Derivation of Oocytes from Mouse Embryonic Stem Cells

Karin Hübner,1 Guy Fuhrmann,3 Lane K. Christenson,4 James Kehler,1 Rolland Reinbold,1 Rabindranath De La Fuente,2 Jennifer Wood,4 Jerome F. Strauss III,4 Michele Boiani,1 Hans R. Schöler1*

Continuation of mammalian species requires the formation and development of the sexually dimorphic germ cells. Cultured embryonic stem cells are generally considered pluripotent rather than totipotent because of the failure to detect germline cells under differentiating conditions. Here we show that mouse embryonic stem cells in culture can develop into oogonia that enter meiosis, recruit adjacent cells to form follicle-like structures, and later develop into blastocysts. Oogenesis in culture should contribute to various areas, including nuclear transfer and manipulation of the germline, and advance studies on fertility treatment and germ and somatic cell interaction and differentiation.

In the early mammalian embryo, the germ line and soma are indistinguishable from each other. In the mouse, germ cell competence is induced at embryonic day 6.5 in proximal epiblast cells by signals emanating from the extraembryonic ectoderm (1, 2). Even during the specification period, precursor cells give rise to primordial germ cells and certain somatic cells, such as extraembryonic mesoderm and allantois. The potential of embryonic stem (ES) cells to generate all lineages of the embryo in vivo has been widely reported in the literature, in striking contrast to the lack of data describing the derivation of germ cells from ES cells in vitro. We attributed the inability to demonstrate the derivation of germ cells from ES cells in culture to the lack of a suitable reporter system for the noninvasive visualization of germ cell formation.

Induction of germ cells in culture. Elucidation of the various known regulatory elements within the germline-specific gene

1Germline Development Group, 2Female Germ Cell Biology Group, Center for Animal Transgenesis and Germ Cell Research, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, 382 West Street Road, Kennett Square, PA 19348, USA. 3Centre de Neurochimie, Laboratoire de Neurobiologie du Développement et de la Régénération, FRE 2373 CNRS, 5 Rue Blaise Pascal, 67084 Strasbourg Cedex, France. 4Center for Research on Reproduction and Women’s Health, School of Medicine, University of Pennsylvania, 1349 Biomedical Research Building 2/3, 421 Curie Boulevard, Philadelphia, PA 19104, USA.

*To whom correspondence should be addressed. E-mail: Scholer@vet.upenn.edu

Fig. 1. Analysis of early stages of germ cell formation. (A) Schematic representation of the Oct4 reporter gene (gcOct4-GFP) showing four conserved sequences (CR1 to 4) in the 5′ regulatory region with the deleted area boxed. The conserved sequences overlap with two regulatory elements: a distal enhancer (DE, germ cell–specific) and a proximal enhancer (PE, epiblast-specific) that have been described previously (5). (B) Phase contrast image of E14 ES cells transformed with gcOct4-GFP and growing on an embryonic fibroblast feeder layer (42). The clone presented here resulted in 3.5 dpc and 6.5 dpc mice embryos that lacked GFP expression but showed specific expression in germ cells of 12.5 dpc fetuses. (C) Merged fluorescent and phase contrast image of gcOct4-GFP ES cells 7 days (d7) after ~10^4 to 2.5 × 10^4 cells/cm² were plated without feeder cells or growth factors in ES cell medium Bar scale for (B) and (C), 75 μm. (D) Expression analysis of four distinct cell populations of d7 cultures sorted by fluorescence-activated cell sorting (FACS) using markers GFP and c-kit. RT-PCR results for Oct4, the Oct4-GFP reporter, c-kit, Vasa, DMC1, SCP3, and β-actin are shown. (E) Scheme of differentiating early germ cells in vitro. Only positive markers are presented. (F) FACS analysis of a d9 culture using the gcOct4-GFP reporter. The R3 population was further sorted in (G) on the basis of c-kit staining, which shows that, at d9, only a minor fraction of gcOct4-GFP+ cells are also positive for c-kit. R5 represents the population of c-kit negative cells, whereas R2 denotes cells in which c-kit is weakly or moderately expressed.

