Sex determination in mammals has been defined as those events that fix the nature of the germ cell and the gonad, while other processes influencing sexual form and function such as hormone action constitute sex differentiation (Austin et al., 1981). Such determination involves the separation of germline and somatic, controlled initially by maternal genes and then by the zygotic genome after fertilization and early cleavage (Braude et al., 1988). A cascade of events in the genital ridge initiates the differentiation of the gonads and secondary sex characteristics. In this article, we correlate embryological and genetic evidence and propose that germ cell could be allocated by the 4-cell stage and regulated by oocyte polarity, controlled cleavage planes and early transcription of sex-determining genes from pronuclear stages. Subsequent steps including germ line segregation in early embryos and migration of primordial germ cells, and their possible role in the initiation of gonadal sex differentiation in the genital ridge are discussed.

Germline and polarization of maternal transcripts in oocytes and fertilized eggs

Maternal germplasm, an ooplasmic region containing polarized determinants for germ line, is fundamental to germ cell determination in many animals. Its maternal regulation is achieved via the translation of vegetally-localized mRNAs (Micklem, 1995; Grunert and St Johnston, 1996; Macdonald and Smibert, 1996). Genes associated with vegetal germplasm in oocytes of three different animals and their known homologies are shown in Table I. A continuous sequence of development from germplasm to germ cell can be traced from the unfertilized egg to the genital ridge of the differentiating embryo in Zebrafish, Drosophila and other species using markers such as vasa protein in oocytes and migratory primordial germ cells (Figure 1) (Komiya et al., 1994; Warrior, 1994; Yoon et al., 1997). This clearcut sequence is not apparent in mammals, where germ cells positive for alkaline phosphatase emerge in epiblast (Pedersen, 1986; Ginsburg et al., 1990; Lawson and Hage, 1994), leaving a lineage gap in the cleavage stages when the absence of recognizable germ cell markers leaves the early origins of germ line an open question.

Genes regulating germplasm and germ line in Drosophila and amphibians have been classified into class I (specifying germplasm formation and spatial distribution), class II (assembling germplasm) and class III (specifying germ cell segregation) (Wakahara, 1996). Since there is no clear evidence for germplasm in mammalian oocytes (Gardner 1996; Edwards and Beard 1997), class II genes may be inactive in mammals, a concept urgently in need of verification. Recently it has been proposed that vasa proteins may be involved throughout the life cycle in a common mechanism of germ cell segregation in organisms with or without germplasm (Ikenishi, 1998). A clear marker for germ line in Caenorhabditis elegans, Xenopus, Drosophila and Zebrafish (Komiya et al., 1994; Warrior, 1994; Ikenishi and Tanaka, 1997; Yoon et al., 1997), homologues of vasa gene have now been identified in germ line of mice (Fujiwara et al., 1994) and rats (Komiya and Tanigawa, 1995). Further work is obviously needed in these species to trace protein and mRNA expression in developing embryos.

It is not clear how germplasm controls germ line development, whether through germ line ‘determinants’ (Davidson, 1986) or by protecting it from somatic differentiation (Smith, 1986; Dixon, 1981). Germplasm is damaged and sexual determination and differentiation are impaired in Xenopus by exposing embryos to UV irradiation (Dixon, 1981), fertility being restored by transferring putative germplasm (Wakahara, 1978). Irradiating Drosophila pole plasm also invokes sterility unless donor pole plasm is given (Okada et al., 1974), and when oskar mRNA is injected at the opposite pole in the presence of vasa and tudor proteins germplasm forms ectopically (Ephrussi and Lehmann, 1992). Oocyte cortex is essential for the polarization of regulatory maternal transcripts (Kloc and Etkin, 1995). X-cat2 is one clear germ line marker in Xenopus, homologous to the Drosophila gene nanos, and it encodes an RNA-binding protein which associates with the mitochondrial cloud, migrates to vegetal cortex, then forms islands of germplasm. Its movements are probably regulated by microfilaments and microtubules (Forristall et al., 1995; Micklem, 1995) and the kinesin superfamily of motor proteins (Mowry, 1996; Robb et al., 1996). Oocyte cortex may be the site of post-translational modifications, as in the activation of Vg1 protein by bone matrix protein (BMP) (Vize and Thomsen, 1994).

