COMMUTING THE DEATH SENTENCE: HOW OOCYTES STRIVE TO SURVIVE

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Programmed cell death claims up to 99.9% of the cells in the mammalian female germ line, which eventually drives irreversible infertility and ovarian failure — the menopause in humans. New insights into the mechanisms that underlie germ-cell apoptosis have been provided by the study of oocyte death in lower organisms and in genetically manipulated mice that lack apoptosis-regulatory proteins. With new therapeutic tools to control fertility, oocyte quality and ovarian lifespan on the horizon, understanding how and why the female body creates, only to delete, so many germ cells is imperative.

OOGENESIS
The process of gamete formation in the female, which in mammals leads to the production of germ cells that are arrested in prophase-I of meiosis (oocytes) from a pool of mitotically active germ cells (oogonia).

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One of the earliest descriptions of programmed cell death was by Walther Flemming who, in 1885, published detailed observations on cellular degeneration in the rabbit ovary[1]. This cell death, he noted, was characterized by chromatin condensation along the inner side of the nuclear envelope, followed by nuclear fragmentation (events he termed 'chromatolysis') and the ultimate budding of the cell into small remnants. This study — probably the first to illustrate the degradation of DNA during apoptosis — was published almost a century before Kerr, Wyllie and Currie would contribute what many consider to be a landmark paper[2] in which the term 'apoptosis' was first used. It took even longer for interest in cell death within the ovary to pick up again[3,4], and this stimulated research that has now solidified a central role for apoptosis in female development, gonadal function and fertility.

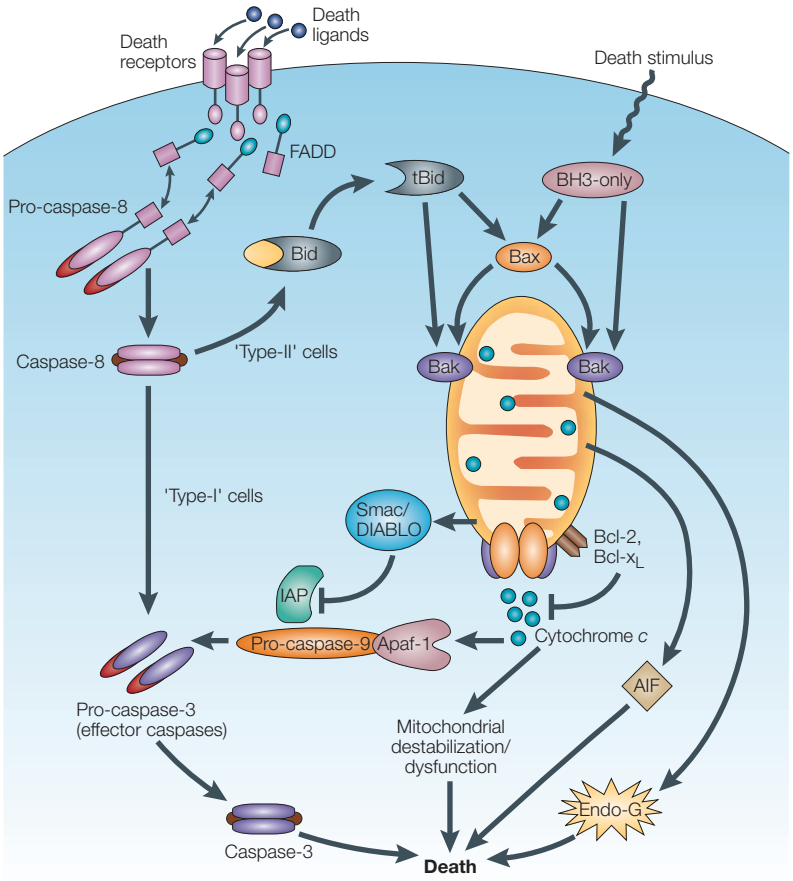
Although apoptosis occurs almost incessantly in the ovaries throughout fetal and postnatal life, much of what we have learned about this process in vertebrates has been derived from *in vitro* studies of cell lines or fractionated and reassembled cellular components, rather than from ovaries. However, reports of new genes that are associated with apoptosis have slowed, and much of the molecular framework for how various proteins — such as Bcl-2 family members, mitochondrial-derived factors, Apaf-1 and caspases (BOX 1) — determine cell fate has been worked out in detail[5–10]. So what can we now do with all of this information?

One priority is to work out the functional significance of conclusions from *in vitro* studies to normal organ biology. These types of investigations are valuable in determining whether a given cell lineage responds similarly to survival/death stimuli in its normal microenvironment as it does when isolated from its neighbours and cultured in a dish. Indeed, studies of apoptosis *in vivo* offer insight into whether cell death can be manipulated in an organ or tissue and what its potential therapeutic value might be.

This is where the ovary provides a powerful model that permits *in vivo* testing of the conclusions from studies that use *in vitro* organ or cell-culture systems. Such studies have already affected many aspects of biology and medicine — from advances in understanding mitochondrial inheritance and function in development, to therapeutic approaches to combat infertility. Furthermore, recent studies of germ-line development in less genetically complex organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have provided insights into germ-cell death that have begun to change long-standing concepts that are related to OOGENESIS in mammals.

This review will summarize what is known about the occurrence, regulation and functions of pro-

Box 1 | **The molecular basis of apoptosis**



The core intracellular machinery that regulates and executes apoptosis in vertebrates comprises families of proteins that interact with each other and with intracellular organelles[5–10]. In many cases, information from the environment is relayed to a central 'apoptosis rheostat' at the mitochondrion by stimulus-specific induction, modification or movement of pro-apoptotic 'BH3 domain-only' members of the Bcl-2 family (including Bid, Bad, Bim, Bik/Nbk, Blk, Hrk, Bnip3, Nix, NOXA, PUMA and Bcl-G_S).

Once 'activated', these proteins are thought to facilitate, through many mechanisms, the assembly of pro-apoptotic 'multi-domain' members of the Bcl-2 family (Bax, Bak, Bok/Mtd, Bcl-rambo) into heterodimeric units or 'pores' in the outer mitochondrial membrane. This causes the release of numerous 'apoptogenic' factors, which include cytochrome *c*, Smac/DIABLO, apoptosis-inducing factor (Aif) and endonuclease-G (endo-G), from the mitochondria into the cytosol through a process that can be blocked by anti-apoptotic multi-domain Bcl-2 family members (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1/Bfl1 and Bcl-B).

