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The developing ovary of the South American plains vizcacha, *Lagostomus maximus* (Mammalia, Rodentia): massive proliferation with no sign of apoptosis-mediated germ cell attrition

N P Leopardo, F Jensen, M A Willis, M B Espinosa and A D Vitullo

Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico, CEBBAD, Universidad Maimónides, Hidalgo 775, C1405BCK Buenos Aires, Argentina

Correspondence should be addressed to A D Vitullo; Email: vitullo.alfredo@maimonides.edu

N P Leopardo and F Jensen contributed equally to this work
F Jensen is now at Experimental Obstetrics and Gynaecology, Medical Faculty, Otto-von-Guericke University, Magdeburg, Germany

Abstract

Apoptosis-dependent massive germ cell death is considered a constitutive trait of the developing mammalian ovary that eliminates 65–85% of the germinal tissue depending on the species. After birth and during adult lifetime, apoptotic activity moves from the germ cell proper to the somatic compartment, decimating germ cells through follicular atresia until the oocyte reserve is exhausted. In contrast, the South American rodent *Lagostomus maximus* shows suppressed apoptosis-dependent follicular atresia in the adult ovary, with continuous folliculogenesis and massive polyovulation, which finally exhausts the oocyte pool. The absence of follicular atresia in adult *L. maximus* might arise from a failure to move apoptosis from the germinal stratum to the somatic compartment after birth or being a constitutive trait of the ovarian tissue with no massive germ cell degeneration in the developing ovary. We tested these possibilities by analysing oogenesis, expression of germ cell-specific VASA protein, apoptotic proteins BCL2 and BAX, and DNA fragmentation by TUNEL assay in the developing ovary of *L. maximus*. Immunolabelling for VASA revealed a massive and widespread colonisation of the ovary and proliferation of germ cells organised in nests that disappeared at late development when folliculogenesis began. No sign of germ cell attrition was found at any time point. BCL2 remained positive throughout oogenesis, whereas BAX was slightly detected in early development. TUNEL assay was conspicuously negative throughout the development. These results advocate for an unrestricted proliferation of germ cells, without apoptosis-driven elimination, as a constitutive trait of *L. maximus* ovary as opposed to what is normally found in the developing mammalian ovary.

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Introduction

Development of the mammalian female germ line begins with the migration of the primordial germ cells (PGCs) from the base of the allantoids to the genital ridge (Lawson *et al*. 1999, McLaren 1999, Matova & Cooley 2001). During migration, and after the colonisation of the primordial gonads, the PGCs proliferate and differentiate giving rise to a large number of oogonia. Mitotic proliferation is followed by the entrance of germ cells in the prophase of the first meiotic division to produce a pool of oocytes arrested at the diplotene stage of prophase I (Rucker *et al*. 2000, De Felici *et al*. 2005). As long as germ cells proceed through prophase I, they enter in close contact with somatic cells that surround the oocytes to form primordial follicles initiating the folliculogenesis (Baker & Franchi 1967). Depending on the species, development through mitotic proliferation, meiosis entrance and folliculogenesis may occur sequentially or as overlapping processes during gestation (Kurilo 1981). In any case, an extensive constitutive degeneration of germ cells affecting proliferating PGCs, oogonia, and mainly the oocytes at the zygotene and pachytene stages of prophase I, decimates the germinal reserve (Baker 1963, Baker & Franchi 1967). It has widely been accepted that massive constitutive elimination is a general feature of the dynamics of mammalian female germ cell development whose significance may be an extreme control selection process to preserve good

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quality oocytes and maximise the chances of reproductive success (Tilly 2001). Programmed cell death (PCD) or apoptosis is responsible for the loss of germ cells in the developing mammalian ovary (Peters & McNatty 1980, Hsueh *et al*. 1996, Tilly 1996*a*, 1996*b*, De Felici 1999, De Felici *et al*. 2005), and genes belonging to the *BCL2* family play an essential role in germ line development and establishment (Tilly *et al*. 1997, Tilly 2001). Studies mainly performed in mice, rats and humans have shown that ovarian germinal tissue displays an enhanced expression of apoptosis-inducing *BAX* and low levels of apoptosis-inhibiting *BCL2*, which are responsible for the high rate of apoptosis that characterises the developing mammalian ovary (Kim & Tilly 2004, Albamonte *et al*. 2005, 2008, Hussein 2005). Apoptotic activity in the developing ovary is restricted to the germ cell proper, both PGCs, oogonia and oocytes, rather than to pregranulosa/granulosa cells, a process referred to as germ cell attrition. After birth, once the primordial-follicle enclosed oocyte pool has been established, apoptotic activity moves to the somatic compartment involving primarily the granulosa cells of resting follicles (Vaskivuo *et al*. 2001), and oocyte loss continues throughout reproductive lifetime as the result of follicular atresia.

