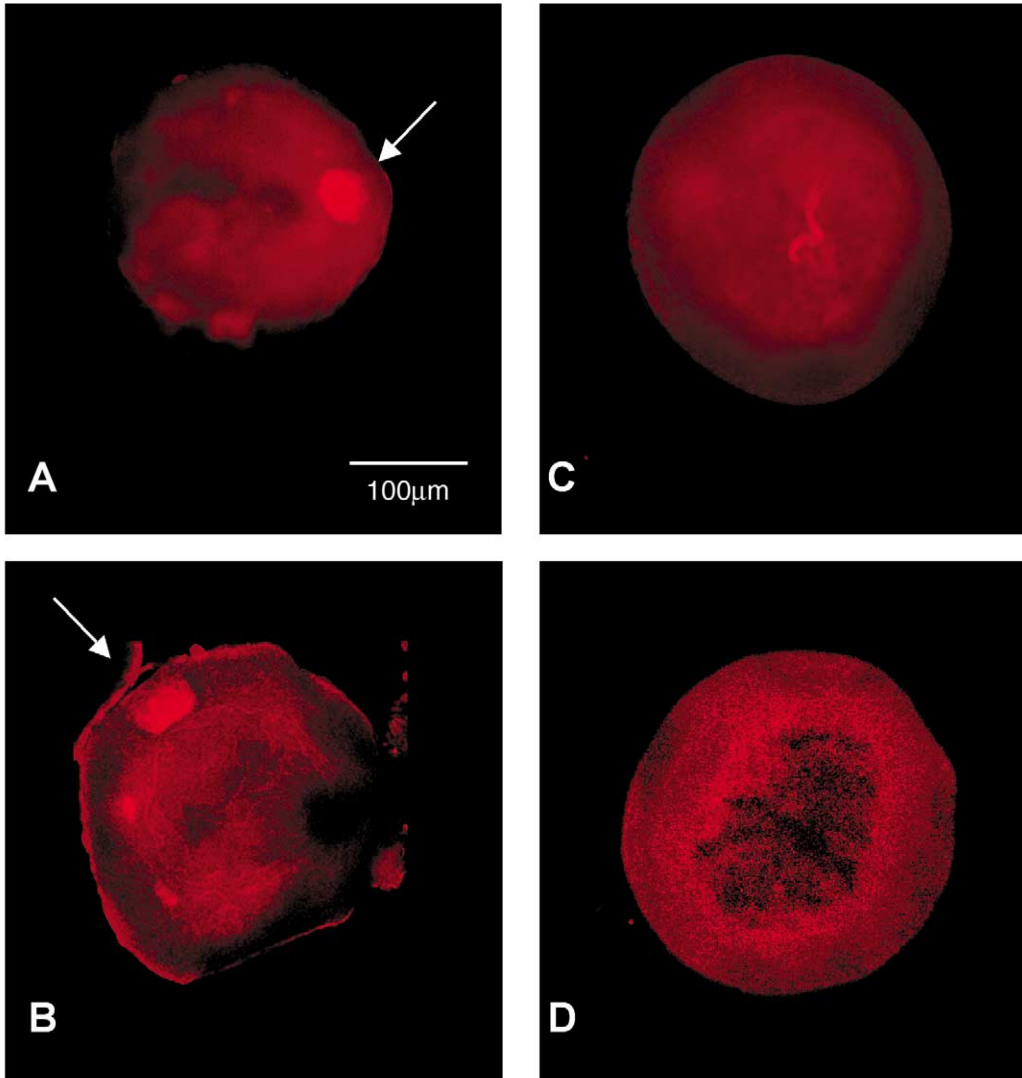
Within 24 h of establishment of the mouse germ cell lineage, *Fragilis* is downregulated and the cluster begins to fragment. By 8.5 dpc, the endoderm is invaginating to form the hind gut, and the PGCs are carried along with the endoderm cells. Eventually, when the hind gut is fully extended, the PGCs lie along its length, and only a few alkaline-phosphatase-positive germ cells remain in the cluster location, at the base of the allantois which by now is growing up towards the chorion. Although initially located in a ventral position in the hind gut wall, the PGCs move dorsally, then into the body wall towards the notochord and the dorsal aorta, round the coelomic angle on each side, and into the two nascent genital ridges. A few end up in the adrenal (initially part of the same primordium as the urogenital ridge).