Cytochrome *c* release is essential for the generation of the 'apoptosome', containing Apaf-1 and pro-caspase-9. Once formed, the apoptosome initiates the effector caspase cascade (which usually involves caspases-2, -3, -6 and/or -7) after auto- or transcatalytic activation of the most apical (initiator) enzyme, caspase-9. A checkpoint, which is composed of a family of gene products referred to as inhibitor-of-apoptosis proteins (IAPs), prevents premature or unwanted activation of apoptosis by repression of caspase activation/activity. But this survival checkpoint is subverted by the mitochondrial release of Smac/DIABLO, which dissociates IAPs from caspase-9 and permits it to activate effector caspases.

In the case of death-receptor-initiated apoptosis, two distinct intracellular pathways can be engaged after pro-caspase-8 becomes activated[103]. In 'type-I cells', sufficient levels of caspase-8 are generated to directly initiate the effector caspase cascade. In 'type-II cells', low levels of caspase-8 cleave the pro-apoptotic Bcl-2 family member Bid to a truncated protein (tBid) that works with Bax and Bak to 'damage' mitochondria, promote formation of the apoptosome and trigger effector caspase activation.

grammed cell death in the female germ line during embryonic and postnatal life, and how this information is being used for therapeutic purposes to try to control the ageing process and infertility.

Oocytes: 'seeded' under a sword on a thread

The main objectives of each reproductive cycle in female mammals are to ovulate an egg(s) that is fully competent for fertilization and embryonic development, and to prepare the accessory reproductive organs (the oviducts and uterus) for the gestation and birth of healthy offspring. The egg that is selected for ovulation and potential fertilization must beat hundreds of competitors along the way, through, as yet, poorly described selection mechanisms[11]. So, each oocyte exists under a continuous threat of death, and this feature — along with the fact that female germ cells outlive almost all other cells in the body — makes oocytes a unique model system for studying programmed cell death. With these points in mind, each main developmental checkpoint that an oocyte must pass to survive to ovulation will be explored below.

Setting the stage — fetal development

In most mammals, after primordial germ cells migrate

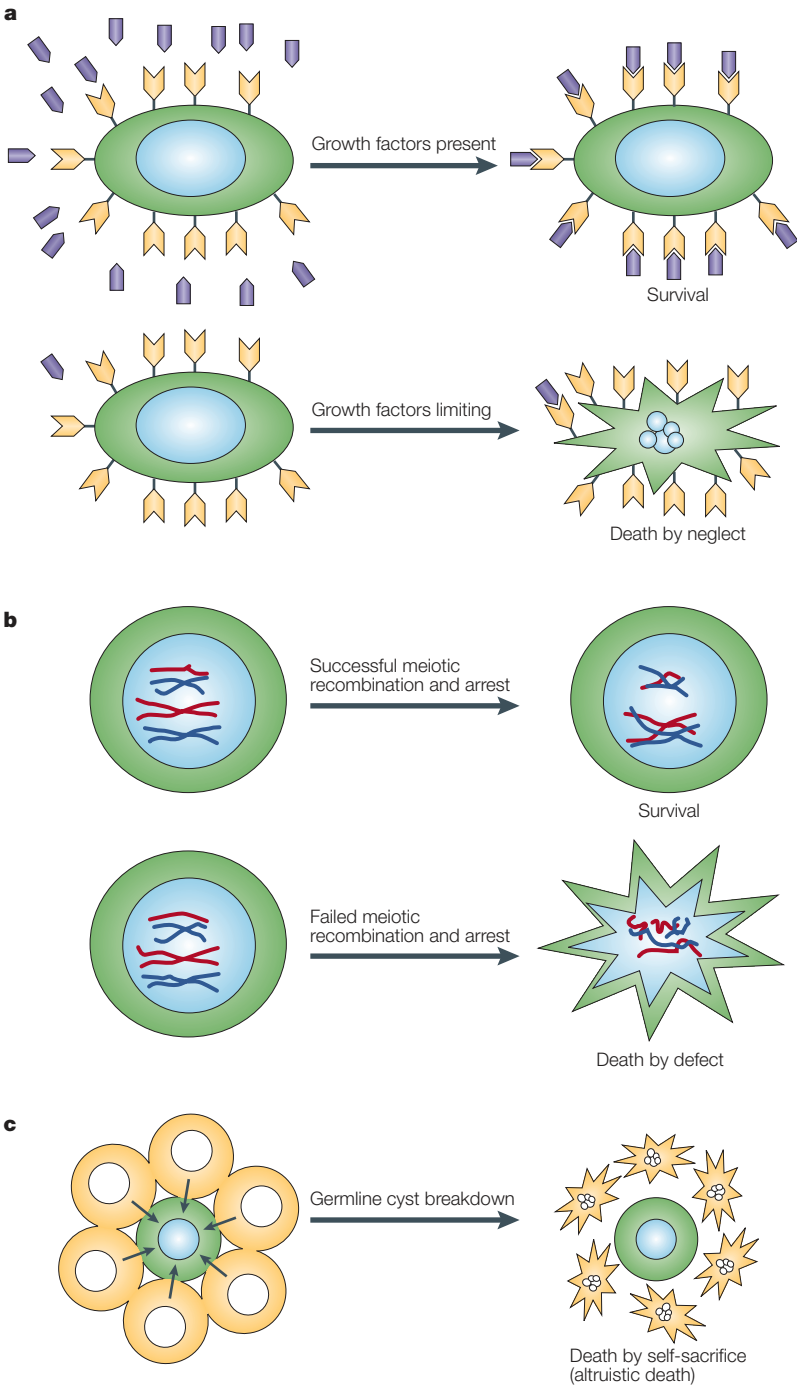


Figure 1 | **Hypotheses to explain mammalian fetal ovarian germline death.** **a** | Apoptosis is actively suppressed by growth factors which act through receptors that are expressed on the germ-cell surface; however, if growth-factor availability is low, intracellular survival signals no longer keep apoptosis repressed and death by neglect ensues. **b** | Oocytes that successfully complete meiotic recombination become arrested in prophase I and are used to form primordial follicles; however, failure of meiotic recombination results in germ-cell death by defect. **c** | In germ-cell cysts that form during development, 'nurse' germ cells (yellow) transport macromolecules and organelles (arrows) into one germ cell, which is destined to become the oocyte (green), to perhaps coordinate meiotic events or mitochondrial inheritance. Coincident with primordial follicle formation, the cyst breaks down through a mechanism which involves nurse germ-cell death by self-sacrifice for the benefit of their sister germ cell — the oocyte. In mammals, more than one oocyte per 'cyst' might survive.

to and colonize the as-yet-undifferentiated fetal gonadal tissues, the developmental programmes of oogenesis (XX individual) or spermatogenesis (XY individual) are initiated. In the female, pools of mitotically active germ cells, termed oogonia, expand the size of the germ line during fetal development until the germ cells enter meiotic prophase, which marks the end of female germ-cell production[12,13]. These germ cells, now referred to as oocytes, pass through the first four stages of meiosis I and become arrested in DIAKINESIS, at which time they are enclosed by a specialized lineage of ovarian somatic cells — the pre-granulosa cells — to form PRIMORDIAL FOLLICLES. Once they become enclosed, the oocytes remain arrested, resuming and completing meiosis only after ovulation and fertilization of the egg, respectively, at some point in adult life[12,13].