The South American plains vizcacha, *Lagostomus maximus*, is a fossorial rodent belonging to the suborder hystricognatha, distributed in the Southern region of the Neotropics. This is a seasonal breeder, medium-sized caviomorph with a 4 kg body mass in adult females and 6.3 kg in males (Jackson *et al*. 1996). Gestation lasts around 154 days (Weir 1971*a*), a very unusually long period for a rodent and one of the longest recorded in hystricomorphs. In addition, *L. maximus* shows the highest ovulation rate described for a mammal, releasing between 400 and 800 oocytes at each oestrous cycle (Weir 1970, 1971*a*, 1971*b*). However, it seems that just a few (10 to 12) oocytes are fertilised and only one to two embryos are gestated to term (Weir *et al*. 1971*b*). Massive polyovulation in *L. maximus* has classically been explained as the result of a highly convoluted ovary that increases the organ surface for ovulation and the lack of detectable follicular atresia by standard staining (Weir 1971*b*, Mossman & Duke 1973). More recently, we have shown that the apoptosis-inhibiting *BCL2* gene is highly overexpressed from primordial to fully mature antral follicles in the adult ovary of *L. maximus*, while apoptosis-inducing *BAX* gene shows low levels of expression or is not detected, leading to a strong suppression of apoptosis-dependent follicular atresia. Suppression of apoptosis-dependent follicular atresia in *L. maximus* opposes to what is commonly found in other mammals, and it seems to relate directly to the massive ovulatory phenotype and the maintenance of pregnancy to term (Jensen *et al*. 2008). The absence of follicular atresia in the mature ovary of the plains vizcacha could be attributed to a failure in moving apoptosis from germ cells to the somatic compartment after birth (described earlier). If this is the case, it could be anticipated that the developing ovary should be subjected to considerable levels of germ cell attrition as it occurs in most mammalian species. Alternatively, if the absence of germ cell dismissal through apoptosis is a constitutive trait of the ovarian tissue in *L.maximus*, one could expect no massive germ cell degeneration in the developing ovary leading to an overpopulated germ line at birth. In such a case, massive ovulation might act as the alternative mechanism of oocyte loss in this species.

In order to discern between these two possibilities, we have investigated the dynamics of germ cell formation in the developing ovary of *L. maximus*, through the analysis of the distribution of the specific germ cell marker VASA to evaluate colonisation and proliferation in the foetal gonad, through the evaluation of apoptotic germ cell activity by a DNA *in situ* labelling assay, and through the expression balance of *BCL2* and *BAX* genes from early to late development in order to encompass the transition from oogonial mitotic proliferation to the entrance to meiosis, the course of prophase I, and the primordial follicle formation.

Results

General morphology and histology of the foetal ovary in L. maximus

Foetal ovary in *L. maximus* showed a typical oval shaped, compact and solid morphology, with no sign of invagination of the surface epithelium deep into the organ as described in the adult ovary. Early developing ovaries were abundantly packed with germ cells, mainly oogonia at interphase or undergoing mitosis (Fig. 1). Although oogonia were distributed in the entire ovary among somatic cells, two different regions were clearly distinguished according to germ cell density and organisation. The cortical external area was very rich in germ cells with oogonia organised in ovarian cords (Fig. 1B) with a low proportion of somatic cells, whereas in the internal region somatic cells prevailed in the face of germ cells (Fig. 1A). Oogonia in mitosis (Fig. 1C) and oocytes in the early stages of meiosis organised in small nests of two–eight cells, which often displayed a similar stage of cell cycle. Mid developing ovary showed a considerable proportion of primary oocytes that had entered early stages of prophase I (Fig. 1D), distributed mainly in the internal region often close to the area connected to the mesonephros by the rete ovarii. Proliferating oogonia remained in the cortical external area. At late development, oocytes were still organised in nests (Fig. 1E), and the ovary was plentiful of incipient primordial follicles with oocytes in different meiotic stages (Fig. 1F). Isolated oogonia/oocytes still remained at the external cortical region.

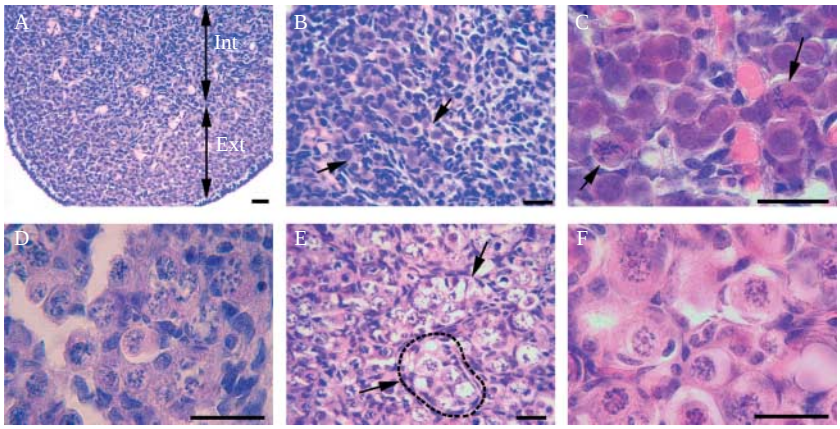

Figure 1 General histology of the developing ovary in *L. maximus*. (A) Panoramic view of an early developing ovary abundantly packed with oogonia distributed in the entire organ. Note the difference in germ cell density between the external (ext) and internal (int) areas. (B) Detail of the cortical external area at early development showing oogonia organised in cords (arrow) surrounded by somatic cells. (C) Detail of germ cells organised in a nest; note the presence of proliferating oogonia (arrows). (D) Mid developing ovary showing primary oocytes that have entered prophase I. (E) Nest-organised distribution (arrows) of oocytes at mid development; the dotted area indicates one nest. (F) Characteristic view of the late developing ovary plentiful of incipient primordial follicles. Bar=20 μm.