A number of mutants interfere with germ cell migration, including knock-outs of *β2 integrin* (Anderson et al., 1999), *Fgf8* (Sun et al., 1999), and the insertion mutation *germ cell deficient* (Pellas et al., 1991). In particular, the c-kit/Steel Factor (SF) signal transduction pathway is required for normal migration. The downstream c-kit signalling pathway in PGCs has recently been dissected, using a retroviral-mediated gene delivery system (De Miguel et al., 2002). Mutations in either *Steel* (which codes for SF) or *W* (which codes for c-kit, the receptor for SF) interfere with both germ cell proliferation and migration (Mintz and Russell, 1957; Donovan, 1994). In extreme mutants (e.g., W^e homozygotes), few if any germ cells reach the genital ridges (Buehr and McLaren, 1993). Establishment of the germ cell lineage appears to occur normally, followed by a modest increase in numbers, for which the c-kit/SF pathway is presumably not required; but after 8 dpc, no further increase occurs. The PGCs form clumps in the hind gut, suggesting an abnormality in either cell surface properties or in motility, and some fail to leave the base of the allantois. Nonetheless, the reduced number of germ cells become distributed along the entire length of the hind gut, as normal, indicating that the hind gut endoderm carries the germ cells along passively, as it invaginates (Fig. 5). Mostly they remain in the ventral portion of the hind gut, few enter the body wall or travel up the dorsal mesentery, some end up in ectopic sites. Death of the germ cells in both *W* and *Steel* mutants is presumably by apoptosis: Steel Factor (hence also its receptor c-kit) is necessary to suppress apoptosis in PGCs, and PGCs in extragonadal sites die by apoptosis (Pesce et al., 1993).

Further information on the role of growth factors during migration are to be found in a valuable review by Wylie (1999), whose group has more recently used a GFP (green fluorescent protein) tagged *Oct4* construct, to make time-lapse videos of PGCs throughout the migration pathway (Molyneaux et al., 2001). The germ cells show active locomotory movements at all times until they enter the genital ridges. It is not clear whether the spreading of the germ cells from the cluster into the extraembryonic endoderm and thence to the hind gut is an active migration or part of a morphogenetic tissue movement.

The videos confirm that the germ cells in the hind gut, although motile, do not migrate anteriorly in a directed manner. Distribution around the hind gut from the initial ventral location to the dorsal side may be due to random dispersion, since the cells are moving very actively, but it is perhaps more likely to be due to directed migration, since by 9.5 dpc, the majority of the PGCs are moving out of the hind gut and into the dorsal body wall. The videos show that movement at this time is initially random with respect to the future genital ridges, but by 10.5 dpc, the PGCs in the body wall as well as the minority that are in the newly formed hind gut mesentery, are predominantly showing directed movement. Most of the PGCs reach one or other of the genital ridges, but those that remain in the midline position or lag behind in the mesentery are lost (Molyneaux et al., 2001).