Most female germ cells produced during oogenesis die, with up to two-thirds of the oocytes being lost before or shortly after birth[11]. In humans, it has been estimated that the ~7 million germ cells in the fetal ovaries at around week 20 of gestation are decimated to 1–2 million viable oocytes in early neonatal life[14]. Comparable proportions of germ cells are lost during fetal ovarian development in other vertebrate species[15], yet it remains a mystery why so many germ cells are lost. There are three hypotheses to explain the basis of fetal germline death in females, and each is discussed below.

Death by neglect. In other examples of developmental cell death — such as the high loss of neurons during the formation of the central nervous system[16] — competition between cells for a limiting amount of growth ('survival') factors has been proposed as a key component of organogenesis[17]. Cells that receive insufficient support from their environment simply wither and die (FIG. 1a). Evidence for 'death by neglect' in the developing female germ line comes mainly from studies of gametogenic failure in mutant female mice that lack germ-cell survival factors, such as stem-cell factor (SCF)[18] or interleukin-1α/β[19]. Furthermore, *in vitro* culture of fetal mouse ovaries in the absence of serum or cytokines leads to a rapid induction of germ-cell apoptosis, and germline death in this artificial situation of acute neglect can be prevented by adding exogenous survival factors[20]. In addition, fetal ovarian germ-cell death both *in vivo* and *ex vivo* is attenuated in female mice by deletion of either a key stress sensor (ceramide[21]) or a downstream executioner of apoptosis (caspase-2; REF. 22). In fact, the increased numbers of oocytes that are lost from the ovaries of mice that are deficient in germ-cell survival factors can be rescued from death by the inactivation of the *caspase-2* gene[19]. However, despite mounting evidence for death by neglect, there is no proof that it is a normal component of oogenesis.

Death by defect. A second theory for why so many germ cells are lost during fetal ovarian development is that apoptosis eliminates oocytes with meiotic pairing or recombination anomalies (FIG. 1b). The idea is that a surveillance (or quality-control) mechanism detects and removes defective oocytes and retains meiotically com-

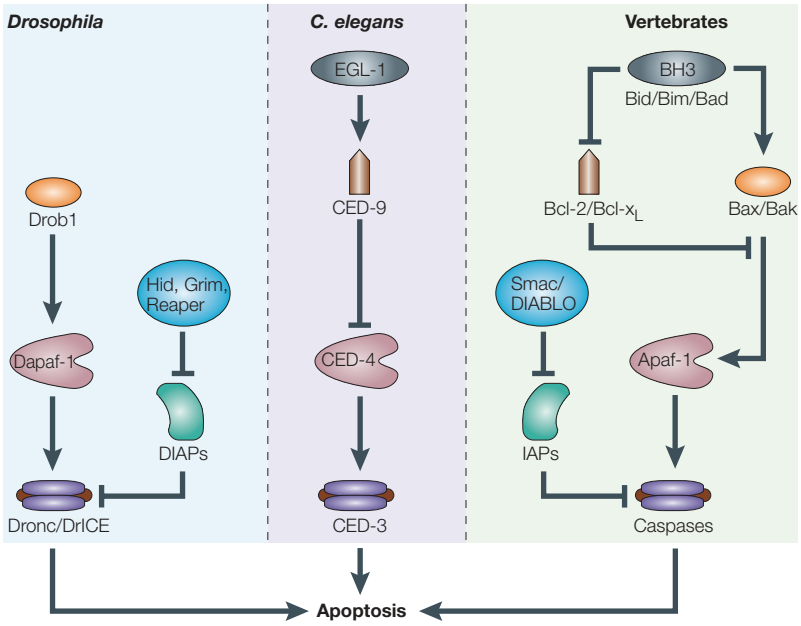


Figure 2 | **Evolutionary conservation of the regulation of apoptosis.** The core intracellular framework that controls activation and execution of cell death in *Drosophila melanogaster*, *Caenorhabditis elegans* and vertebrates (also see BOX 1) consists of several families of structurally and functionally related proteins (represented by the same colour). Note that in *Drosophila*, Drob1 is also named dBorg-1, Debcl or DBok, whereas Dapaf-1 is also referred to as HAC-1 or Dark. In addition to Dronc and drICE, the *Drosophila* caspase family also consists of DCP-1, DCP-2/Dredd and Decay.

DIAKINESIS
The point of the first meiotic block after the diploid germ cell in females (oogonium) progresses through the leptotene, zygotene and pachytene stages of prophase-I to become arrested in diplotene. In mammals, an oocyte reinitiates and completes meiosis-I after the preovulatory follicle that houses the oocyte receives the stimulus for ovulation.

PRIMORDIAL FOLLICLES
In vertebrates, each oocyte becomes enclosed by supportive somatic cells to form the most basic functional unit of the ovary, a follicle. The least differentiated type of follicle is referred to as primordial, being characterized by the presence of a single layer of squamous pregranulosa somatic cells and its existence in a 'resting' state.

petent oocytes for the formation of primordial follicles. Evidence for 'death by defect' comes mainly from studies of impaired germline development in mice with genetic mutations that cause abnormalities in either chromosomal recombination or pairing during meiosis. For example, inactivation of the *ataxia telangiectasia-mutated* (*Atm*) gene causes massive germ-cell apoptosis in both sexes at, or shortly after, prophase I of the first meiotic division. As a consequence, *Atm*-deficient female mice are born with ovaries that lack oocytes[23]. Interestingly, this model of death by defect is unaffected by the simultaneous inactivation of the gene that encodes either caspase-2 or its upstream activator Bax[19]. This indicates that fetal oocyte apoptosis occurs by more than one pathway, and that death by defect might not be amenable to control by experimental manipulation or therapeutic intervention.

Mutant mice with X-chromosome defects have also been used to support the death by defect hypothesis. Female mice that lack a second X chromosome (XO) or that harbour a large X-chromosome inversion (InX/X) show gametogenic failure[24], presumably due to failed-chromosome pairing, which leads to increased germline death during the development of the fetal ovaries. A similar situation occurs in Ullrich–Turner (XO) syndrome in humans[25]. Unfortunately, cytogenetic evidence from investigations of oocyte attrition in human fetal ovaries has not clearly shown whether meiotic defects are a cause or a consequence of germline apoptosis[26,27]. Therefore, the jury is still out on the contribution, if any, of death by defect to the overall number of oocytes that are eliminated by apoptosis before birth under normal physiological conditions.