Germ cell distribution and progression through oogenesis: VASA expression

Cytoplasmic VASA immunolabelling was detected in oogonia, primary oocytes and primordial follicle-enclosed oocytes, at all developmental ages analysed (Fig. 2). In early developing ovary, VASA showed a bright, punctuated distribution in oogonia that clearly highlighted the organisation of germ cells in nests with no clear-cut among cells, revealing cytoplasmic interconnection (Fig. 2A and B). Signal intensity was still bright and punctuated in mid developing ovaries with a clear-cut limit among germ cells (Fig. 2C and D). Germ cell nests or cysts could be still observed clearly delimited by somatic cells that had also invaded the nest (Fig. 2C and D). A closer inspection of nests showed the coexistence of interconnected oogonia distributed in the periphery of the nest and well-defined primary oocytes dispersed among somatic cells in the internal area (Fig. 2D). Late developing ovaries showed a more homogeneous VASA distribution covering the entire cytoplasm of oocytes, which had entered in close contact with somatic cells in incipient primordial follicles (Fig. 2E and F). In all gestational ages analysed, ovarian sections incubated in the absence of primary antibodies did not exhibit detectable immunolabelling (Fig. 3G). Widespread expression of VASA throughout ovarian development was confirmed by conspicuous detection in western blot (Fig. 2H).

Immunohistochemical localisation and expression of BCL2 and BAX proteins

Immunodetection of BCL2 protein was positive in early, mid, and late developing ovaries. At early development, BCL2 positive immunostaining was particularly strong, displayed a decreased intensity in mid developing ovaries and became stronger again in late developing ovaries (Fig. 3A–C). It is interesting to note that the decrease in immunostaining intensity for BCL2 was coincident with the entrance in meiosis. The pattern of BAX immunodetection was quite different from that of BCL2 and displayed a time-restricted expression. Positive slight immunostaining was just detected in early developing ovary (Fig. 3D) and became negative, except for occasional isolated germ cells, in mid and late developing ovaries (Fig. 3E and F). The same pattern of strong immunostaining for BCL2 and slight or negative staining for BAX was reproducible in all samples analysed from different embryos. Western blot analysis was consistent with immunohistochemical (IHC) observations. Enhanced amounts of immune-reactive protein corresponding to BCL2 (26 kDa) were found in early, mid and late gestating ovaries (Fig. 4A) compared with those in the control tissues. In agreement with IHC observations, a slight decrease in BCL2 detection was evident in mid developing ovary (Fig. 4B). In contrast, BAX significantly decreased from early to late development (Fig. 4B).

Apoptosis in the developing ovary of L. maximus*: TUNEL assay*

Apoptosis studied by TUNEL technique showed to be conspicuously negative in sections belonging to early, mid, and late developing ovaries (Fig. 5A, C, and E). Occasionally, a few isolated germ cells were found to be positive (Fig. 5B, D, and F) in all developmental time points. In order to confirm negative TUNEL results, treated sections were incubated with DNase and reprocessed by TUNEL protocol. After this treatment, all nuclei become positive, confirming that DNA was originally not fragmented in the samples (Fig. 5G).

Discussion

In contrast to what is normally found in studied mammalian species, the results presented in this study indicate that the establishment of the female germ line in *L. maximus* takes place in the absence of germ cell attrition, representing the first description of abolished massive germ cell elimination in the developing mammalian ovary. Over expression of apoptosis-inhibiting *BCL2* gene together with the absence of