Many aspects of early development in mammals resemble that of lower animal forms. In mammals an anterior/posterior axis extends meridionally from the polar body (Gardner, 1996,
and an anterior/posterior polarity was identified in the distribution of the leptin (cytokine), and STAT3 (signal transducer and activator of transcription), in a thin cortical rim on either side of the meiotic spindle at the animal pole of unfertilized and newly fertilized mouse and human oocytes (Figure 2) (Antczak and Van Blerkom, 1997). Products of several other genes with widely differing functions, including Bax and Bclx (apoptosis proteins), TGF-β2 (transforming growth factor), VEGF (vascular endothelial growth factor), c-kit (stem cell factor receptor) and c-erB (epidermal growth factor receptor), each had similar distributions to those of leptin and STAT3 (Antczak and Van Blerkom, 1999), an interesting finding as yet unexplained. Other maternal proteins are radially distributed in cortex of mouse oocytes, including α1 and α3 subunits of Na/K–ATPase (Betts et al., 1998), and Dazla protein (Ruggiu et al., 1997). The symmetry of Dazla protein, an autosomal gene product essential for germ cell differentiation, must be confirmed since oocyte sections did not pass through germinal vesicle (Ruggiu et al., 1997). This gene is a homologue of the *Drosophila* gene *boule* although it has a slightly different action since the fruit fly gene is essential for spermatogenesis but not oogenesis (Eberhardt et al., 1996). Recent studies identified a cortical equatorial polarization of calcium-channel regulating receptors for type I inositol 1, 4, 5-triphosphate in human oocytes (Goud et al., 1999), and the astonishing tight distribution of myosin IIA to the cortex overlying the meiotic spindle in human and rhesus oocytes which may even mark the exact location of the animal pole, (Hewitson et al., 1999). Transcripts of other regulatory factors, such as the nuclear multipotency factor *oct-4*, are not polarized (Palmieri et al., 1994).

Cytoplasmic movements imposed by microtubules from the sperm centriole occur soon after fertilization in human eggs, just as in *C. elegans* (Van Blerkom et al., 1995; Goldstein and Hird, 1996). Two membrane-bound pronuclei form some hours later. A larger paternal pronucleus forms initially followed by a smaller maternal pronucleus located near the polar body and marking the animal segment (Dieguez et al., 1995). In video sequences of human eggs after intracytoplasmic sperm injection (ICSI), rotation placed paternal pronuclei adjacent to vegetal cortex in some cases (Edwards and Beard, 1997; Payne et al., 1997). Further video sequences showed how rotation to the polar axis occurred in other eggs as the first cleavage spindle was being formed (Payne, 1998).

Rotation could have important consequences in realigning the polar axis preparatory to germline formation. We postulate that, following fertilization (Figure 3a–c), rotation positions the paternal pronuclei adjacent to cortically localized proteins (Figure 3d,e). At this time, chromatin packaging is reorganized and protamines are replaced by histones in the paternal pronuclei, which outcompete maternal pronuclei for the hypercetlylated histone H4 pool until close to syngamy (Thompson et al., 1998). Paternal pronuclei are accordingly 5-fold more active transcriptionally throughout virtually all the 1-cell period in mice (Figure 3e) (Ram and Schultz, 1993; Wiekowski et al., 1993, 1997; Matsumoto et al., 1994). After syngamy (Figure 3f) all regulators would then be distributed to individual blastomeres by successive divisions, and based on a *C. elegans* model undergo meridional and equatorial cleavages resulting in a single germline precursor at the 4-cell stage (Edwards and Beard, 1997) (see Figure 3g–i). Regulatory maternal factors are gradually replaced by zygotic transcripts during zygotic gene expression (Schultz, 1993; Nothias et al., 1995; Wang and Latham, 1997).

**Table I. Genes contributing to the assembly and function of germplasm: P granules in Caenorhabditis elegans, pole plasm in Drosophila and germplasm in Xenopus**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Gene</th>
<th>Localized product</th>
<th>Typea</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. elegans</em></td>
<td>Mex-3</td>
<td>RNA, protein</td>
<td>ND</td>
<td>(Draper et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Par-1,Par-2</td>
<td>RNA, protein</td>
<td>II</td>
<td>(Kemphues, 1989)</td>
</tr>
<tr>
<td><em>Drosophila</em></td>
<td>Oska, vasa*, tudor</td>
<td>RNA, protein</td>
<td>II</td>
<td>(Ephrussi and Lehmann, 1992)</td>
</tr>
<tr>
<td></td>
<td>Nanosb</td>
<td>RNA</td>
<td>ND</td>
<td>(Kobayashi et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Cappuchino, spire, stauufen</td>
<td>protein</td>
<td>I</td>
<td>(Ephrussi and Lehmann, 1992)</td>
</tr>
<tr>
<td></td>
<td>Valois, mugo nashi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pgc</td>
<td>non-translatable RNA</td>
<td>ND</td>
<td>(Nakamura et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Germ-cell less</td>
<td>RNA</td>
<td>III</td>
<td>(Jongens et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial large rRNA</td>
<td>RNA</td>
<td>III</td>
<td>(Kobayashi and Okada, 1989)</td>
</tr>
<tr>
<td><em>Xenopus</em></td>
<td>X-catab</td>
<td>RNA</td>
<td>ND</td>
<td>(Forristal et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>XVLCIb</td>
<td>protein</td>
<td>II</td>
<td>(Komiya et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Xklpb</td>
<td>protein</td>
<td>II</td>
<td>(Robb et al., 1996)</td>
</tr>
</tbody>
</table>