After leaving the hind gut, the germ cells tend to contact each other by extending processes, to form a network (Gomperts et al., 1994). Cell adhesion to laminin may also play a role in PGC guidance, perhaps involving the regulation of expression of integrins and/or proteoglycans on the cell surface (Garcia-Castro et al., 1997). However, the changed behaviour of the germ cells at 10.5 dpc, once the genital ridges have started to form, strongly suggests that they are responding to a chemotropic signal. The nature of the signal in mice is not known, but in zebrafish, there is now strong evidence that PGC migration is guided by a pair of evoluntionarily conserved molecules: the chemokine SDF-1 (stromal cell-derived factor 1), which is expressed in locations towards which the cells migrate, including the site of the future gonad, and its receptor CXCR4, expressed on germ cells (Doitsidou et al., 2002; Knaut et al., 2003). Knock-downs of either SDF-1 or its receptor CXCR4 produced very aberrant PGC migration. SDF-1 and CXCR4 are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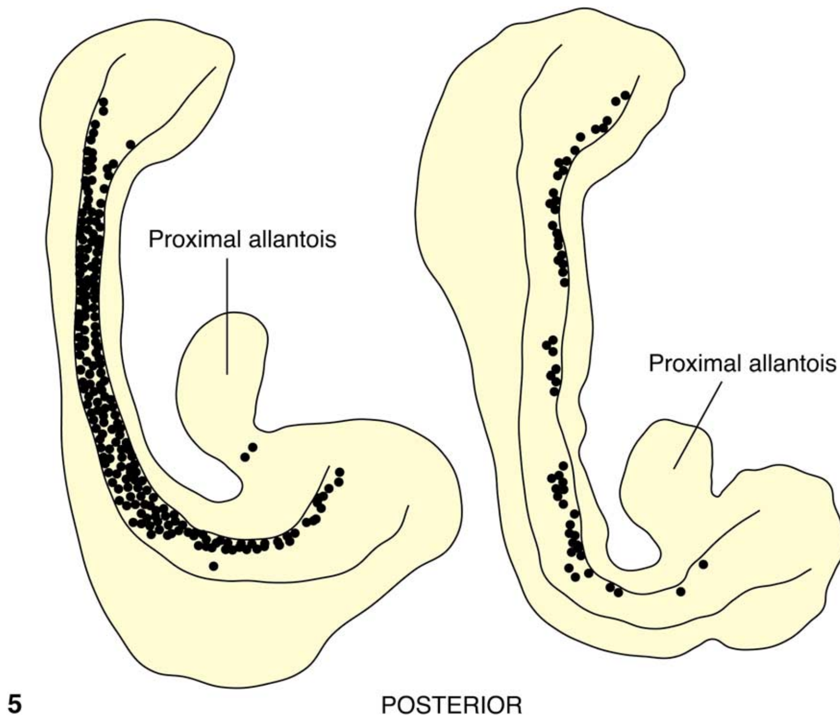
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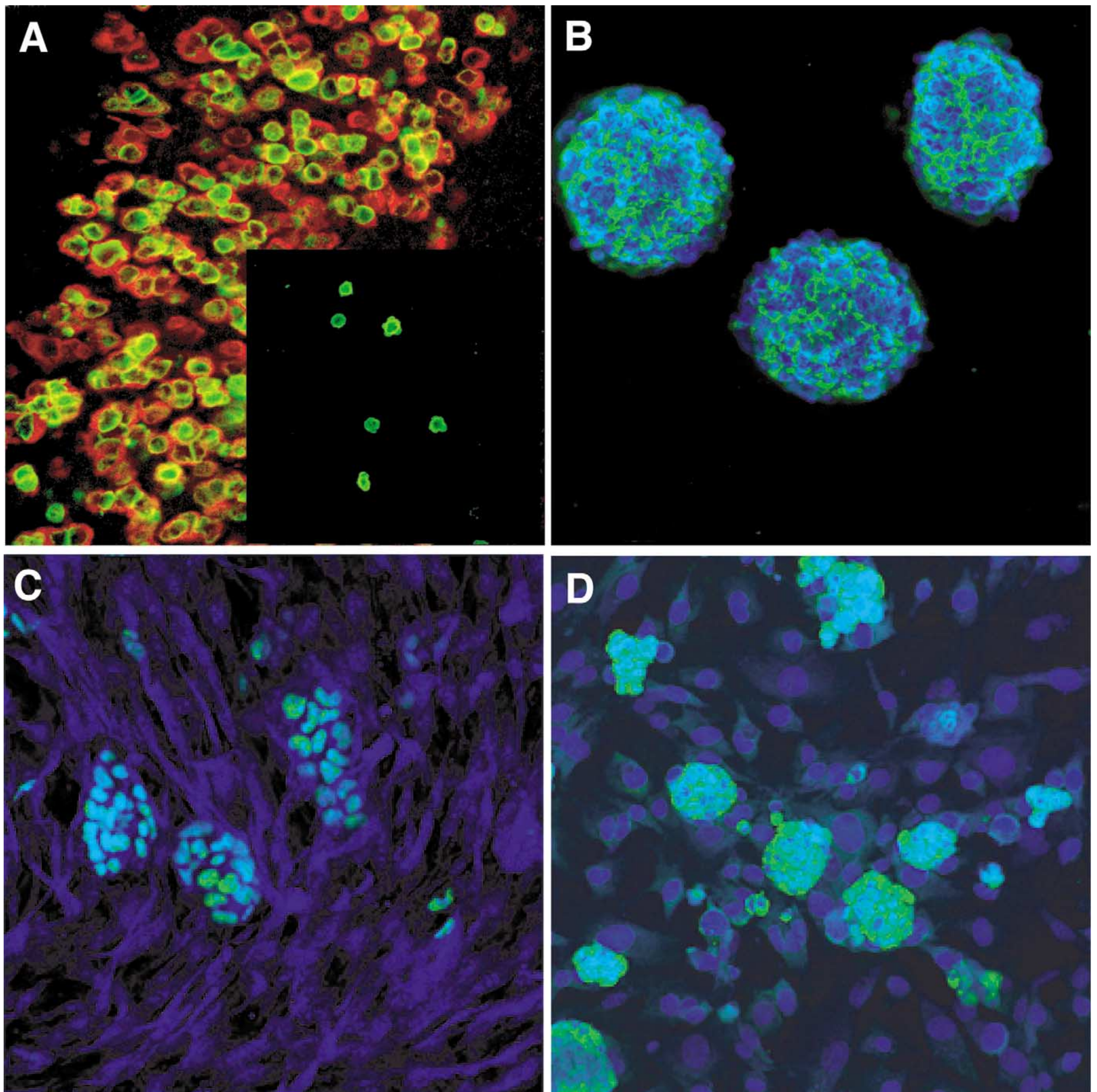