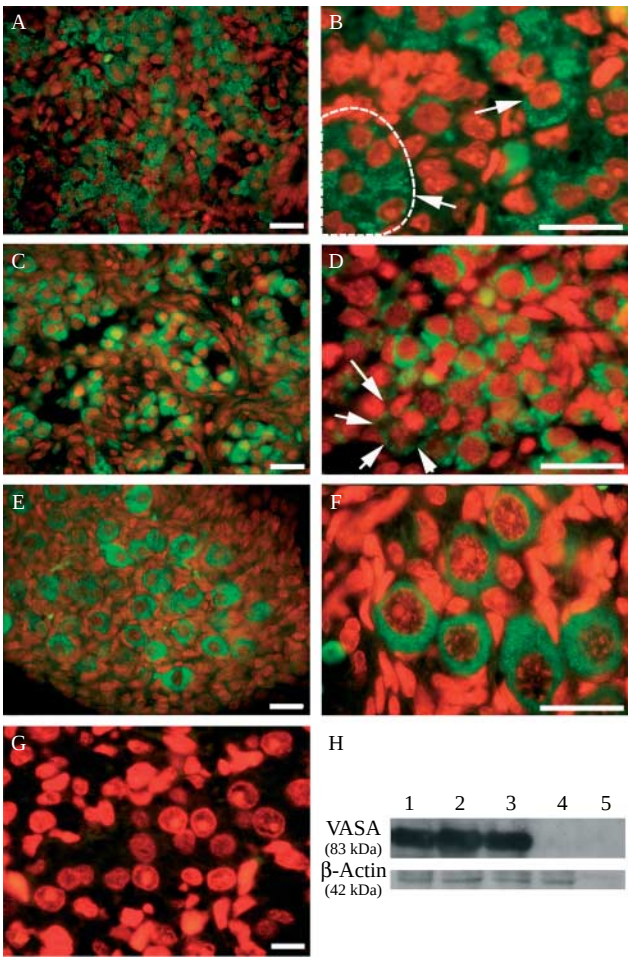

Figure 2 Immunodetection of the specific germ cell marker VASA (green staining) in developing ovary of *L. maximus*. (A) Oogonia in early developing ovary displaying bright and punctuated VASA immunolabelling with no clear-cut distribution among cells. (B) Detail of nest-organised germ cells (arrows) showing no cell limits in VASA signal (dotted area). (C) Brighter and still punctuated distribution of VASA immunolabelling in germ cells in mid developing ovary organised in nests. Note that VASA signal became mostly cell-contained. (D) Detail of a germ cell nest showing cell-contained VASA signal in the centre of the nest and still interconnected oogonia in the nest periphery (arrows). (E) Panoramic view of homogeneously distributed VASA immunolabelling in the entire cytoplasm of oocytes at late development. (F) Detail view of VASA signal in diplotene-arrested oocytes in incipient primordial follicles at late development. (G) Negative control in early developing ovary. (H) Expression in western blotting for early (lane 1), mid (lane 2) and late developing ovary (lane 3) protein extracts and control homospecific tissues, spleen (lane 4) and muscle (lane 5). Bar=20 μm. Red staining: nuclear DNA counterstained with propidium iodide.

apoptosis-inducing *BAX* gene expression and the low level of apoptotic germ cell nuclei detected by TUNEL assay support the idea that abolished germ cell attrition in the developing ovary of *L. maximus* arises from a strong prevention of the intrinsic apoptosis pathway. A similar situation has been described in the adult ovary of *L. maximus* in which the *BCL2* gene is overexpressed throughout folliculogenesis accompanied by a scarce detection of apoptosis-inducing BAX protein and a rare and occasional detection of TUNEL-positive follicles (Jensen *et al.* 2006). Prevention of germ cell demise in the foetal ovary and scarce follicular atresia in the adult indicate that the absence of germ cell dismissal through apoptosis is a constitutive trait of the ovarian tissue in *L.maximus.* Moreover, prevention of apoptosis through *BCL2* overexpression not only involves the germ cell proper and the supporting cells, granulosa and theca cells, but also extends to the corpora lutea preserving its structural integrity and functionality during the whole gestation (Jensen *et al.* 2008), reinforcing the hypothesis that the *BCL2* gene family shows an ovary-specific regulation in order to maintain low levels of apoptosis-dependent germ cell elimination in this mammalian species.

Apoptosis-dependent massive germ cell death has been regarded as a constitutive trait of the developing mammalian ovary that eliminates 65–85% of the germinal tissue depending on the species (Flaws *et al.* 2001, Guigon & Magre 2006). Much of our understanding of this process has emerged from studies performed in mice, rats and humans, and to a lesser extent in other mammals. Although multiple genes are known to regulate apoptosis, *BAX* and *BCL2* genes play an essential role in the mammalian ovary since they function as a rheostat determining cell death or survival through the expression balance between the proteins they encode (Tilly *et al.* 1997, Tilly 2001). In fact, pro-apoptotic *BAX* seems to be up-regulated by default in the mammalian ovary, being responsible for massive germ cell degeneration by attrition of foetal germ cells and apoptosis-dependent follicular atresia in the adult (Kim & Tilly 2004, Albamonte *et al.* 2008). The central role of the *BCL2/BAX* balance in prevention of apoptosis has been supported through experimental BCL2 transfection of cell lines that protect cultured cells from death (Reed 1994). Moreover, *Bcl2*- and *Bax*-deficient knockout mice have decreased or increased primordial follicles respectively (Knudson *et al.* 1995, Ratts *et al.* 1995). Our results indicate that *BCL2/BAX* are expressed in the developing ovary of *L. maximus* in the opposite way from that found in mice (Kim & Tilly 2004), rats (Tilly *et al.* 1995), monkeys (Uma *et al.* 2003), quail (Van Nassaw *et al.* 1999) and humans (Vaskivuo *et al.* 2001, Albamonte *et al.* 2008), with a biased expression in favour of the apoptosis-inhibiting BCL2 protein and a time-restricted or absent expression of BAX. This seems to preserve the germinal reserve from apoptosis with little or no restriction to proliferation, a situation that reminds the pattern of enhanced *BCL2* expression contributing to uncontrolled proliferation and cell survival in many cancers (Kirkin *et al.* 2004). The very low levels of apoptosis revealed by TUNEL assay indicate a strong prevention of germ cell attrition and little or no restriction to proliferation. However, it