aAccording to Wakahara (1996).
bHomologous genes.
ND = not defined.

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**Early transcription of sex-determining and other genes from pronucleate stages**

Genes which become active in the first wave of zygotic transcription initiated in the pronucleate egg immediately post-fertilization include *Sry*, *Zfy*, *Zfx*, *Sox-2*, *MHC class I* and *hsp 70.1* (Sprinks et al., 1993; Zwingman et al., 1993; Ao et al., 1994; Christians et al., 1995; Fiddler et al., 1995, Collignan et al., 1996). *Sry* is believed to be a primary sex regulator, located on Yp. Injections of a *Sry* 14.6 kb fragment into pronuclei reverses female development in mice, to produce sex-reversed transgenic XX males with no signs of hermaph-
Sex determination and germline formation—new hypothesis

Among the X- and Y-linked zinc finger genes, ZFY, is located on Yp and encodes a protein with 13 cys-cys/His–His zinc-fingers with nucleic acid binding motifs at the carboxy-terminal region (Schneider-Gadicke et al., 1989). Its X-linked homologue ZFX has similar introns and exons while its protein contains 383 out of 393 similar amino acid residues, with 13 zinc fingers at the carboxy-terminal region, and may bind the same nucleic acid sequences.

The role of Y-linked sex-determining genes so early in development is intriguing. In mice, Sry and Zfy transcripts were identified by nested polymerase chain reaction (PCR) in mRNA isolated from pooled batches of mouse embryos between the 2-cell and blastocyst stage (Zwingman et al., 1993). Transcripts of Zfx, Zfa, Ubely-1 (a sex-determining gene encoding ubiquitin-activating enzyme E1), and AIS9 (an autosomal gene encoding anti-Mullerian hormone) were not identified in blastocysts, perhaps overlooked by inadequate technology as suggested by the authors. Earlier embryonic stages were not examined. The specific Zfy gene (Zfy-1 or Zfy-2) transcript they searched for was not identified (Zwingman et al., 1993). These authors postulate that sexual dimorphism began in mammals before morphological gonadal differentiation as in marsupials (Zwingman et al., 1993). Both linear and circular Sry transcripts were identified between the 2-cell and blastocyst stage in mouse embryos (Boyer and Erickson, 1994), with the linear product remaining constant and apparently being synthesized in very few cells. These authors also speculate on an early regulatory role for Sry in sex determination. Cao et al. (1995) did not find Sry transcripts in unfertilized eggs or single female mouse blastocysts but using Northern blotting with an RNase protection step identified Sry transcripts, some circular, in single male blastocysts. Amounts increased between early and late blastocysts, reaching 40–100 copies per cell in late blastocysts assuming a uniform distribution between cells. Pronuclear and cleavage stage embryos were not investigated.

Nested PCR and sequencing of the amplified products was used to identify SRY, ZFX and ZFY transcripts in pooled preimplantation human embryos 19–20 h post-insemination and in blastocysts (Ao et al., 1994). Maternal ZFX transcripts were identified in oocytes, but ZFY and SRY transcripts were not detected. ZFX and zygotic ZFY transcripts were found from the 1-cell up to the blastocyst stage, and those of SRY from the 2-cell to the blastocyst stage. It was not possible to identify the parental origin of ZFX transcripts in their pooled embryos. These authors commented on the possible relationship between SRY expression and the faster growth of male versus female embryos.

For ethical reasons, another study using PCR analysis on single human embryos was confined to uni- or tripronucleate human eggs. SRY transcripts were not detected in unfertilized oocytes, spermatozoa or female leukocytes, but were identified in four out of six tripronucleate eggs at 12–18 h post-insemination (as expected for Y-linked transcription beginning in male pronuclei) (Fiddler et al., 1995). SRY transcripts were detected in two out of three 2- and 4-cell embryos, and in 10 out of 15 embryos between the 5-cell and blastocyst stages (Fiddler et al., 1995). Six out of 12 embryos between the 2-
Figure 2. A newly fertilized mouse egg stained with an antibody to leptin and viewed using a scanning laser confocal microscope. (a) Fully compiled image. (b) Optical section. Reproduced with the permission of Johnathan Van Blerkom and Molecular Human Reproduction (Antczak and Van Blerkom, 1997). PB2 = second polar body. *Indicates leptin domain. Arrows delineate borders of leptin domain.