www.sciencemag.org SCIENCE VOL 300 23 MAY 2003 1251
Oct4 (3, 4) was instrumental in the development of a system that can visualize the initial steps of germ cell formation in vitro. Comparative analysis of the mouse, human, and bovine Oct4 genes highlighted three conserved regions, CR2 to CR4, that lie within the known germ cell (DE) and epiblast (PE) enhancers (Fig. 1A) (5). To restrict expression of an Oct4-based reporter to germ cells during mouse development, we deleted CR2 and CR3 from a genomic Oct4 fragment driving an inserted green fluorescent protein (GFP) rather than Oct4 (gcOct4-GFP, Fig. 1A) (6). ES cells were transfected with gcOct4-GFP, and three positive clones were expanded and tested for specific expression in transgenic animals. Two transgenic lines showed specific gcOct4-GFP expression in germ cells and no signal in blastocysts or epiblast-stage embryos (7).

The same gcOct4-GFP ES cells (Fig. 1B) that had been used to generate transgenic mice were subsequently used to establish the conditions required to induce germ cell differentiation in vitro. ES cells were plated on tissue culture dishes and maintained in ES cell medium without any feeder cells or growth factors besides the factors present in the heat-inactivated serum. Expression of gcOct4-GFP was detected in some cells after 4 days (d4), in ~25% of all cells at d7 (Fig. 1C), and in 40% of all cells at d8 (not shown). Day 7 cultures were sorted into four cell populations on the basis of the levels of expression of the gcOct4-GFP reporter and endogenous c-kit, both being markers of early germ cells (Fig. 1D). After sorting, we further characterized cells by determining the mRNA expression of Oct4, gcOct4-GFP, c-kit, Vasa (a marker of postmigratory germ cells) (8), and two meiosis-specific markers, namely the synaptonemal complex protein 3 (SCP3) and the mouse homolog of the yeast meiosis-specific homologous recombination gene (DMCI) (9). The SCP3 protein is part of the axial-lateral element of the synaptonemal complex to which the chromatin loops are attached and is an excellent marker for detection of the meiotic transition in mammals because its expression is required for the onset of the first meiotic division (10). The DMCI protein is supposed to function during chromosome synapsis and homologous recombination events (11, 12).

Three of the sorted cell populations (GFP+/c-kit+, GFP+/c-kit−, and GFP−/c-kit−) contained cells at different stages of germ cell development (Fig. 1D and E). The GFP+/c-kit− fraction expressed Oct4, gcOct4-GFP, and c-kit but little Vasa mRNA (Fig. 1D, lane 2). Because Vasa is a marker for postmigratory germ cells until postmeiotic stages (8), these results suggest that these cells correspond to premitotary or migratory primordial germ cells. Cells sorted as GFP+/c-kit− (Fig. 1D, lane 3) were found to express Oct4, gcOct4-GFP, and Vasa, but not c-kit, and thus may represent cells of an early postmitotary germ cell stage. GFP+/c-kit− cells did not express Oct4, gcOct4-GFP, or c-kit mRNA but expressed Vasa mRNA (Fig. 1D, lane 4). These cells likely represent postmitotary germ cells that are about to enter meiotic prophase I, because these cells did not express the meiotic markers DMCI and SCP3 (9). GFP−/c-kit− cells seem therefore to exhibit the same expression pattern as that in vivo, in which both Oct4 and c-kit are down-regulated in female germ cells before the zygotene-pachytene stage of meiotic prophase I, around 15.5 days postcoitum (dpc) (13). The fourth group (GFP+/c-kit−) was composed of cells that were not part of the germ line (Fig. 1D, lane 1) (6, 14). Further analyses are required to define the identity of these c-kit− cells that 2 days later were almost absent (d9) (Fig. 1, F and G).

**Early germ cell differentiation.** In our cultures, colonies of variable size had formed by d12 (Fig. 2, A to C, represent a large colony). Large colonies in general exhibited reduced GFP expression but contained a high percentage of Vasa− cells (Fig. 2B). Within these colonies, three distinct types of cells were identified: (i) cells only expressing GFP, (ii) cells expressing both GFP and Vasa, and (iii) cells only expressing Vasa. Strings of GFP+ and Vasa− cells were also found in the cultures, reminiscent of migratory germ cells (Fig. 2D) (15). GFP+/Vasa− cells were always found in the vicinity of both GFP+/Vasa− and GFP−/Vasa− cells and may correspond to early postmitotary germ cells in vivo (Fig. 2B). GFP-expressing cells had nuclei with a more diffuse DNA staining, whereas cells solely expressing Vasa were more condensed, round, and physically separated from each other (Fig. 2A), which is typical of postmigratory germ cells. Outside the colonies, all cells were negative for germ cell markers and therefore most likely represent somatic cell types.