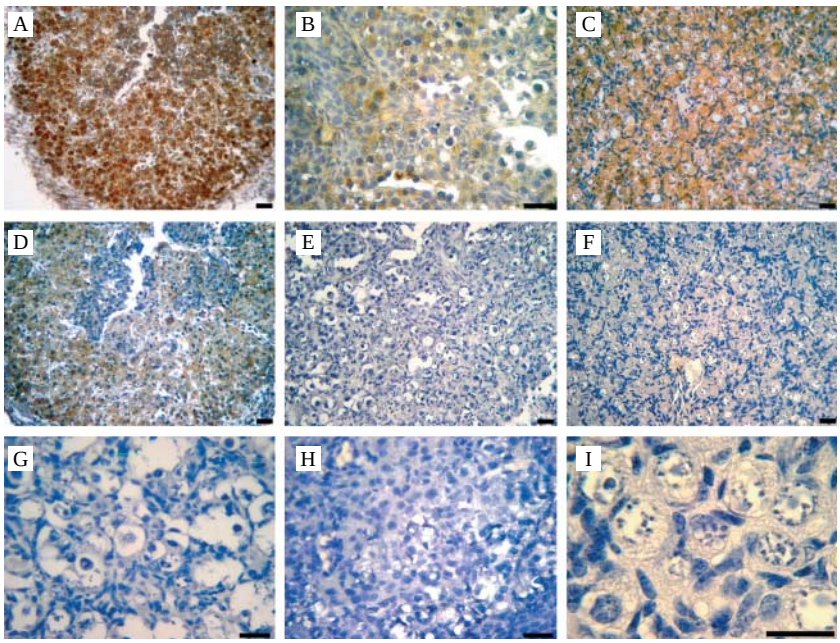

Figure 3 BCL2 and BAX protein immunohistochemical detection in the developing ovary of *L. maximus*. (A) Apoptosis-inhibiting BCL2 protein showed a strong immunolabelling at early development, (B) decrease in intensity in mid developing ovary and (C) becoming stronger again by late development. (D) Apoptosis-inducing BAX was slightly detected in early-developing ovary and (E) became undetectable at mid and (F) late development. (G–I) Representative negative control performed by omitting the primary antibody. Bar = 20 μm.

is worthwhile to consider that other mechanisms may be acting in the developing ovary of *L. maximus*. For instance, it has been shown that germ cell exfoliation from the ovarian surface contributes to eliminate PGCs, oogonia and oocytes in the developing human ovary (Motta *et al*. 1997).

General histology and the pattern of VASA expression that we found in the developing ovary of *L. maximus* are commensurate with a high level of colonisation through proliferation. Although just three time-points have qualitatively been analysed through the 5-month-long gestation of the plains vizcacha, it is worth to note that the genesis of the germ line proceeds through the overlapping of the main processes involved in the gonadal development: mitotic proliferation, entrance to meiotic prophase I and germ cell–somatic cell association to form primordial follicles. All those essential changes that occur in ovarian organogenesis were reflected by the stage-specific immunodetection pattern displayed by VASA protein. In the early developing ovary, VASA showed a widespread distribution in proliferative germ cell nests reflecting the interconnection of cells. Once germ cells entered meiosis and folliculogenesis in mid and late development, VASA displays a cell-restricted immunostaining pattern. In all cases, however, immunoreaction extended to the entire cytoplasm. In mice and humans, once the oocytes enter prophase I and folliculogenesis, VASA protein displays a restricted aggregation in a cytoplasmic paranuclear localisation corresponding to the Balbiani's vitelline space (Balbiani 1864), a cytoplasmic complex composed of mitochondria, endoplasmic reticulum and lamellar aggregates (Hertig & Adams 1967, Pepling *et al*. 2007). In the developing human ovary that displays the highest germ cell elimination reaching 85% of the germinal reserve before birth (Baker 1963, Forabosco *et al*. 1991), most of the primordial follicle-enclosed oocytes show VASA protein associated to Balbiani's body (Castrillon *et al*. 2000), and it has been estimated that only around 10% of the oocytes display