Figure 3. Effect of polarization on germline determination in the 4-cell mammalian embryo. This diagram shows the hypothetical stages occurring between fertilization and early segregation of germline in the 4-cell embryo. (a) Gamete fusion; sperm attachment at surface of metaphase II oocyte. Polar body shown at 1 o’clock. (b) Fertilization and formation of second polar body. (c) Pronucleus formation; the male pronucleus is slightly larger. (d) Pronuclear rotation. (e) Activation of transcription of the paternal pronucleus, shown shaded. (f) Syngamy and spindle formation. Note that the axis of the cleavage is meridional. (g) Products of the first cleavage division and axis for division of the first dividing blastomere of the second cleavage division. (h) Products of the second division and axis for division of the second dividing blastomere of the second cleavage division. (i) Third cleavage division, leading to uneven distribution of germline determinants. Note that the axis of cleavage is drawn slightly eccentric to the polar axis. Shading in (g–i) represents the location of germline determinants.

Cell and blastocyst stages derived from unipronucleate eggs expressed SRY transcripts, again a ratio to be expected. ZFX transcripts in pooled ovine oocytes and ZFX and ZFY transcripts in embryos were identified at 24 h post-insemination (Bernardini et al., 1996). SRY transcripts were absent at all stages even when 60 blastocysts were pooled. Their findings were in agreement with previous authors (Koopman et al., 1989; Hacker et al., 1995) who failed to detect Sry in mouse blastocysts. Both of these investigators studied Parkes outbred strain mice.

These somewhat controversial conclusions, summarized in Table II, could result from problems in detecting transcripts in single embryos, or reflect species differences. Detecting gene expression may be hampered by different initiation sites in Sry (Clepet et al., 1993) or by the existence of circular and linear forms (Boyer and Erickson, 1994). These studies are incomplete in the sense that mRNA expression and not protein synthesis were analysed. Despite such varied results, positive identifications of Sry, Zfx and Zfy transcripts in preimplantation embryos by independent groups merits further investigation.

The activity of sex-determining genes in preimplantation embryos may be quite distinct from their later actions in genital ridge, although it has been suggested (Erickson, 1997), and others that their expression in preimplantation embryos is part of pre-gonadal sex differentiation, bringing mammals closer to marsupials. Male preimplantation embryos of several species are known to grow faster than females (Tsunoda et al., 1985; Mittwoch, 1988; Bourgoyne, 1993; Pergament et al., 1994) and the possibility that Sry and other sex-determining genes may act as growth regulators has been discussed (Ao et al., 1994; Fiddler et al., 1995). In contrast, expression of the Ped gene, which controls growth rate in mice and is linked to the Q region of the major histocompatibility locus, is not affected by the sex of the embryo (Warner et al., 1998). Another possible function of these early acting sex-determining genes might be a role in placental development (Norris et al., 1990); this study showed defective trophoectodermal development in gynogenic mouse embryos derived by pronuclear transfers. We suggest however that these sex-determining genes are more likely to be concerned with events which specify the germline, and the question raised in this paper is whether their early expression may be linked with a primary function in development, namely germ cell allocation in preimplantation stages.
Germline formation between the 1-cell stage and blastocyst

The segregation of germline from soma during embryo development is fundamental to the survival of the species and might be expected to be an early priority. The concept of its origin in early cleavage stages should come as no surprise since many early investigators believed that as few as 2–9 cells formed germline in allogenic (chimaeric) mice (Mintz, 1974), in genetic mosaic mice with rare spontaneous marker mutations (Russell, 1964), in transgenic mice (Wilkie et al., 1986), and in recombinant virus-labelled mice (Soriano and Jaenisch, 1986).

In animals where expression of germline genes can be traced from germplasm in the oocyte through early cleavage and development to adulthood (see Figure 1), an early segregation of germline is easily followed. In *C.elegans*, germplasm is confined to a single germline precursor cell at the first cleavage, regulated by microfilaments and the par genes, while mes-1 regulates germline segregation with P granules during later divisions (Hird et al., 1996). In *Xenopus*, segregation of germplasm between individual blastomeres at the 4–8- stage can vary (Akita and Wakahara, 1985) and a similar variable pattern was found for the segregation of the germline gene X-crat2 (Forristal et al., 1995). A novel type of segregation occurs in Zebrafish where vasa mRNA localizes to the cleavage planes during the first two divisions, appears in subcellular clumps in the first four blastomeres, where it persists in subsequent development, to maintain four germline precursor cells while other cells apparently ‘continue to divide around them’ (Yoon et al., 1997).