Fig. 6. (A, B) Reproduced from McLaren and Durcova-Hills (2001): (A) Primordial germ cells (PGCs) in a female genital ridge, 12.5 days postcoitum (dpc), stained with antibodies to both mouse vasa homologue (MVH;red) and germ cell nuclear antigen 1 (GCNA1;green). (Inset) Isolated PGCs expressing stage-specific nuclear antigen 1 (SSEA1;green). (B–D) EG cell colonies. Nuclei are stained with TOTO-3 (blue). EG cells expressing (B) SSEA-1 (green), (C) Oct4 (green), (D) GCNA (green). Samples were observed under a confocal microscope. Magnification: ×400 in A, ×200 in (B–D).

Fig. 4. Reproduced from Johnson et al., (2001). Previtellogenic axolotl oocytes ranging from 170 to 390 μm in diameter and *Xenopus* oocytes ranging from 140 to 290 μm were incubated in Mito Tracker Red and examined either by Quantitative Fluorescence Microscopy (A, C) or as optical sections with a confocal microscope (B, D). (A, B) Examples of *Xenopus* oocytes showing large aggregates of mitochondria corresponding to the mitochondrial cloud (arrow). (C, D) Examples of axolotl oocytes lacking a mitochondrial cloud.

Fig. 5. Modified from Buehr et al. (1993). Diagrammatic reconstruction (based on camera lucida drawings) of the anterior–posterior distribution of germ cells in the hindgut of a $W^e/^+$ embryo (18 somites) and a W^e/W^e embryo (15 somites). Migration along the hind gut is passive: germ cells in embryos with migration defects still extend along the hind gut.

known to play a role in leukocyte movements in mammals, but no reports of their involvement in mouse PGC migration have yet appeared.

During the migratory period, PGCs with two X chromosomes have one X randomly inactivated, like XX somatic cells (McMahon et al., 1981). The proportions of cells with one or the other X chromosome inactivated were compared in various somatic tissues and in the germ cells, within and between embryos (the two Xs carried different X-coded iso-enzyme alleles). The correlations observed were then used to calculate statistically the likely number of cells in each founder population at the time of X-chromosome inactivation. The estimated number for each tissue (including PGCs) was 193 (McMahon et al., 1983). Nesbitt (1971), using late replication of a translocated X chromosome as a cell marker, had calculated smaller primordial precursor pool sizes (21–58) for a number of tissues. These estimates, which are not dissimilar to the estimate of 45 progenitor cells derived from the subsequent clonal analysis of Lawson and Hage (1994), constituted the first indication that mouse PGCs were derived from a relatively large pool of cells, hence relatively late in embryogenesis. An alternative model, of early germ line allocation (Soriano and Jaenisch, 1986), does not fit well with the more recent findings.

Germ cells in the genital ridge

Once the germ cells are in the genital ridge, they start to express new germ-cell-specific genes (Fig. 6), including the highly conserved *mouse vasa homolog (Mvh)* (Toyooka et al., 2000), *germ cell nuclear antigen 1 (Gcna1)* (Enders and May, 1994), and *germ cell-less (Gcl)* (Kimura et al., 1999). Subsequently (Kimura et al., 1999), *Tnap* and other genes such as *Ssea1* are downregulated. These changes in gene expression are part of the general reprogramming process that germ cells undergo at this time.

Sex determination

In most mouse strains, PGCs enter the genital ridges between 10 and 11 dpc. From this time on, they are no longer locomotory, and are sometimes termed gonocytes rather than PGCs. They undergo two or three further rounds of mitosis, but by 12.5 dpc in both female and male embryos they enter a premeiotic stage and upregulate meiotic genes such as *Scp3* (Di Carlo et al., 2000; Chuma and Nakatsuji, 2000). In the male genital ridge, meiosis proceeds no further, *Scp3* is downregulated, and the germ cells (whether XY or XX in sex chromosome constitution) enter mitotic arrest as GO/G1 prospermatogonia (McLaren, 1984). Mitosis in the male genital ridge is not resumed until after birth.