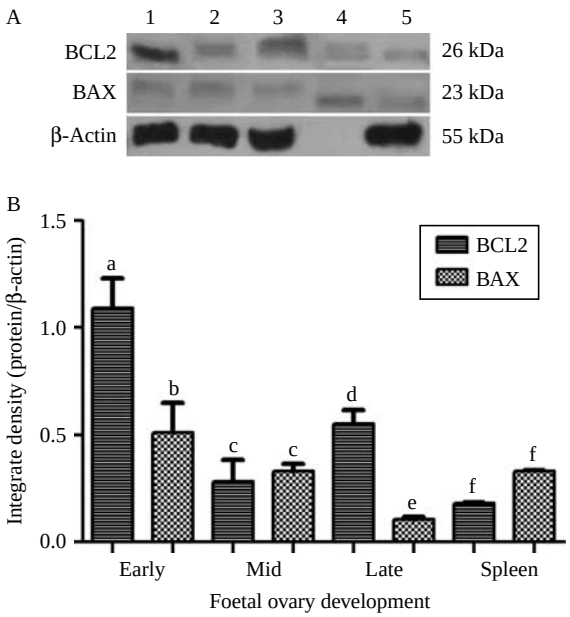

Figure 4 Western blot analysis for BCL2 and BAX proteins in the developing ovary of *L. maximus*. (A) Early (lane 1), mid (lane 2) and late developing ovary (lane 3), and control homospecific tissues, muscle (lane 4) and spleen (lane 5). (B) Integrate density calculated against control load (actin) showing enhanced expression of BCL2 protein, especially at early and late development. Different letters above bars indicate significant differences between groups ($P<0.05$).

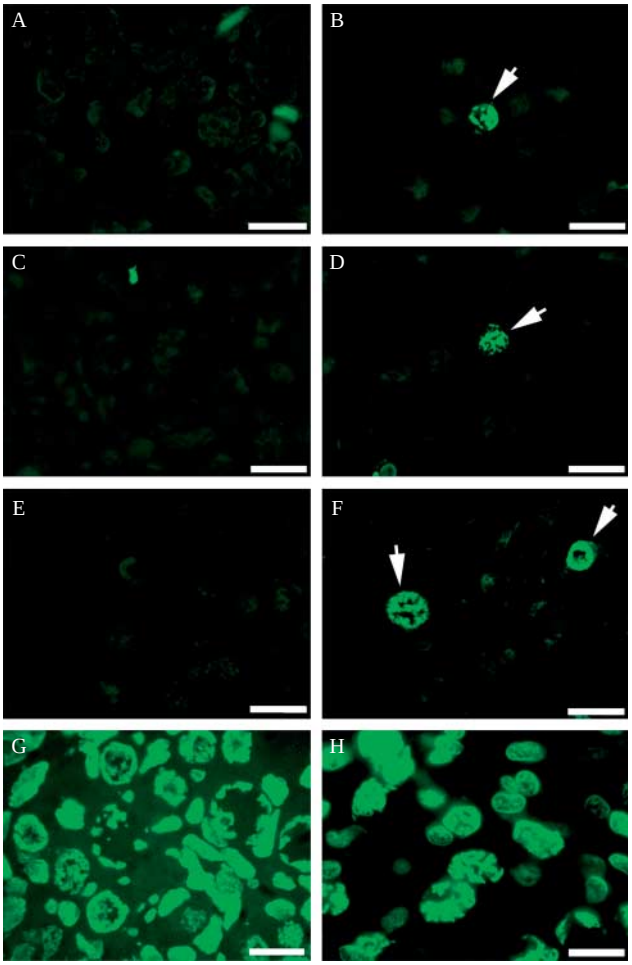

Figure 5 Apoptosis-related DNA fragmentation detected by TUNEL assay in the developing ovary of *L. maximus*. Ovaries were conspicuously negative during early (A), mid (C) and late development (E). A closer inspection at a higher magnification revealed rare and isolated TUNEL-positive germ cell nuclei for early (B), mid (D) and late developing ovaries (F). (G) Negative TUNEL sections treated with DNase and re-processed by TUNEL protocol confirmed true negative results, since all nuclei became positive indicating intact DNA prior to enzyme digestion. (H) Positive control (see Materials and Methods). Bar=20 μm.

a widespread cytoplasmic distribution of VASA (Albamonte *et al.* 2008). We did not detect VASA aggregated to the Balbiani's space in any sample analysed in the developing ovary of the plains vizcacha. It is worth to note that developing ovaries of human and *Lagostomus* show an opposite behavior, the former having almost 85% of germ cell dismissal by birth, the latter having abolished apoptosis and germ cell elimination throughout foetal development as shown in this study. Considering that VASA is a DEAD box, RNA-binding protein involved in the assembly and transport of mRNAs during oogenesis (Castrillon *et al.* 2000) and Balbiani's body is viewed as a structure related to universal mechanisms of RNA metabolism (Pepling *et al.* 2007), it is tempting to propose that the association or not of VASA protein to the Balbiani's space may relate to the final fate of the oocyte, death or survival, in the light of the differences found between humans and *L. maximus*.