Germplasm or early germline markers remain unidentified in mammals, although segregation at the 2-cell stage has been proposed in a model of cell fate generated on the basis of the existence of oocyte polarization and maternal control of early development in mammals (Edwards and Beard, 1997). Early indications of polarization in mammalian oocytes and embryos (Gardner, 1996; Edwards and Beard, 1997) has been supported by the observed distribution of leptin/STAT3 proteins in 2- and 4-cell mouse and human embryos (Figure 4; Antczak and van Blerkom, 1997). Located cortically, these proteins are derived exogenously in the developing follicle, and are hemispherically but slightly erratically polarized in the oocyte. The first cleavage plane differs slightly from the polarity of the egg, so that leptin and STAT3 are unequally segregated between daughter blastomeres during the first (Figure 4A) and second cleavage divisions (Figure 4B).

Further evidence for polarization in the cortex of the mammalian egg comes from studies on fragmentation in human embryos as described earlier. Polarized domains similar to those found for *leptin* and *STAT3* were identified for several other regulatory genes including *Bax*, *Bclx*, TGF-α2, VEGF, c-kit and c-erb (Antczak and van Blerkom, 1999). The similar localizations for all these proteins may reflect a common phenomenon perhaps involving cortical receptors since many of these molecules originate exogenously from the oocyte. Such a possibility is supported by a recent study (Goud et al., 1999) which traces the dynamic polarization of the cortical calcium channel regulating type I inositol 1,4,5-trisphosphate receptors in immature and mature human oocytes.

Recently published experiments (Zernicke-Goetz, 1998) purport to deny any role of polarity in the regulation of early mouse development. Animal, vegetal or longitudinal segments of cytoplasm were excised from late pronuclear eggs to produce blastocysts with reduced cell numbers without jeopardizing their development to full-term, albeit at very low efficiency (5–11% live births per embryo transferred). This study almost certainly failed to achieve its intentions. It assumed that polarized regulators are discretely located exactly on the polar axis, instead of being cortical, eccentric, hemispherical and
reconsideration of early events in mammalian development. A general form of damage seems insufficient cells in blastocysts and could apparently be restored by fusing two operated embryos. The equally valid explanation of a restoration of polar determinants closer to their original levels was not considered. A general form of damage seems to be the best explanation for this work with a remarkable level of repair occurring following operation. Thus there appears to be growing evidence to support reconsideration of early events in mammalian development.

Figure 4. Distribution of leptin in a mouse 2–4-cell embryos; (a) 2-cell embryo showing distribution of leptin staining to both blastomeres; white asterisk indicates an exposed region of cortical cytoplasm (b) 4-cell embryo in which one blastomere is brightly stained, two are less bright and one is unstained. Black asterisk indicates leptin rich domain. Reproduced with the permission of Johnathan Van Blerkom and Molecular Human Reproduction (Antzak and Van Blerkom, 1997). PB2 = second polar body.

Our original model (Edwards and Beard, 1997) aligned closely with some developments in *C.elegans*, isolated germline to one blastomere at the first cleavage division, and again to one blastomere at the 4-cell stage following spindle rotation in the second dividing cell at the 2-cell stage. We believe this remains the best model, since it is consistent with the pattern of leptin/STAT3 distribution in mouse and human embryos, where one equatorial and one meridional cleavage at the 2-cell stage results in a single cell largely devoid of leptin/STAT3 in 4-cell embryos, (Figure 4B). This cell is a possible germline precursor since later inner cell mass, unlike trophectoderm, lacked these markers (Antczak and Van Blerkom, 1997). Nevertheless different models could be constructed. While there is good evidence for controlled cleavage planes in the germline of *C.elegans* (Goldstein, 1995), the situation in mammalian cells is not clear. The crosswise pattern observed in 4-cell embryos of many species including mice, rabbits and humans (Lewis and Wright, 1935; Gulyas, 1975; Edwards and Beard, 1997) involves rotation about the polar axis but it is not certain whether this is spindle rotation or blastomere rotation as cleavage progresses (Gulyas, 1975; Graham and Deussion, 1978). Neither is it known whether segregation of maternal determinants is regulated by controlled cleavage planes as it is in other animals. The distribution of germline markers such as X-cat2 mRNA in *Xenopus* is an active process involving elements of the cytoskeleton (Forristall et al., 1995) and similar processes could be active in mammalian blastomeres. It is possible therefore to derive several feasible models in which mammalian germline originates in 1–4 cells or even a variable number in this range during initial cleavage divisions, possibly having features in common with various other animals from *C.elegans* to *Xenopus* and Zebrafish.