Death by self-sacrifice. In *Drosophila*, *Xenopus laevis* and, very recently, in mice, it has been shown that female gametogenesis involves the breakdown of small clusters of interconnected germ cells formed by the incomplete cytokinesis of synchronously dividing progenitor cells, which are termed germline cysts[28,29]. In the *Drosophila* ovary, in which the process has probably been best studied, each cyst is composed of 16 'cystocytes', which are surrounded by a single somatic-cell layer of follicular epithelium. The cystocytes are connected by specialized intercellular bridges, called ring canals. During oogenesis, one germline cell of the cyst develops into an oocyte, whereas the remaining 15 cystocytes serve as nurse cells, transporting organelles, messenger RNA and proteins into the oocyte to support its development and maturation. Close to the final stage of oogenesis, the nurse cells transport most of their cytoplasm into the oocyte and degenerate, at which time the cyst breaks down to produce a single mature oocyte[30].

In keeping with reports that, during cyst breakdown, the nurse cells degenerate by apoptosis, several key regulators of this process in *Drosophila* somatic cells — including the *reaper* (*rpr*), *head-involution defection* (*hid*), *grim*, *Drosophila inhibitor-of-apoptosis protein-1* (*DIAP1*) and *DIAP2* genes[31] (FIG. 2) — are actively transcribed during oogenesis[32]. Surprisingly, however, oogenesis is normal in flies with the *Df(3)H99* deficiency that deletes *rpr*, *hid* and *grim*[32]. By comparison, egg chambers in females with a germline mutation in the *Dcp-1* gene (a *Drosophila* caspase) show the 'dumpless' phenotype in which the nurse cells fail to transfer their cytoplasmic components to the oocyte[33]. The nurse cells cannot 'sacrifice' themselves to ensure proper development of the oocyte, and this leads to infertility (FIG. 1c).

A similar case has been made for this type of altruistic death in the germ line of the *C. elegans* hermaphrodite. Here, large numbers of mitotic and meiotic germ-cell nuclei are partially enclosed by a single plasma membrane to form a syncytium[34]. Over half of these germ-cell nuclei ('oocytes') are eliminated by programmed cell death during meiotic maturation. Interestingly, a key upstream regulator of the *C. elegans* somatic-cell death pathway[31,35] — namely EGL-1, which is orthologous to vertebrate 'BH3-only' Bcl-2 family members (FIG. 2) — is not involved in controlling apoptosis[34]. However, the downstream executioners of this pathway, CED-4 and CED-3 (REFS 31,35) — which are orthologous to Apaf-1 and caspases, respectively, in vertebrates (FIG. 2) — are required for oocyte death in *C. elegans*[34].

Despite the similarity in regulation of germ-cell death during oogenesis in *Drosophila* and *C. elegans*, the *C. elegans* hermaphrodite does not generate true germ-line cysts or morphologically distinct nurse cells. Nonetheless, oocytes in *C. elegans* are still thought to require some type of nurse cells for their final development and maturation — a function that

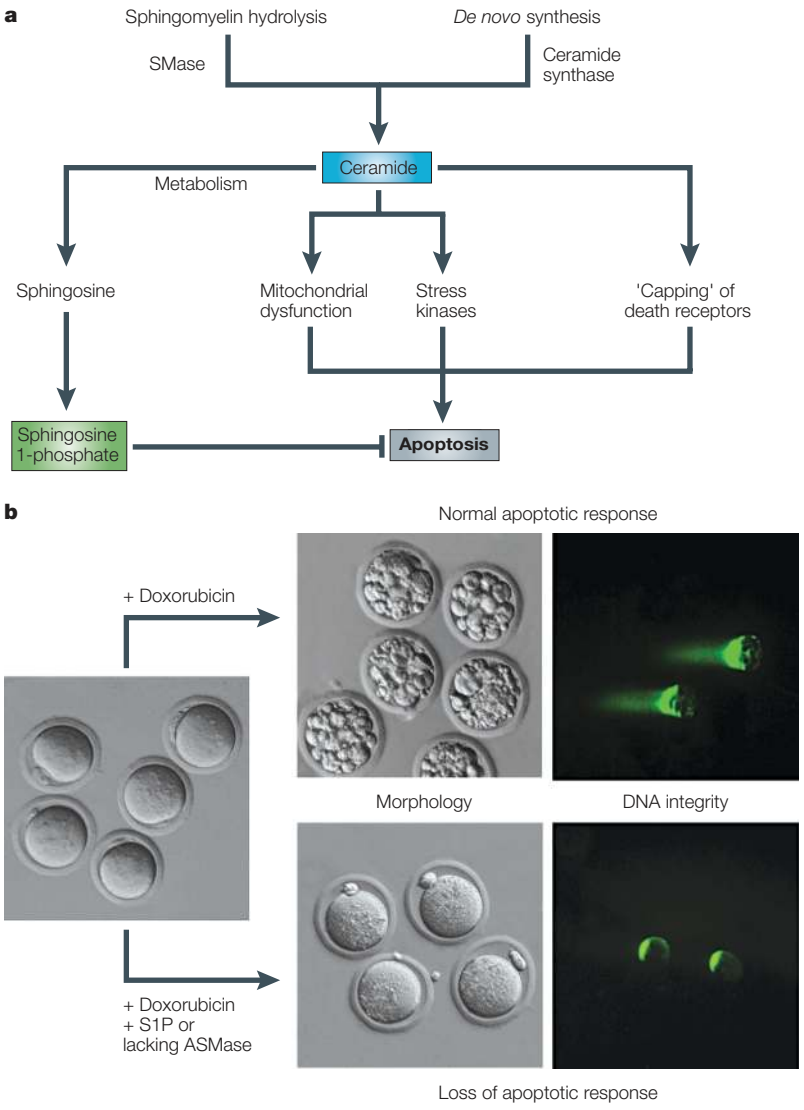


Figure 3 | **The sphingomyelin pathway and fertility preservation. a** | Ceramide generation by membrane hydrolysis (through a sphingomyelinase or SMase) or *de novo* synthesis (through ceramide synthase) can initiate apoptosis by several mechanisms. These include mitochondrial membrane destabilization, stress kinase (such as c-Jun amino-terminal kinase and p38) activation and super-aggregation of trimerized death receptors (for example, Fas). However, the actions of ceramide are kept in check by its anti-apoptotic metabolite, sphingosine-1-phosphate (S1P). **b** | The apoptotic response of mouse oocytes — as assessed by both light microscopic analysis of cellular morphology and a microelectrophoresis-based ('comet') assay of DNA integrity — to doxorubicin can be thwarted either by S1P treatment or by disruption of the gene that encodes acid sphingomyelinase (ASMase). (Part of this figure was reprinted with permission from REF. 21 © 2000 Macmillan Magazines Ltd.)