In conclusion, we have shown that germ cells may not be necessarily committed to death through massive apoptosis as a constitutive feature of the developing mammalian ovary. Unrestricted proliferation of germ cells, without apoptosis-driven elimination, in the developing ovary of *L. maximus* reveals the existence of alternative strategies for oocyte production in mammals and bolsters our contention that mechanisms shared by a few species not necessarily apply to all mammals.

Materials and Methods

Animals

The experimental protocol described in this study was reviewed and authorised by the Ethics and Research Committee of Universidad Maimónides, Argentina. Handling and killing of captured animals were performed in accordance with the standards defined in the guide for the care and use of laboratory animals (CCAC 2002), and guidelines on the care and use of wildlife (CCAC 2003) from the Canadian council of animal care. Plain vizcachas, *L. maximus*, were captured from a natural population at the Estación de Cría de Animales Silvestres (ECAS), Ministry of Agriculture, Villa Elisa, Buenos Aires province, Argentina, at three different time points during the main breeding season (from March–April to August–September), in order to obtain early, mid and late gestating embryos during the 5.5-month length gestation period. A total of 13 samples classified as early ($N=6$), mid ($N=4$) and late gestating ($N=3$) embryos were collected from ten pregnant females. Female gestational age was estimated on the basis of capture time, examination of foetal morphology, foetal weight and crown–heel length.

Tissue collection and processing

Gestating females were intraperitoneally anesthetised with ketamine (10 mg/kg body weight; ketamine clorhydrate, Holliday Scott S.A., Buenos Aires, Argentina) and xilacine (1 mg/kg of body weight; xilacine clorhydrate, Laboratorios Richmond, Buenos Aires, Argentina), and immediately killed by trained technical staff by an intracardiac injection of Euthanyle

Table 1 Foetus growth and foetal ovary size in *Lagostomus maximus* throughout development.

		Foetus		Foetal ovary	
Gestation (days)	***N***	Length (mm)	Weight (g)	Length (mm)	Width (mm)
Early (50±15)	6	30.4±5.3	3.1±1.2	1.3±0.4	NR
Mid (90±15)	4	87.0±7.0	52.0±16.0	4.5±1.4	2.6±0.7
Late (140±15)	3	185.0±1.5	90.0±10.0	7.9±1.2	4.5±0.6

NR, not registered. Data are expressed as mean±S.E.M.

(0.2 ml/kg of body weight; sodium pentobarbital, sodium diphenyl hydantoinate; Brouwer S.A., Buenos Aires, Argentina). Uterine horns were exposed, and embryos were removed, weighed and measured (Table 1). Left and right ovaries were dissected out from the embryos under a stereomicroscope, and width and length were immediately recorded (Table 1). For histochemical analysis, one ovary was fixed in cold 4% neutral-buffered paraformaldehyde (PFA) for 24 h, and the other one was frozen at −80 °C until used for protein isolation. When more than one foetus was implanted in the same uterine horn (especially at early and mid gestation stage) only the distal foetuses were used for further analyses since it is known that only the embryos nearest to the cervix develop normally to term (Weir 1971*a*, 1971*b*, Jensen *et al*. 2008). PFA-fixed tissues were dehydrated through a graded series of ethanol and embedded in paraffin, and the whole sample was serially sectioned at 5 μm and mounted onto cleaned coated slides. For each specimen, at least three to five slides were stained with haematoxylin–eosin for general histological inspection. The remaining serial-sectioned slides to those used for haematoxylin–eosin staining were stored at room temperature until used for immunofluorescence, immunohistochemistry or TUNEL assay.

Immunofluorescence analysis of VASA protein

Mounted paraffin sections were dewaxed in xylene (Sigma Chemical Co.) and re-hydrated through a decreasing series of ethanol. After two 10 min rinses in PBS, they were incubated with a blocking solution containing 10% bovine foetal serum (BFS)/15% BSA in PBS, pH7.4, for 1 h at room temperature. Then, the slides were thoroughly rinsed in PBS and incubated with polyclonal rabbit anti-*Mvh* (mouse Vasa homologue) primary antibody (1/500), kindly provided by Dr Noce (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan), overnight at 4 °C. Although the primary antibody has been raised against mouse VASA protein, inmunodetection of *L. maximus* VASA protein can be anticipated since the high degree of conservation of this protein among different species (Lasko & Ashburner 1988). Moreover, sequencing of *L. maximus* VASA homologue protein showed 90% homology with rat and mouse (F Jensen, ML Muscarsel Isla, ML Bacigalupo, G Wöllmer & AD Vitullo 2007, unpublished observations). After incubation with anti-*Mvh*, the slides were incubated with goat anti-rabbit IgG–FITC-conjugated antibodies (Santa Cruz Biotechnology, CA, USA) for 60 min at room temperature. All the slides were counterstained with 1 mg/ml propidium iodide, washed for 30 min in PBS, and mounted with VectaShield mounting medium (Vector). Negative controls were performed by omitting the primary antibody. Samples were examined under an Olympus BX40 microscope by conventional epifluorescence with u.v. illumination, and images were captured with an Olympus Camedia C-5060 camera.