If germline blastomeres are ‘allocated’ purely by the distribution of maternal determinants when do they become ‘committed’ to germline? A study of mosaic mice created by injecting blastocysts with cloned epiblast cells indicated that some cells isolated from late mouse blastocysts were still capable of producing fetal soma and germline (Gardner et al., 1985). Little is understood about ‘totipotency’ and plasticity in blastomere differentiation in preimplantation embryos. As far as we know, the bovine 4-cell stage is the final reported stage for cellular (as opposed to nuclear) totipotency in mammals (Johnson et al., 1995). Cell potency is more limited after this stage. Yet some apparently fully-differentiated adult somatic cells can partly dedifferentiate and redifferentiate in culture. One example is the reversal of pigmented epithelial cells *in vitro* when placed on matrices containing phenylthiourea and hyaluronidase. They down-regulate some specific genes and up-regulate others as they redifferentiate into lens cells (Agata et al., 1993; Mazaki et al., 1996). The culture of chondrocytes and other cell types on specific matrices may involve some de- and redifferentiation as they form organ grafts such as ears and thumbs for human autografting (Vacanti, 1988; Cao et al., 1997). These cytological events could represent initial nuclear dedifferentiation steps which could progress to full dedifferentiation as occurred during the cloning of Dolly (Wilmut et al., 1997).

In view of this evidence, allocated but not committed,
embryonic blastomeres in initial phases of differentiation may retain the capacity to partially dedifferentiate, e.g. in response to stress as blastomeres are excised for preimplantation diagnosis. Flexibility characterizes early germline segregation in different colonies of Xenopus, as illustrated by the uneven segregation of Xcat2 (Forristal et al., 1995) and by the correlation of germ cell formation with total germplasm rather than with amounts of germplasm in individual 8-cell blastomeres (Akita and Wakahara, 1985). Perhaps the multipotency gene Oct-4 normally stabilizes allocated germ cell blastomeres within embryos until they are ‘committed’ in mesoderm as morphologically-identifiable primordial germ cells in proximal epiblast close to extraembryonic ectoderm in pre-gastrulae and primitive streak stages (Pedersen, 1986; Lawson and Hage, 1994).

If our interpretation of embryological events leading to germline formation is correct, the rate of germ cell multiplication from the 4-cell stage will be determined by the mitotic rate and the frequency of rotational divisions confining it to one daughter cell. Drosophila germline replicates more slowly than soma (Su et al., 1998). Total germline cells in mouse embryos as indicated by brightly-fluorescing oct-4 cells in primitive endoderm (Palmieri et al., 1994), do not exceed a dozen at 4.5 days, i.e. 3–4 cleavages. By 7.2 days (7–8 divisions), mouse germline has increased to 45 alkaline phosphatase-positive cells (Lawson and Hage, 1994). These estimates of germline expansion indicate that all descendents of the original germ line form germ cells, and that rotational cleavage is rare or absent after the 4-cell stage. Primordial germ cells migrating to genital ridge continue their proliferation, regulated by c-kit/Steel (McCoshen and McCallion, 1975), and by inhibitory effects of TGF-β1 (Goddin and Wylie, 1991).

**X-inactivation and early expression of sex-determining genes**

Concepts on early germline formation must be related to the onset of X-inactivation in female blastocysts. Initially transcribed in 4–8-cell mouse embryos, Xist (X-inactive specific transcript) expression is unstable until the blastocyst stage, when it becomes regulated by a second promoter. It then persists for 2–3 days before down-regulation as it targets the maternal X chromosome inactivation centre between days 5.5 and 8.5 in mice (Sheardon et al., 1997; Panning and Jaenisch, 1998). Although many genes on the X chromosome are silenced, others escape inactivation, including ZFY and SOX-3, which are homologous with the Y chromosome genes ZFY and SRY respectively (Schneider-Gadicke et al., 1989; Stevanovic et al., 1993).

Escape from inactivation may be restricted to specific sites associated with evolutionary losses in function of corresponding Y genes. Methylation of CpG islands was used to measure the inactivation of ZFX, RPS4X and SMCX among several primates, 11 orders (19 species) of eutherians and four myomorph species (including mouse) (Jegalian and Page, 1998). Myomorphs were the only group where Zfx was X-inactivated. This group also lacked the homologous Y-linked Zfy nucleotide sequences found in other species, and it has been proposed that this finding supports an evolutionary link between these X and Y genes (Jegalian and Page, 1998). Such species differences in X-inactivation may explain differences in expression of sex-determining genes in preimplantation embryos in mouse, sheep and human embryos. X-inactivation is clearly controlled independently in germlinal and somatic cells at different phases of development (Byskov and Hoyer, 1988; Surani, 1998), and further work may help to integrate X-inactivation into our proposals for early germline formation.