MENOPAUSE
A clinical diagnosis that is based on the permanent cessation of menses. In human females, the peri-menopausal period is usually heralded by infertility, near exhaustion of the oocyte reserve, a cessation of ovarian function and a loss of menstrual cyclicity. Only humans and nonhuman primates have menses, so other animal species cannot have a 'true' menopause.

might be performed by transcriptionally active germ cells at early stages of meiosis in the syncytium[34]. If this is the case, cell death during oogenesis in *C. elegans*, like that in *Drosophila*, might have a homeostatic role in eliminating excess germ-cell nuclei after they have done their job as 'nurse cells' for those germ cells that are selected to become mature oocytes. Indeed, the abnormal accumulation of germ-cell nuclei in the gonadal syncytium of *ced-4*- or *ced-3*-mutant animals eventually reduces the area that can be occupied by (and hence the total number of) mature oocytes in the hermaphrodite gonad[34].

In light of these observations, we need to uncover the function of germline cysts in mammals (FIG. 1c). For example, perhaps nurse cells are needed to control the rate or timing of meiotic events in the cystocyte that is destined to become the oocyte by production of a certain hormone or second messenger. Alternatively, a unidirectional transfer of 'nutrients' — metabolic or otherwise — from the nurse cells might be needed to ensure that the oocyte survives from when it becomes physically dissociated from its supporting sister cells until it becomes fully re-enveloped by the supportive pre-granulosa cells during primordial follicle formation.

An intriguing role for ovarian germline cysts in mitochondrial sorting and inheritance has been proposed, based on observations in mice that, just before the cyst breaks down, the germline mitochondria increase in number and are found in the ring canals of interconnected cystocytes[29]. These observations could reflect the random segregation of mitochondria among sister cells, or they might reflect active selection to explain the 'genetic bottleneck' for mitochondrial inheritance in the female germ line. If the latter is true, then a principal function of apoptosis could be to delete oocytes with inferior or mutant mitochondrial genomes, which ensures that only 'superior' mitochondria pass from mother to offspring. Evidence for such a hypothesis has come from comparative analyses of the severity of the mitochondrial bottleneck, the total number of offspring produced and the incidence of embryonic oocyte loss in various animal species[36]. These studies, along with preliminary findings indicating the selection of oocytes with superior quality mitochondria for fertilization in humans[37], support a role for female germline apoptosis in the maintenance of the mitochondrial genome.

No 'mouseapause' — is no menopause next?

The female germ line continues to die throughout postnatal life as most follicles never ovulate, but degenerate by a process called atresia. In human ovaries, the reserve of 1–2 million oocyte-containing primordial follicles at birth is reduced to ~300,000 by puberty[38]. So, by puberty, over 95% of a female's potential germ-cell stockpile has been lost through programmed cell death, with no prospect for renewal. Furthermore, only ~400 of the oocytes that are present at puberty will survive long enough to complete development and be released for potential fertilization. In other words, over 99% of the oocytes that are present at puberty therefore face death as well[11], as the ovaries are essentially devoid of viable oocytes at MENOPAUSE[39]. But what would happen if this postnatal germline death were attenuated? Could menopause be delayed? Recent studies in mice indicate that this is plausible.

Female mice also have a finite reserve of oocytes at birth, which dwindles away during pre-pubertal development (when up to 80% of the reserve is lost) and adult life. Although female mice do not undergo a 'true' menopause like that of humans and some non-human primates, the oocyte stockpile is practically exhausted about half to two-thirds of the way through the lifespan

HORMONE REPLACEMENT THERAPY (HRT). A therapeutic preparation of oestrogens, usually combined with a small amount of progestins, that is prescribed to alleviate some of the physical and psychological manifestations of menopause which are believed to result from the loss of ovarian function.

ASSISTED REPRODUCTIVE TECHNOLOGIES (ART). These are clinical procedures that are designed to help achieve pregnancy. Most of these procedures include the retrieval and fertilization of a woman's eggs outside the body (*in vitro* fertilization or IVF) followed by return of the embryos to the uterus (embryo transfer or ET) for implantation.

of the animal, which leads to ovarian failure[40]; or, in other words, the 'mouseapause'.

There is evidence in mammals that the pro-apoptotic Bcl-2 family member, Bax (BOX 1), mediates ovarian germ-cell demise[41]. In *Bax*-deficient female mice, the loss of Bax function does not alter the numbers of oocytes at birth, but it markedly reduces the rate at which the oocytes are eliminated by apoptosis during pre-pubertal and adult life[42]. As a result, ovaries from aged *Bax*-deficient females contain many oocytes at all stages of development, in contrast to their wild-type sisters, whose senescent ovaries are devoid of oocytes. In addition, the uterus — a target for ovarian steroid action — shows a striking level of hypertrophy in aged *Bax*-mutant females, which indicates that ovarian function is sustained. This study therefore concluded that the mouse equivalent of menopause could be prevented[42].

What, then, are the prospects for postponing menopause in humans? One cannot simply knock out the *Bax* gene, so there is no immediate clinical application of the results from the *Bax*-mutant mice. Nonetheless, perhaps the age at which menopause occurs could be modulated by some as-yet-undiscovered therapeutic compound that can antagonize the programmed-cell-death pathway in human oocytes.

What would the benefits of such a strategy be? Unfortunately, conclusions that are drawn with respect to this question are based solely on results from HORMONE REPLACEMENT THERAPY (HRT) trials in post-menopausal women[43,44]. Nonetheless, it is logical to assume from the results of these trials that the onset or severity of health problems that result from the ovaries ceasing to function — such as increased risks of osteoporosis, cardiovascular disease and neurodegeneration — would be similarly postponed or lessened. It is also possible that the fertile lifespan of women might be increased. The obvious drawback is the possibility of an increased risk of cancer in steroid-sensitive tissues such as the breast and uterus[45].

It should be stressed, however, that little is known about the consequences of sustaining ovarian function into old age in any species. And we still do not know how well current HRT regimens 'replace' the beneficial factors lost from a woman's body when the ovaries fail. Considering that HRT does not alleviate the physical or psychological effects of menopause in all women, there might be more to ovarian failure and menopause-related health problems than the simple loss of oestrogenic support. This is where models such as the *Bax*-mutant mouse might be valuable, as we can now study the effects of prolonging ovarian function, rather than simply restoring oestrogen levels, on the ageing body.