In the female genital ridge, in contrast, germ cells, whatever their sex chromosome constitution, enter meiotic prophase as oocytes, and pass through leptotene, zygotene, and pachytene stages before arresting in diplotene at about the time of birth. Germ cells enter meiotic prophase at about the same time not only in the female genital ridge, but also outside the genital ridge, for example, in the adrenal gland of either the female or male embryo (Upadhyay and Zamboni, 1982; McLaren, 1995), or in a cultured reaggregate of fetal lung cells (McLaren and Southee, 1997), or isolated onto a feeder layer in vitro (Chuma and Nakatsuji, 2000). We assume therefore that all germ cells, whatever their sex chromosome constitution, are programmed to develop as oocytes. The timing of meiotic entry appears not to be an induced response, but is cell-autonomous, related perhaps to the interval since the establishment of the germ cell lineage, or since departure from the initial cluster. Only in the male genital ridge does this prenatal entry into meiosis fail to occur.

The block to meiotic entry in the male genital ridge occurs at about 12.5 dpc, the stage at which Sertoli cells have differentiated and testis cords have formed. From 12.5 dpc on, the germ cells are committed to spermatogenesis, but if they are removed before that time, and mixed with either 12.5 dpc female genital ridge cells (Fig. 7) or fetal lung cells, they enter meiosis. If a male genital ridge is disaggregated and reaggregated at 11.5 dpc, testis cords do not develop nor does the block to meiosis: all the germ cells enter the oogenesis pathway (McLaren and Southee, 1997). Germ cells from a female genital ridge can also be inhibited from entering meiosis if they are aggregated with 12.5 dpc male genital ridge cells, but only if they are 12.5 dpc or younger; by 13.5 dpc, the germ cells in the female genital ridge are committed to oogenesis and cannot be diverted (Adams and McLaren, 2002) (Fig. 7). Thus, entry into the spermatogenic pathway is not cell-autonomous, but is an induced response.

In a male genital ridge separated at 11.5 dpc from the mesonephric region, Sertoli cells differentiate but testis cords do not form, probably because the progenitors of peritubular myoid cells normally migrate in from the mesonephros after 11.5 dpc. The germ cells develop as prospermatogonia rather than as oocytes (Buehr et al., 1993). This suggests that the meiosis inhibitor is a signalling factor produced by Sertoli cells, probably a diffusible molecule since germ cells outside the testis cords and even in the mesonephric region outside the genital ridge may develop as prospermatogonia (McLaren, 1985). Possible candidates include Prostaglandin D2 (Adams and McLaren, 2002) and TDL (Yamamoto and Matsui, 2002). In XX Sxr (sex-reversed) male mice, all the XX germ cells in the genital ridge develop as prospermatogonia (McLaren, 1981), but die soon after birth. XY germ cells in a female genital ridge enter meiosis and undergo oogenesis, but few form mature oocytes.

X-chromosome reactivation

XX germ cells have one X chromosome randomly inactivated during the migratory period (McMahon et al., 1981).

However, both X chromosomes are active during oogenesis, since the silent X is reactivated on entry into the genital ridge. This was first shown by Monk and McLaren (1981), using a sensitive assay to measure the ratio between an X-coded and an autosomal enzyme (HPRT and APRT), both for XX, XY, and XO PGCs in female genital ridges, and for XY and XX Sxr (sex-reversed) PGCs in male genital ridges (McLaren and Monk, 1981). This result was later confirmed by Tam et al. (1994), using a LacZ transgene on the X chromosome, and more recently by a study of *Xist* expression (Nesterova et al., 2002). *Xist* codes for a stable RNA, which coats the inactive X chromosome in migrating germ cells (just as in XX somatic cells), but disperses shortly after the germ cells enter the genital ridge. Some X-borne genes show biallelic expression on entry into the genital ridge, even before loss of the *Xist* RNA (N.R. Nesterova, personal communication).

Although an exact time cannot be given, X-chromosome reactivation appears to begin soon after entry into the genital ridge, certainly before the PGCs enter meiosis. Whether the silent X chromosome is reactivated cell-autonomously, as for entry into meiosis, or in response to some signal from the neighbouring somatic cells, like the block to meiosis, is not yet known.

Methylation and imprinted genes

The level of global methylation of CpG sites is markedly low in blastocysts and in germ cells once they have entered the genital ridge (Monk et al., 1987). Whether the level of methylation in the germ cell lineage is higher in the intervening period, perhaps more similar to the level seen in somatic lineages, or whether it remains low throughout, has not been established.