**The role of Oct-4 in the regulation of germline**

Oct-4 is a 5-exon multifaceted member of the family of Oct genes. The Oct-4 protein is multifunctional and consists of 352 amino acids, of which 150 are located in its so-called POU domain which binds to the DNA octamer ATGCAAAT. Although sparse in unfertilized eggs, its protein is present in at least one pronucleus and evenly distributed in nuclei from 2-cell embryos to morulae as zygotic transcripts replace maternal transcripts (Yeom et al., 1991; Palmieri et al., 1994; Abdel-Rahman et al., 1995; Ovitt and Scholer, 1998). It is not confined to particular cells in 8-cell embryos as suggested previously (Edwards and Beard, 1997). By 3.5 days, Oct-4 is down-regulating in trophectoderm and remains expressed in only a few cells of the inner cell mass (Scholer et al., 1989; Yeom et al., 1996). It is then successively restricted to epiblast, to specific restricted areas of primitive endoderm, and finally to germline and primordial germ cells after day 8 (Palmieri et al., 1994; Ovitt and Scholer, 1998). It is developmentally regulated by two promoters, a distal promoter in germline and a proximal promoter in epiblast (Yeom et al., 1996).

A final location in germline implies that Oct-4 is a germline organizer. However, its expression and multiple activities are more typical of a multipotency gene. It lacks a TATA box, has multiple transcription start sites and maps close to the mouse t-complex and the human major histocompatibility complex (MHC) (Scholer et al., 1990, 1991; Ovitt and Scholer, 1998). The Oct-4 protein is a transcriptional activator of the platelet-derived growth factor (PDGF)-α receptor gene and a transcriptional repressor of the human chorionic gonadotrophin (HCG) gene in non-trophoblastic tissues. It enhances transcription of the osteopontin (opn) gene, together with the regulatory genes EIA and Rex-1. Oct-4 is co-expressed with Sox-2 in preimplantation mouse embryos enabling both proteins to act synergistically in activating the fgf-4 gene to sustain the inner cell mass (Yuan et al., 1995; Zwilling et al., 1995). In contrast, by day 4.5, Oct-4 has up-regulated opn, acting antagonistically with Sox-2, and opn expresses an extracellular phosphoprotein which functions in migrating parietal endoderm by mediating adhesion to integrins (Botquin et al., 1998). Thus via several interactions Oct-4 could enable cells to escape from somatic differentiation. This functional role was suggested for germline genes more than three decades ago (Smith, 1966; Dixon, 1981).

**Is sexual differentiation in the genital ridge regulated by soma or germline?**

Zfx and Zfy apparently regulate germ cell differentiation and gametogenesis (Sinclair et al., 1990; Gubbay et al., 1990; Luoh et al., 1997), as indicated by the observation that Zfy-1 transcripts are absent in mouse genital ridge until migratory germ cells arrive (Koopman et al., 1989). The brief expression
of Sry in mouse genital ridge at 10.5–12.5 days apparently regulates Sertoli cell differentiation (Koopman et al., 1990) and expression of Mullerian inhibitory factor (MIF) (Hacker et al., 1995; Jeske et al., 1995; Swain et al., 1998). Although not found in primordial germ cells in the genital ridge, SRY expression occurs in human spermatids but not spermatocytes (Fiddler et al., 1995; Hacker et al., 1995). Interactions between Sry and Sox genes are proposed to regulate sex determination (Graves, 1998).

Sox-1, 2 and 3 qualify as sex-determining genes expressed in somatic cells of the genital ridge during sex differentiation, and may be co-expressed there with Sry (Collignon et al., 1996). Sox-9, a transcription factor, may act immediately downstream of Sry in Sertoli cells (da Silva et al., 1996), and may regulate Amh expression in the genital ridge. Sox-9 is then down-regulated in fetal ovaries and coelomic epithelium and up-regulated in fetal testes after Sry is activated (da Silva et al., 1996). Sox genes are also active in various somatic tissues.