Mitochondria and the jump-start to life?

Apoptosis — and mitochondria — might also be at the heart of the age-related decline in fertility in women. After the age of 40, the chances of a successful pregnancy drop considerably[46,47], and conventional ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) do little to overcome this[47–49]. Older women show higher pregnancy rates if oocytes that are donated from a younger woman are used in the

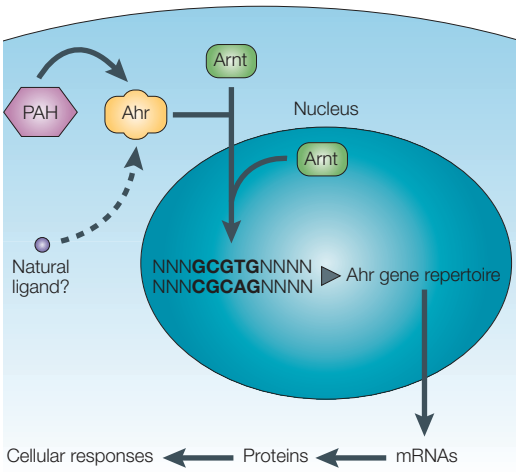


Figure 4 | **Mechanism of Ahr action.** Ligand activation of the cytoplasmic Ahr results in its nuclear translocation where the protein, once complexed with its heterodimeric binding partner Arnt, regulates transcription of target genes that contain Ahr-response-element motifs (the core pentapeptide sequence for Ahr binding is highlighted in bold text).

ART procedure[50,51], which indicates the involvement of some change in the oocyte itself that results in poor embryonic developmental potential[52]. An alternative idea is the 'production-line' hypothesis[53–55], whereby the cohort of oocytes that remain in the ovaries of older women might have all been produced at a particular point during development, and the reduced germ-cell quality reflects a problem with oocyte formation at that particular time. In either case, women can give birth to healthy infants long after the natural menopause if the pregnancy is established through oocyte donation[56].

What, then, is the problem with oocytes of ageing women? Several seemingly unrelated findings offer clues. First, although reduced numbers of oocytes are usually retrieved from 'older' women for ART, their *in vitro* fertilization (IVF) rates are comparable to those obtained using oocytes of younger women[52]. So the main obstacle to successful pregnancy is thought to be a reduced developmental potential of the fertilized eggs. Embryos that are conceived from oocytes of older women are more prone to degenerate *in vitro* before intra-uterine transfer is attempted, or they do not implant (or remain implanted) in the uterus once they are transferred[47,49,57]. Human embryos often arrest and degenerate at the 4- to 8-cell stage *in vitro*[58], and implantation rates of human embryos with less than 20% cellular fragmentation is about fivefold higher than that of embryos that show 30–50% fragmentation[59]. Given that blastomere fragmentation occurs with characteristic features of programmed cell death[60,61], the developmental potential of an embryo might be determined, at least in part, by the inherent 'apoptosis susceptibility' of the oocyte that was fertilized.

The second clue comes from clinical observations on a controversial new ART procedure referred to as ooplasmic transfer[62]. As its name implies, this entails the micro-injection of cytoplasm which has been aspirated

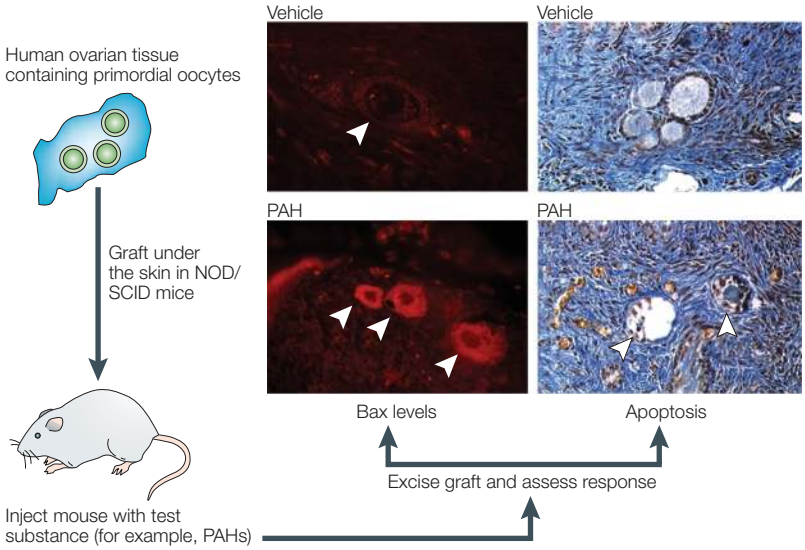


Figure 5 | **Use of a xenograft model to study human primordial oocyte function *in vivo*.** Immature oocytes within fragments of human ovarian tissue, which were grafted subcutaneously in immunodeficient mice, respond to Ahr activation after polycyclic aromatic hydrocarbon (PAH) exposure, *in vivo*, by an increase in Bax production (red immunofluorescence), which leads to apoptosis. For 'Bax levels', the arrowheads demarcate oocytes in both vehicle-treated and PAH-treated human ovaries. For 'apoptosis', the arrowheads point to debris that is left from an oocyte that has completely degenerated and an oocyte caught in the process of apoptotic death. (Part of this figure was reprinted with permission from (REF. 93) © 2001 Macmillan Magazines Ltd.)

from an oocyte ('ooplasm') of a donor female — with presumably normal fertility — into an oocyte of another woman with compromised fertility to 'rejuvenate' the recipient's egg. Several pregnancies and live births have resulted from ooplasmic transfer, although it is still not clear what factor(s) in the donor ooplasm provides the developmental boost to the recipient egg[62].

This is where the third clue might be significant. Micro-injection of oocytes with small numbers of mitochondria, which are derived from non-apoptotic somatic cells, markedly suppresses the death susceptibility of the oocytes[37]. These results highlight a paradoxical role of mitochondria in both the inhibition and promotion of cell death, the latter through the release of 'apoptogenic' factors (BOX 1). They also indicate that the mitochondria within ooplasmic extracts could provide the 'jump-start' to life for a newly conceived embryo. Indeed, micro-injection of mitochondria into zygotes (one-cell embryos) enhances embryonic development *in vitro*[63]. Furthermore, mitochondrial heteroplasmy — the presence of both donor and recipient mitochondria — has now been documented in children who were conceived from the use of ooplasmic transfer, which indicates that donor mitochondria were introduced into the recipient oocyte[64].