For imprinted genes where maternal and paternal alleles are differentially expressed (see Reik and Surani, 1997; Arney et al., 2001), differential methylation acts as a convenient marker of imprinting status, whether or not it plays a primary causal role. The distinction between the maternal and paternal alleles has to be erased in the germline, and reimposed according to the sex of the fetus. The time of erasure of differential methylation is most reliably determined by the technique of bisulphite sequencing. Recent results (Hajkova et al., 2002; Lee et al., 2002) suggest that the rate and timing are different for different imprinted genes (Fig. 8). Erasure may start before entry into the genital ridges, or somewhat later, but for most it is complete within the next few days, in both sexes. Thus, demethylation at this stage of development is genome-wide, affecting both imprinted and nonimprinted genes. Transcription of some imprinted genes has been shown to be no longer parent-specific (monoallelic) once the germ cells have entered the genital ridge (Szabo and Mann, 1995). There is evidence that some new imprints in the male germline are imposed in prospermatogonia before birth, or in spermatogonia after birth but before entry into meiosis (Ueda et al., 2000; Jue et al., 1995). Most imprinted genes, however, acquire their methylation in the female germ line, during oogenesis, rather than during spermatogenesis (Reik and Walter, 2001). Nongrowing primary oocytes (20 μm), such as those in newborn mice, have been shown to lack differential methylation at several imprinted loci (Kono et al., 1996; Obata et al., 1998); new imprints are imposed later at different stages of oogenesis for different genes, from very early (30 μm) to antral follicle stage (70 μm) (Obata and Kono, 2002). The DNA methyltransferase Dnmt3L is required for maternal methylation of imprinted regions, though not for global methylation (Bourc'his et al., 2001).

EG cells

Although isolation and culture of mouse primordial germ cells dates back 20 years (De Felici and McLaren, 1982, 1983), attempts to derive long-term germ cell cultures met with repeated setbacks (for reviews, see Buehr, 1997; Donovan, 2001). However, in 1992, both Hogan's and Donovan's lab (Matsui et al., 1992; Resnick et al., 1992) reported that provision of bFGF (basic fibroblast growth factor), LIF (Leukaemia Inhibitory Factor), and membrane-bound SF (Steel Factor) allowed long-term survival and proliferation of PGCs in vitro. The resulting embryonic germ (EG) cell lines proved to be pluripotent: they differentiated into a range of tissue types in embryoid bodies (Matsui et al., 1992) and in chimeras, including into the germline (Labosky et al., 1994). EG cells and PGCs are very similar in their size, appearance, proliferation rate and antigenic properties (McLaren and Durcova-Hills, 2001), but PGCs neither form embryoid bodies nor contribute to chimeras. The emergence of pluripotency may take place after relatively short periods of culture (Durcova-Hills and A.M., unpublished observations).

EG cells are very similar in their properties to the embryonic stem (ES) cells derived from blastocysts. The major difference is that the cells from which EG cells are derived may have undergone the epigenetic changes that characterise the germ cell lineage, such as demethylation, and in particular the erasure of allele-specific differential methylation characteristic of imprinted genes. EG cell lines have been made from PGCs isolated throughout the period during which imprint erasure is believed to take place: before migration (8.0, 8.5 dpc), during migration (9.5, 10.5 dpc), and after entering the genital ridge (11.5, 12.5 dpc). Before the development of bisulphite sequencing, which enables DNA methylation to be examined on small numbers of cells, the most reliable method of assessing methylation was Southern blotting. Because of the difficulty of collecting enough PGCs for Southern blotting, several studies have been carried out on EG cells, on the assumption that the differential methylation of an imprinted gene in an EG cell line would reflect that in the PGC population from which the EG cells were derived. For *Igf2r*, Labosky et al. (1994)