IHC detection of BCL2/BAX

Dewaxed and re-hydrated ovarian sections were subjected to blocking of endogenous peroxidase activity with 3% H_2O_2 for 20 min at room temperature. They were then blocked with 10%BFS/15%BSA in PBS pH7.4 for 1 h at room temperature and incubated overnight at 4 °C with specific rabbit polyclonal anti-BAX (1/500 Santa Cruz Biotechnology) or anti-BCL2 (1/500 Santa Cruz Biotechnology) antibodies. Antibodies employed were cross-reactive with mouse, rat and human proteins, and previously shown to recognise the targeted proteins in the vizcacha (Jensen *et al* 2006, 2008). Immunoenzymatic reactions were performed with HRP and revealed with 3′,3′-diaminobenzidine using the LSAB 2 System (DAKO Cytomation, Carpinteria, CA, USA). Procedure was as per the suppliers' recommendations. Staining for each antibody was repeated at least three times in separate assays for each specimen, using a minimum of two slides per assay. Sections assayed were entirely inspected under the microscope. Repetitions of assays performed in different days confirmed that staining was reproducible. Negative controls were performed by omitting the primary antibody or presorption with the synthetic peptides. All the slides were counterstained with haematoxylin.

Western blot analysis

Ovaries were immersed in five volumes of lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM Nα-p-tosyl-lysine chloromethyl ketone and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone). Then, samples were homogenised with a high-speed homogeniser, centrifuged at 10 000 ***g*** for 10 min at 4 °C, and pellets were discarded. Protein content of the supernatant was determined by the Bradford assay (Bio-Rad). Aliquots of proteins (100 μg) were boiled for 5 min, resolved by SDS-PAGE (15%) at 150 V, 1 h at 4 °C, and transferred onto nitrocellulose membranes in transfer buffer containing 20% methanol (vol/vol), 0.19 M glycine and 0.025 M Tris-base (pH 8.3) at 250 mA during 70 min at 4 °C. Molecular weight (MW) of corresponding proteins was determined in relation to MW Rainbow Markers (Amersham). Equal loading was confirmed by Ponceau S staining. After blocking unspecific binding with PBS/5% skim milk overnight at 4 °C, the nitrocellulose membranes were allowed to react with rabbit polyclonal anti-BAX (1/1000, Santa Cruz Biotechnology), rabbit polyclonal anti-BCL2 (1/1000, Santa Cruz Biotechnology) or polyclonal rabbit anti-*Mvh* (VASA) as primary antibodies for 2 h at room temperature, followed by two extensive washes with 0.1% Tween 20 in PBS. Blotted proteins were detected by a polyclonal antibody HRP-conjugated (1:10 000) using the ECL western blot detection reagent (Amersham). Adult ovary and muscle protein extracts of *L. maximus* were employed as BCL2 and BAX controls (Jensen *et al*. 2006, 2008). Monoclonal antibody anti-β-actin was assayed to normalise the protein loading. Relative expression of BAX and BCL2 was quantified at each developmental time point by integrate density against the load control, with Scion Image Software (Scion Corporation, Frederick, MD, USA). Data are expressed as mean and s.e.m., plotted with GraphPad Prism Software, version 5 (San Diego, CA, USA) and statistically analysed by the Newman–Keuls test. Differences were considered significant when $P<0.05$.

TUNEL assay

Detection of apoptosis-associated DNA fragmentation was performed in paraffin-embedded sections by TUNEL, using the '*In Situ* Cell Death Detection Kit' (Roche Diagnostics) with fluorescein-tagged nucleotides. The procedure was done following the suppliers' recommendations. Treated sections were examined in an Olympus BX40 microscope by conventional epifluorescence with ultraviolet illumination. In order to confirm negative results, TUNEL-processed sections were incubated with 10 IU/ml DNaseII (Sigma Chemical Co.) in 50 mM Tris–HCL pH 7.5, 10 mM Mg_2Cl and 1 mg/ml BSA for 10 min at room temperature. After incubation, the slides were thoroughly rinsed and treated again according to the TUNEL protocol. Negative control was done by omitting the deoxynucleotidyl transferase enzyme. A 20-week-old human foetal ovary section was included as a positive control at each TUNEL reaction. At this time-point, human ovary displays the highest TUNEL positivity throughout foetal development (Albamonte *et al.* 2008).

IHC and immunofluorescence analyses

In total, six sections were analysed at each IHC or immunofluorescence (IF) experiment for VASA, BCL2, BAX and TUNEL assays. The six sections were selected for every five consecutive cuts; thus, they were separated by a 30 µm distance. If sections 1, 6, 12, etc. were used for VASA, then sections 2, 7, 13, etc. were used for BCL2, and so on for BAX and TUNEL. This enabled us to have the three antibodies and TUNEL in a comparable 20 µm matched area, in consecutive cuts. All IHC, IF and TUNEL assays were repeated at least three times in separate and independent experiments. Treated samples were examined in an Olympus BX40 microscope attached to a photo camera Olympus Camedia C-5060 for image capture.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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