Dax1 has many characteristics of a female-determining gene. Its activity in genital ridge parallels that of Sry. This gene consists of 160 kb and maps to Xp21 (Bardoni et al., 1994; Swain et al., 1998), while the protein has no zinc fingers or any other obvious DNA binding motif, yet binds to DNA and represses the transcription of the steroidogenic acute regulatory protein (StAR) gene (Zazopoulos et al., 1997). It has not been studied in preimplantation embryos. In contrast to Sox-9, it is down-regulated in the differentiating testis but up-regulated in the formative ovary.

Intersexual and sex-reversed states evidently arise as these genes interact in the genital ridge, and from hormonal and other factors downstream of this stage. Interactions have been described between weak or strong Sry alleles, determined by their expanded numbers of polymorphic CAG trinucleotide repeats (Coward et al., 1994); 11 repeats invoke complete sex reversal, 12 produce normal differentiation and 13 a partial reversal. Strong expression of Dax-1 is associated with its duplication in some X chromosomes (Swain et al., 1998).

Much is known about genetic activity during sexual differentiation and gonadal formation. Emphasis is usually placed on sex-determining activities of genes as primary regulators in Sertoli cells. This concept should be re-examined since it implies that primordial germ cells are merely passengers in the genital ridge. Their importance at these stages may have been discounted too easily. During their 2-day migration from the hind gut to genital ridge they apparently organize some of their own locomotion via a close mobile network rather than being transported passively (Gomperts et al., 1994). They remain connected as they enter mesentery. Germ cells then aggregate 1 day later in closely-apposed clusters as the first-entering cells apparently draw others into the genital ridge, assisted by the incomplete separation of epithelium from underlying tissue and the absence of a fully intact basal lamina. Coelomic epithelium and mesonephric cells co-ordinate with the entry of germ cells into the developing ovary, as some are trapped within epithelial cell processes as they arrive at coelomic epithelium while others enter underlying tissue and initiate oogenesis (Byskov and Hoyer, 1988).
Sex determination and germline formation—new hypothesis

References


Christians, E., Capp, E., Thompson, E.M. and Renard, J.P. (1995) Expression of HSP 70:1 gene, the major inducible hsp gene, a landmark along several cords simultaneously (Henderson and Edwards, 1968). It also explains the ‘neckling’ of follicles in stimulated human ovaries (Delavigne et al., 1993) and why oocytes control most (if not all) granulosa cell functions in growing follicles (Eppig et al., 1997b; Lanuza et al., 1998).

Clinical syndromes can be explained from the above proposals. In females, if merely a few germ cells enter genital ridge and colonize the developing ovary, the natural onset of oogenesis, folliculogenesis and atresia in fetal ovaries will rapidly deplete them. Tubular systems will have been induced, but a very early form of primary amenorrhea will ensue. This condition could also occur after birth, and is well known to arise in some girls at pre- and post-menarchical ages, apparently because their low initial store of germ cells becomes exhausted (Edwards, 1980). Such hypogonadal forms could well stem from reductions in numbers of migratory germ cells, whereas earlier damage to germplasm or germ cell formation would cause agonadism. In males, deletions arising in Y-linked genes such as Sry or AZF in pronucleate eggs or early cleavage stages, could explain how sexual differentiation and gametogenesis might be impaired (Edwards and Bishop, 1997). Deletions arising in early germline precursor cells could also explain how a normal father produced a sex-reversed XY daughter carrying mutated forms of SRY (Koopman et al., 1991).

Conclusions

Embryological evidence indicates that mammalian germline is ‘allocated’ between pronuclear and cleavage stages in mammalian embryos as genes involved in sex determination and differentiation, including Sry and Zfy, and Sox-3 and Zfx, are transcribed. Initial aspects of sex determination could involve germline formation during preimplantation stages, but species differences and other differences in expression of these genes call for caution against too-easy generalizations. The early expression of these genes from pronuclear stages might be concerned with faster cleavage rates in male embryos, or the control of placenta formation, although direct links with either of these processes have not been identified.

More knowledge is needed about mammalian germplasm and germline, and about interactions between primordial germ cells and fetal gonadal tissues. Basic questions such as why sex-determining genes on the Y chromosome are expressed in paternal pronuclei, and whether the paternal X is active in paternal pronuclei of female embryos, must be answered. The significance of the activity of sex-determining genes in preimplantation embryos and of X-inactivation in germline requires further analysis. Primordial germ cells do not seem to be inert during migration and may have an essential role in gonadal differentiation. Since germ cells are the custodians of the genetic code for the next generation, and segregate in the first cleavage division in many lower animals, we question whether mammals can afford to wait until the inner cell mass or epiblast before forming germline, and call for a close re-examination of the regulation of early mammalian development.
of early zygote activity in the mouse embryo is restricted to the first burst of transcript. Development, 121, 113–122.


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