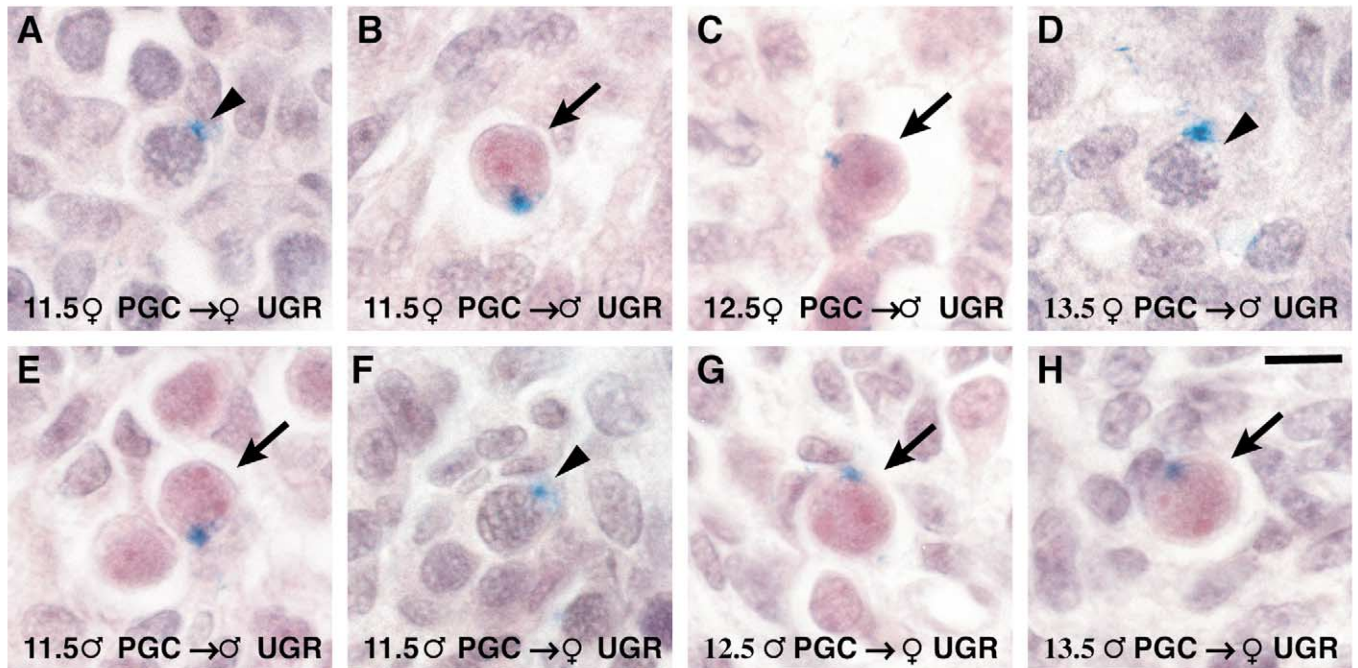

Fig. 7. Reproduced from Adams & McLaren (2002). Development of PGCs in urogenital ridge aggregates. Donor PGCs carrying a *lacZ* transgene were isolated from 11.5 dpc (A, B), 12.5 dpc (C), or 13.5 dpc (D) female genital ridges, and from 11.5 dpc (E, F), 12.5 dpc (G), or 13.5 dpc (H) male genital ridges, as indicated. The donor PGCs were aggregated with male (B–E) or female (A, F, G, H) 12.5 dpc recipient urogenital ridges (UGR) and cultured for 3–4 days. Donor PGCs are identified by a cyan perinuclear dot. Donor PGCs developing as oocytes (arrowheads) have condensed chromatin staining. Donor PGCs developing as prospermatogonia (arrows) have diffuse chromatin staining and prominent nucleoli. Scale bar, 10 μm.

found some degree of erasure in EG cells derived from 8.5 dpc PGCs, and more in EG cells derived from 11.5 dpc PGCs. Tada et al. (1998) looked at a number of imprinted genes in EG cell lines derived from 11.5 and 12.5 dpc male and female PGCs, and found that differential methylation in their 11.5 and 12.5 dpc EG cells was almost completely erased. EG cell lines cannot be made from germ cells later than 12.5 dpc, but the methylation status of imprinted genes in 15.5 dpc male and newborn female germ cell nuclei has been examined by nuclear transfer experiments (Kato et al., 1999; Kono et al., 1996). Complete erasure of differential methylation was observed.

In the 11.5 and 12.5 dpc EG cells, the only genes to have retained some differential methylation were *Igf2* and *H19*. Tada et al. concluded that either this closely associated pair of genes were unusually late in their erasure, or new methylation had been imposed. New EG cell lines made from male 11.5 and also from 9.5 dpc male PGCs (Durcova-Hills et al, 2001) confirmed their observations for the 11.5 dpc lines but in the 9.5 dpc lines almost complete lack of differential methylation was found for several imprinted genes, including *Igf2* and *H19*. This suggested that Tada et al.'s second hypothesis was more likely than their first: i.e., new methylation was imposed on *H19* and *Igf2* DNA after entry of the germ cells into the genital ridge.

The new methylation imposed on *H19* and *Igf2* showed an unexpected sex difference, with higher levels of methylation when the germ cells were derived from male embryos (Tada et al., 1998). By extending the study to include EG cell lines made from sex-reversed embryos (XX males, XY females), it was established that the new methylation was imposed according to the cell's own sex chromosome constitution, and was not a response to the sex of the genital ridge from which the germ cells had come (G. Durcova-Hills, P. Burgoyne, and A.M., unpublished observations).