These findings support the idea that mitochondrial dysfunction is at least one cause of the decline in oocyte quality with age. In addition, during ooplasmic transfer, the donor mitochondria probably provide a much-needed developmental boost to the recipient egg that would otherwise have been prone to embryonic failure after fertilization.

The untimely death of innocent bystanders

Exposure of the ovaries to anticancer therapies — either chemotherapeutic drugs or radiation — accelerates the rate at which oocytes are lost. Indeed, infertility and premature menopause are well documented in young girls and reproductive-age women who have been treated for cancer[65,66]. Given that up to 1 in 52 human females between birth and age 39 (the pre-reproductive and reproductive years) are diagnosed with cancer[67], many people are potentially affected. Unfortunately, attempts to preserve fertility and ovarian function in cancer patients have met with little success.

A recent study showed that mouse oocytes that were exposed to doxorubicin, a widely prescribed chemotherapeutic drug, did not degenerate in a pathological fashion, but initiated programmed cell death[68]. That is, the unwanted destruction of oocytes by anticancer therapy occurs by the same mechanism that is clinically desirable in tumour cells. So, controlled manipulation of the oocyte apoptosis programme emerged as a tantalizing prospect for therapeutic development to protect the germ line from anticancer treatments[69]. Indeed, targeted disruption of the *Bax* gene in mice or, more recently, targeted expression of the Bax-antagonist Bcl-2 to the female mouse germ line protects oocytes from doxorubicin[68,70]. Unfortunately, comparable genetic technologies are not yet feasible in humans.

To circumvent this limitation, experiments in mice tested the possibility that a small-molecule inhibitor of a key step in the oocyte-death programme could be administered *in vivo* to protect the ovaries from radiotherapy-induced damage[21]. However, a crucial question was which step to target for optimal therapeutic outcome. Previous work had shown that suppression of caspase activity in models of Bax-driven apoptosis eventually activates a default pathway of cell death, which is more akin to primary necrosis, probably because the mitochondria are still 'damaged' by Bax in a caspase-independent manner[71]. As oocyte death that is induced by anticancer therapy is Bax-dependent[68], caspase inhibition was ruled out as a possibility. From this, a 'pre-Bax' step in the oocyte-death programme was chosen — the pro-apoptotic molecule ceramide (FIG. 3a).

Many somatic cell types generate ceramide, by either synthetic or hydrolytic mechanisms, in response to stresses such as radiation and chemotherapy[72]. Ceramide can act at many points in the programmed-cell-death pathway, such as 'capping' of death receptors[73] and helping Bax to insert into mitochondrial membranes[74]. However, the actions of ceramide do not go unchecked. In haematopoietic cells, a ceramide metabolite called sphingosine-1-phosphate (S1P) counteracts its pro-apoptotic effects (or those of the stresses that generate it)[75,76]. S1P also inhibits doxorubicin-induced oocyte death *in vitro*[21,68] (FIG. 3b). Furthermore, oocytes that lack acid sphingomyelinase — a hydrolytic enzyme that generates ceramide[72] — are resistant to doxorubicin-induced apoptosis *in vitro*[21] (FIG. 3b). Therefore, S1P was tested as a small-molecule inhibitor of ceramide-promoted apoptosis in oocytes that are

HISTOMORPHOMETRY
A process by which an ovary is removed, fixed, embedded in paraffin and serially sectioned. The sections are mounted in order on glass microscope slides and stained with a vital dye, such as haematoxylin–eosin. The total number of healthy versus dead/dying oocytes per section is then determined by light microscopic analysis.

PER–ARNT–SIM (PAS) FAMILY
A group of interacting and structurally related basic helix–loop–helix (bHLH) transcription factors.

exposed to anticancer therapy *in vivo*.

In this study[21], young adult female mice were given a single injection of S1P into the bursal cavity which surrounds each ovary then, two hours later, they were given enough ionizing radiation to kill over three-quarters of the primordial oocyte reserve. Two weeks after irradiation, the ovaries were analysed. No differences were observed between mice that had not been irradiated versus those that had been protected by S1P *in vivo* before irradiation. By contrast, irradiated females that had not been given S1P showed a pronounced loss of oocytes and a reduced embryonic developmental potential of the remaining oocytes[21]. With preliminary data from *in vivo* mating trials supporting the conclusion that S1P preserves a normal level of fertility in female mice that are exposed to anticancer therapy[77], minimizing the gonadal toxicity of such treatments in female cancer patients might one day prove feasible.

The ovary: a 'no smoking' zone

A final model of germline death underlies the early menopause which is caused by tobacco smoke[78,79]. Women who smoke cigarettes are 2.5 times more likely than non-smokers to have a diminished reserve of oocytes[80], which indicates that accelerated oocyte loss is at the heart of the problem. Tobacco smoke also has long-term effects on the developing fetus, with reports of impaired fertility in women whose mothers smoked during pregnancy[81].

Identification of the chemical(s) in tobacco smoke that are responsible for altering fertility and accelerating menopause is complicated by several factors. The first is the complex chemical composition of cigarette smoke, with more than 50 carcinogens — such as polycyclic aromatic hydrocarbons (PAH), aromatic amines and *N*-nitrosamines — identified so far[82]. The second is a lack of suitable approaches for the detection and monitoring of cytotoxic responses of human ovaries *in vivo*, which has limited extrapolation of results from animal models in female reproductive toxicity studies. Indeed, the only reliable method for assessing accelerated primordial oocyte depletion is to remove the ovaries for HISTOMORPHOMETRY-based assays of oocyte counts in serial tissue sections[83,84] — an approach that is not feasible in humans.

There is a case, however, for PAH as the key culprits that accelerate oocyte death. Nearly 25 years ago, it was reported[85] that oocytes are rapidly depleted from the ovaries of young adult female mice that were exposed to PAH *in vivo*. Further studies[79] supported this finding and also showed that *in utero* exposure of female mouse fetuses to PAH or to cigarette smoke is cytotoxic to the developing germ line[86,87]. Unfortunately, little was done to understand the mechanisms by which PAH kill oocytes. Moreover, direct testing of whether human primordial oocytes are killed by PAH *in vivo* seemed an insurmountable obstacle. However, several observations have recently been made that address these issues.

The first is from studies which show that a cytoplasmic 'receptor' for PAH, termed the aromatic hydrocarbon receptor (Ahr)[88], is abundantly expressed in oocytes[89]. The Ahr belongs to the PER–ARNT–SIM (PAS) FAMILY of transcription factors, and evolved in multicellular organisms over 450 million years ago[90]. The natural ligand(s) for the Ahr remains elusive, but the receptor is known to bind, and be activated by, a number of chemicals, which include PAH. Once activated, the Ahr translocates to the nucleus in search of 'target' genes which contain a consensus nucleotide sequence (Ahr response element, or AHRE) that the ligand-activated receptor can interact with[88,90] (FIG. 4). Although the Ahr is best known for its 'toxicological' role in the body, it has a number of biological functions in the absence of chemical exposure. This is illustrated by the phenotypic abnormalities in *Ahr*-mutant mice[91,92], including the birth of females with a twofold larger than normal oocyte reserve due to attenuated fetal germ-cell apoptosis[89].