![Image](image.png)
Individual cells or groups of cells detached simultaneously from the large colonies, but in both cases these predominantly Vasa⁺ germ cells tended to form small aggregates in the supernatant, with very few GFP⁺ cells (Fig. 2, E and F). It is likely that these cells detach because of the loss of cell-cell contacts, as noted in the lower center area of the colony (Fig. 2, A to C). A reduction in cell-cell adhesion among these Vasa⁺ germ cells in culture is quite similar to that of postmitotically germ cells in vivo (Fig. 2, E and F). Interestingly, the aggregates were frequently attached to GFP⁻/Vasa⁺ cells, which often resulted in a more compact structure than that within the original colony (Fig. 2F). These tightly knit structures are quite similar to the histotypic structures obtained after disaggregation by mild trypsin treatment of male or female genital ridges (16, 17). Furthermore, cultures of suspended ovarian cells of newborn mice and rat can also yield primordial follicles (18).

**Formation of follicular structures.** Aggregates were collected by centrifugation and cultured in new plates. Well-organized structures formed, and some of these were morphologically similar to early ovarian follicles (Fig. 3). During the next 2 weeks, advanced follicle-like structures formed both in the master plate and aggregate cultures (Fig. 3, B, C, and H). The majority of these structures degenerated upon further cultivation, and ~20% produced oocytes larger than 40 μm.

In the mammalian female gonad, the development of a meiotically competent oocyte requires the interaction of somatic cells, which work in cooperation to synthesize estradiol. Androgen precursors produced by theca cells are aromatized into estradiol by granulosa cells. Detection of estradiol in our cultures, therefore, provides evidence for functional activity of the somatic cells in these follicle-like structures. We consistently found estradiol in the culture medium by d12, with levels peaking around d20. Thereafter, estradiol levels decreased (Fig. 3A). To determine whether estradiol production was associated with expression of steroidogenic enzymes, we performed quantitative reverse transcription polymerase chain reaction (RT-PCR) for key genes involved in estradiol biosynthesis, including steroidogenic acute regulatory protein (StAR), P450 17α-hydroxylase-17/20 lyase (CYP17), and P450 aromatase (Fig. 3G) (19). The mRNAs of P450 aromatase, CYP17, and StAR were 4.3-, 3.3-, and 1.8-fold greater, respectively, in d22 attached cultures compared with those at d16. Increased expression of these genes correlated with the observed increased concentration of estradiol in the medium. The steroidogenic gene expression profiles for attached cells at d16 were compared with those of replated cells at d16F 4 days after replating. This comparison illustrated pronounced differences between the two cell populations, including increased expression of P450 aromatase (2.4-fold), CYP17 (1.4-fold), and StAR (3.5-fold) in d16F cells. These higher levels are likely the result of an increased proportion of follicular to nonfollicular somatic cells in the replated culture. This is also supported by the sevenfold increase in growth differentiation factor (GDF-9) in the replated cells as compared with attached cells (d16 and d16F, Fig. 3D). Increased expression of the oocyte-specific cell marker GDF-9 in the replated cultures is an interesting phenomenon, because this growth factor is required for ovarian folliculogenesis (20, 21). The expression of steroidogenic enzymes and follicular estrogen synthesis is typically thought to require gonadotropin support. However, expression of steroidogenic enzymes may occur in the absence of gonadotropins, because transfection of ES cells with steroidogenic factor-1 (SF-1), for example, induces steroidogenic enzyme expression (22). In addition, the serum and possibly endogenously produced gonadotropins in the cultures may provide sufficient hormonal support.