The almost complete lack of differential methylation found in the EG cell lines derived from 9.5 dpc germ cells could suggest that imprint erasure was complete before entry into the genital ridges. However, the bisulphite sequencing results on PGCs (Hajkova et al., 2002) indicate that this is unlikely to be so: several of the imprinted genes (including some examined in EG cells) retained some site-specific differential methylation for a day or two after reaching the ridges. Bisulphite sequencing of some imprinted genes in EG cells has confirmed that the difference between PGCs and the EG cell lines derived from them is real, and is not due to any technical difference between bisulphite sequencing and Southern analysis (P. Hajkova and G. Durcova-Hills, unpublished observations). Evidently, the assumption that the methylation status of an imprinted gene in an EG cell line reflects that in the ancestral PGC population is unwarranted. Perhaps the differential methylation is lost very rapidly once the PGCs are put into culture, or alternatively the methylation process may be programmed, so that it follows the same course in vitro as it would in vivo. The somatic pattern of *Igf2r* differential methylation seen in one

Fig. 8. Taken from Fig. 1 of Hajkova et al. (2002) on the dynamics of DNA demethylation in PGCs, from 10.5–13.5 dpc. Relative methylation levels at individual CpG dinucleotides are shown for various imprinted and non-imprinted genes. Each bar represents the sum of methylation of individually sequenced clones at single CpG positions. Each data set represents the sum of at least two independent bisulphite and PCR experiments. The *H19* panels consist of two different parts of the *H19* upstream promoter region, 5′ part (left) and 3′ part (right). The 3′ part represents the so-called imprinting box.

of the EG cell lines derived by Labosky et al. (1994) from 8.5 dpc PGCs could indicate that not all of the germ cells at this stage of embryogenesis have yet started the erasure process.

To what extent does the germ cell lineage follow an intrinsic timing mechanism?

Many aspects of PGC phenotype (morphology, behaviour, gene expression, DNA methylation) change at about the time of entry into the genital ridges. Often it is not known whether these changes are cell-autonomous, programmed according to some intrinsic clock, or whether they are an induced response to the new tissue environment.

Donovan et al. (1986) suggested that the shift in germ cell behaviour and expression of cell surface antigens before and after entry into the genital ridge might be a response to an intrinsic clock that regulates their development, since they behave in culture much as they do in the embryo. More recently, it has been shown that the expression of *GCNA1* (germ cell nuclear antigen 1) in PGCs isolated from the base of the allantois comes on in culture on a temporal schedule similar to that seen in vivo, without the need for either migration or exposure to the gonadal environment (Richards et al., 1999). We have shown that the block to prenatal entry into meiosis and the associated arrest of mitosis is induced by the environment of the male genital ridge. In contrast, entry into meiosis occurs cell-autonomously, and we and others have suggested that the timing of this event is also cell-autonomous, involving an intrinsic timing mechanism (McLaren and Southee, 1997; Ohkubo et al., 1996).

We do not know whether the reactivation of the inactive X chromosome in XX germ cells is cell-autonomous or induced. If it is induced, it must be a response to some general property of the gonadal environment, since it occurs not only in the female but also in the male genital ridge. The loss of differential methylation at some imprinted loci may well be cell-autonomous: EG cell lines derived from 9.5 dpc PGCs in the hind gut, which can have had no contact with the genital ridge, achieve the same total absence of differential methylation that PGCs achieve at the same imprinted loci, after entry into the genital ridge (Durcova-Hills et al, 2001). The report that addition of 5-azacytidine (a demethylating agent) to the culture medium accelerates the induction of GCNA1 (Maatouk and Resnick, 2003), independently of cell proliferation, raises the possibility that an epigenetic process involving DNA and/or histone demethylation may play a role in any timing mechanism that regulates PGC development.

Conclusion

Strictly speaking, germ cells should only be referred to as primordial until they enter the genital ridge. While they are proliferating in the genital ridge, they may be termed gonocytes, and once they enter meiotic prophase (in the female) or mitotic arrest (in the male), they are known respectively as oocytes or prospermatogonia.

However, in accordance with common usage, I have extended the term “PGCs” to include gonocytes, though I have restricted myself to only certain aspects of the complex interactions of somatic and germinal cells in the genital ridge. Even so, it is abundantly clear that questions still outweigh answers. The genetic and epigenetic changes involved in the establishment of the germ cell lineage, its segregation from the neighbouring somatic tissue, the guidance mechanisms during migration that deliver most of the germ cells into the genital ridges, the onset of meiosis (in both sexes) and its inhibition (in one), the reactivation of the silent X chromosome and the (perhaps associated) erasure of genomic imprints, and finally the possibility that some underlying epigenetic timing mechanism underlies this whole developmental process—this is surely enough to justify the enthusiasm of the increasing number of PGC fans all over the world.

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