Assuming that activation of the Ahr in oocytes initiates programmed cell death, a recent study[93] tested the possibility that this transcription factor directly regulates the expression of genes that control apoptosis in the female germ line. Computer-based scanning of available gene-sequence databases for AHREs showed the pro-apoptotic *Bax* gene as a possible target for the Ahr — a hypothesis which was confirmed by testing the *Bax* promoter in reporter assays with micro-injected oocytes. The transcriptional activity of the *Bax* promoter that was induced by the PAH-activated Ahr in oocytes was shown[93] to depend on both functional Ahr and AHREs. Moreover, treatment of female mice with PAH *in vivo* triggers a rapid increase in expression of the endogenous *Bax* gene in primordial oocytes, followed by apoptosis. The cytotoxic responses of oocytes to PAH — increased *Bax* expression and apoptosis — were shown[93] to require both the Ahr and Bax as mediators. Similar data and conclusions were derived from studies of fetal germ-line death in mouse ovaries. In this study[94], it was shown that oocytes in fetal mouse ovaries increase Bax production and undergo apoptosis in an AHR-dependent manner following PAH exposure. Furthermore, Bax is required for the apoptotic response of fetal oocytes to PAH.

But one crucial piece of information was still lacking — do human ovaries respond similarly *in vivo*? To address this, small fragments of adult human ovaries were grafted under the skin of immunodeficient mice and, after two weeks (to allow for neovascularization of the graft), the mice were treated with or without PAH[93]. Primordial oocytes in the human ovarian tissue showed the same response to PAH as that seen in primordial oocytes of mouse ovaries — an increase in *Bax* expression followed by apoptosis[93] (FIG. 5). These studies[93,94] uncover a new and evolutionarily conserved cell-death-signalling pathway, and highlight the value of the human ovarian xenograft model in studies on human primordial oocytes.

Pondering life and death

With these advances in understanding the occurrence and regulation of apoptosis in the germ line, we still do not know why the human female produces so many germ cells during prenatal gametogenesis, when two-

thirds or more of the oocytes that are generated will be lost before birth through apoptosis. And why do more than 95% of the oocytes that survive the perinatal waves of programmed cell death subsequently die in postnatal life?

The answer probably lies, at least in part, in the supposition that not all oocytes are created equal. So oocyte apoptosis can be viewed in a context analogous to Darwin's 'survival-of-the-fittest' theory of evolution[95]. Consider the mechanisms that underlie male versus female germ-cell contributions to the success of natural reproduction. Although some 'quality-control' selection of male gametes is probably made in the testes during spermatogenesis[96], a human male nonetheless ejaculates millions of mature sperm into the lower portion of the female reproductive tract. But these germ cells must overcome many barriers to reach the female's egg in the upper third of the oviduct. In fact, most sperm do not complete this journey because of mobility defects or other problems, which allow only a few (superior?) sperm to reach the egg. Therefore, in those species that ovulate only a single or a few eggs per cycle for fertilization, the continual deletion of presumably inferior oocytes by apoptosis might maximize the chances of reproductive success.

On a more practical note, understanding of the cellular and molecular mechanisms that activate and execute programmed cell death in the female germ line has implications for the therapeutic management of infertility and also, perhaps, of the ageing process itself. Furthermore, by incorporating emerging models — for example, mice that are grafted with human ovarian tissue fragments[93,97] — into laboratory animal studies, conclusions can be made about the efficacy of 'oocyte-preserving' strategies, such as S1P therapy, in humans before deciding whether to start a clinical trial.

However, the implications of oocyte preservation might go beyond the treatment of premature menopause in female cancer patients, if natural oocyte depletion with age occurs by the same basic pathway as the one that underlies pathological germ-cell death. For example, Bax deficiency not only delays the 'mouse-pause' by attenuating the rate of postnatal-oocyte loss through atresia[42], but it also protects the female germ line from cytotoxicity that is caused by anticancer therapies[68] or environmental toxicants[93,94]. Assuming that other steps in the oocyte death programme are similarly conserved, there is the prospect that promising new therapies could be used to prolong the natural lifespan of the ovaries. As such, perhaps strategies that are developed to combat premature menopause will also be useful 'anti-ageing' agents in women, to alleviate the post-menopausal health problems that are attributed to ovarian senescence.

The relationship between mitochondrial homeostasis and susceptibility to apoptosis in oocytes also warrants further study. The possibility that mitochondrial sorting and inheritance are tied to germline death is exciting[29,36,37], given that so little is known about how the integrity of the mitochondrial genome is maintained from one generation to the next[98]. Answers to this question are crucial as the mitochondria in the oocyte are the main — if not the only — source of mitochondria that replicate in the embryo after fertilization[99]. Moreover, there are more mutations in the mitochondrial than the nuclear genome[100], a number that increases considerably with age[101]. Yet diseases that are caused by mitochondrial DNA mutations are relatively rare, which indicates that such a 'cleansing' mechanism must exist before the mitochondria are inherited from mother to offspring. On a more clinical note, the ramifications of using 'donor' mitochondria — in either crude cytoplasmic extracts or purified suspensions — to jump-start an incompetent egg are tremendous, but are tangled in a web of ethical issues[102]. Indeed, with so little known about possible long-term consequences of ooplasmic transfer, even in animal models, we must ask whether the drive for solutions to fertility problems has created a situation in which application has begun to outpace validation.

Nonetheless, the knowledge gained by studies of programmed cell death in the female germ line is important. Not only have broad conclusions that were drawn from studies of apoptosis using cell lines or subcellular fractions been validated (or in some instances refuted), but also exciting research prospects have surfaced. It is to be hoped that future work will complete the molecular blueprint of how female germ cells live and die, and possibly deliver new oocyte-preserving therapies to the clinic. And the answer to the question of why so many oocytes die might also finally be found.

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ERRATUM

GIULIO BIZZOZERO: A PIONEER OF CELL BIOLOGY

Mazzarello, P., Calligaro, A. L. & Calligaro, A.

Nature Reviews Molecular Cell Biology **2**, 776–781 (2001).

The entry in Box 1 corresponding to Samuel August Tissot should have read:
Samuel August Tissot (1728–1797), a clinician famous for his works on nervous diseases.
The online version of this Perspective has been corrected.