**Characterization of oocytes.** As early as d26 of culture, oocyte-like cells were released from the vicinity of their companion somatic cells (Fig. 3I) and found floating in the supernatant. The oocyte-like cells were enclosed in a coat resembling the zona pel-
lucida, which was fragile and easily lost when manipulated with a micropipette (compare Fig. 4, B and C). Most of these cells were 50 to 70 μm in size, which is in the size range of natural oocytes (23). However, some looked swollen, reaching a size of 130 μm, and had a thinned zona (Fig. 4C). They were GFP+, in accordance with Oct4 being reexpressed after birth in diplotene-arrested oocytes (Fig. 4D) (13). The large cells also exhibited cytoplasmic staining for zona pellucida proteins 2 and 3 (ZP2 and ZP3, respectively) (24) at a location predominantly adjacent to or in the cell membrane, which is distinct from that of the GFP signal (Fig. 4E, ZP2 shown in red; ZP3 not shown). To support the immunocytochemical analysis, we examined the cultures for mRNA expression of several oocyte-specific markers, including ZP1, ZP2, ZP3, a factor in the germ line (Fig. 5), and GDF-9 (20, 21, 24–27). Figo, a transcription factor required for the expression of ZP1, ZP2, and ZP3, was absent in the ES control cells (not shown) and expressed at similar levels between d16 and d30 (Fig. 4A). As expected, expression of both ZP2 and ZP3 was also observed; however, ZP1 expression was not detectable (Fig. 4A, lanes 2 to 4). This may indicate that factors required for specific expression of ZP1 are not properly expressed in our cultures. Because ZP1 serves as a linker for ZP2 and ZP3 (28), its absence may account for the fragile zona observed on the ES-derived oocytes. Loss of ZP1 has been reported to perturb but not impair folliculogenesis (28).

Expression of the murine oocyte-specific GDF-9 in the d16 and d22 adherent cultures and the increase in GDF-9 mRNA levels in the replated aggregate cultures (d16F; with a contrast image of a small oocyte-like cell with holding pipette (right)). (C) Phase contrast image of a newborn oocyte-like cell with holding pipette (right) and injection needle (left) for size comparison. (D) Fluorescence image of gcOct4-GFP, and (E) ZP2 immunostaining of the same cell shown in (C).

Expression of SCP3 in d16 and d22 adherent cultures was measured as SCP3/COR1 staining (Fig. 5C), suggesting that these cells are in a stage before leptotene. Germ cells up to 25 μm in size showed SCP3/COR1 staining that colocalized with the nucleus (Fig. 5, A, black-white arrow, and D to F). In contrast, SCP3/COR1 was undetectable in accompanying smaller cells (Fig. 5, D to F) or in germ cell controls in which the primary antibody had been omitted (not shown). In large germ cells (Fig. 5A, black arrow), SCP3/COR1 (Fig. 5, G to I) and SCP1/SYN1 (not shown) were localized predominantly in the cytoplasm, which is indicative of a more advanced meiotic stage (28). Addition of gonadotropins, i.e., pregnant mare serum gonadotropin and human chorionic gonadotropin, i.e., pregnant mare serum gonadotropin, caused folliculogenesis (Fig. 3D).

Evidence of meiosis. DMC1 expression and lack of SCP3 expression as determined by RT-PCR at d16 indicated that the ES-derived germ cells were about to enter meiosis (6). To further substantiate entry into meiosis, we mildly disaggregated d16 cultures with trypsin, replated them, and collected single cells of varying sizes after the majority of the cell population had reattached (Fig. 5A). Expression and distribution of SCP3/COR1 (Fig. 5, B, D, F, and I) or SCP1/SYN1 (not shown) within these cells were analyzed with the use of the respective antibodies (29, 30). Germ cells indistinguishable in size from somatic cells showed SCP3/COR1 staining (Fig. 5, A, white arrows, and B) that was very similar to that of female germ cells isolated from day 15.5 embryos (Fig. 5C), suggesting that these cells are in a stage before leptotene. Germ cells up to 25 μm in size showed SCP3/COR1 staining that colocalized with the nucleus (Fig. 5, A, black-white arrow, and D to F). In contrast, SCP3/COR1 was undetectable in accompanying smaller cells (Fig. 5, D to F) or in germ cell controls in which the primary antibody had been omitted (not shown). In large germ cells (Fig. 5A, black arrow), SCP3/COR1 (Fig. 5, G to I) and SCP1/SYN1 (not shown) were localized predominantly in the cytoplasm, which is indicative of a more advanced meiotic stage (28). Addition of gonadotropins, i.e., pregnant mare serum gonadotropin, caused folliculogenesis (Fig. 3D).
Blastocyst-like structures derived from ES cells. At about d43, structures are found in the cultures that, with respect to morphology (Fig. 6, A to M) and expression of molecular markers (Fig. 6, M and N), are similar to mouse preimplantation embryos. A defined zona around the embryo was only detected in a few cases (for example, Fig. 6B), which is likely a consequence of its fragile structure. A zona was identified after it had detached from the embryo (compare Fig. 6, C and D, showing the same embryo on 2 subsequent days). This embryo had defined features of a 16-cell morula (Fig. 6, E to G). Nuclear staining by Hoechst, the cytoplasmic-to-nuclear ratio, a compacted morphology (Fig. 6, A to M) and expression of Oct4 protein and distribution, which at this stage starts to be localized predominantly to the nucleus [Fig. 5C in (31)], are characteristic for a mouse morula. Several structures were found in our cultures that resembled blastocysts (Fig. 6, H to L, show a representative collection of five different blastocyst-like structures).

It is likely that the structures resembling preimplantation embryos represent parthenogenotes, as suggested by the similarity of our follicle outgrowths (Fig. 6A) to parthenogenetically activated oocytes of the LT/Sv mouse strain (32). Future experiments will address whether cleavage was induced by the culture conditions. It is well known that agents such as ethanol or cations or thermic shock of the oocytes during the observations may induce parthenogenesis with or without extrusion of a second polar body. Addressing these and related questions may provide a better understanding of the physiological problems of parthenogenesis in vivo. In addition, our system may contribute to a better understanding of the regulation of spontaneous ovarian teratocarcinogenesis and the molecular mechanism by which genes such as c-mos block the second meiotic metaphase arrest (22, 33, 34).

Summary and conclusions. It is not surprising that the derivation of oocytes and blastocyst-like structures could be accomplished with both female and male ES cells. In the absence of appropriate SRY expression in the gonads, male primordial germ cells enter the female pathway and often undergo the first step of oogenesis, entering meiotic arrest at prophase I [for review see (35)]. Male-to-female sex reversal even happens in the mammalian embryo when single genes such as Sry, Sox9, or Fgf9 are not properly regulated or deleted (36). Because we detected Sry expression by RT-PCR only in the later stages of our cell cultures (not shown), the gene may not be efficiently regulated in the areas where germ cells differentiated into female germ cells.

The reports from 1978 cited above in the context of dissociation and reaggregation of gonadal cells are interesting also in this con-
Keeping G Proteins at Bay: A Complex Between G Protein–Coupled Receptor Kinase 2 and Gβγ

David T. Lodowski, Julie A. Pitcher, W. Darrell Capel, Robert J. Leffkowitz, John J. G. Tesmer*

The phosphorylation of heptahelical receptors by heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptor kinases (GRKs) is a universal regulatory mechanism that leads to desensitization of G protein signaling and to the activation of alternative signaling pathways. We determined the crystallographic structure of bovine GRK2 in complex with G protein β1γ2 subunits. Our results show how the three domains of GRK2—the RGS (regulator of G protein signaling) homology, protein kinase, and pleckstrin homology domains—integrate their respective activities and recruit the enzyme to the cell membrane in an orientation that not only facilitates receptor phosphorylation, but also allows for the simultaneous inhibition of signaling by Gαs and Gβγ subunits.

G protein–coupled receptors (GPCRs) are integral membrane proteins that respond to specific extracellular signals by activating G proteins within the cell. They represent the largest class of receptors in the mammalian genome and play a fundamental role in the sensing of light, smell, and taste and in the regulation of heart rate, blood pressure, and glucose metabolism (1). For cells to remain responsive to their environment, activated GPCRs must be rapidly desensitized. The best characterized system for receptor desensitization is that of the GRKs and arrestins (2, 3). Activated GPCRs are first phosphorylated by GRKs and are then bound by molecules of arrestin, which block the binding of G proteins, target the receptor to the G protein–mediated endocytosis (4), and serve as adaptors that link receptors to other signaling pathways such as those of the mitogen-activated protein.