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Identification of the proliferative activity of germline progenitor cells in the adult ovary of the bat *Artibeus jamaicensis*

Tania J. Porras-Gómez and Norma Moreno-Mendoza

Department of Cell Biology and Phisiology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apartado Postal 70228 Ciudad de México 04510, Mexico

Research Article

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Corresponding author:
Norma Moreno-Mendoza;
Email: angelica@biomedicas.unam.mx

Summary

Until a few years ago, it was assumed that oocyte renewal did not take place in the ovary of adult organisms; however, the existence of germline progenitor cells (GPCs), which renew the ovarian follicular reserve, has now been documented in mammals. Specifically, in the adult ovary of bats, the presence of cells located in the cortical region with characteristics similar to GPCs, called adult cortical germ cells (ACGC), has been observed. One of the requirements that a GPC must fulfil is to be able to proliferate mitotically, so the evaluation of cell proliferation in ACGC is of utmost importance in order to be able to relate them to a parental lineage. Currently, there are several methods to determine cell proliferation, including BrdU labelling or the use of endogenous proliferation markers. Thus, the aim of this work was to evaluate the proliferative activity of ACGC in the adult ovary of the bat *Artibeus jamaicensis*, using different proliferation markers and correlating these with the protein expression of the transcription factor Oct4 and the germ line marker Ddx4. We found that the expression pattern of the proliferation markers BrdU, PCNA, Ki-67 and pH3 occurs at different times of the cell cycle, so co-localization of two or more of these markers allows us to identify proliferating cells. This allowed us to identify ACGC with proliferative capacity in the adult ovary of *A. jamaicensis*, suggesting that GPCs renew the follicle reserve during the adult life of the organism.

Introduction

It is well known that germ cells define themselves very early in development, and migrate to the embryonic gonads, where their sexual differentiation occurs to become oocytes in the case of the ovary, and spermatozoa in the case of the testis (Saitou and Yamaji, 2012). The mammalian testis is a highly dynamic organ, where the spermatogonial cell population is continuously renewed; not so the ovary, where a mechanism of germline self-renewal remains a matter of debate. In the testes and ovaries of adult mammals the germinal lineage is considered to be progenitor cells, because its function is the periodic regeneration of gametes (sperm and oocytes); highly specialized cells responsible for transmitting genetic information from generation to generation. Therefore, germ stem cells (GSCs), whose function is to renew the pool of germ cells established during the development of the organism, are considered the progenitor cells of the germ line. Among mammals, the information derives from studies carried out mainly on mice, where GSCs known as spermatogonial stem cells have been located and characterized in males. These progeny reside in the basement membrane within the seminiferous tubule, in a microenvironment that contains the necessary elements to induce their death, survival, quiescence, self-renewal and/or differentiation (Greenspan *et al.*, 2015). In the case of the ovary, studies suggest the presence of progenitor cells with mitotic activity that allow them to contribute to the renewal and maintenance of oocytes in adult life (Johnson *et al.*, 2004). This fact has been reported in different organisms such as: prosimians, mice, rats, pigs, sheep, bats and humans (Porras and Moreno, 2017). For such reason, the reproductive biology paradigm that the ovary has no capacity to renew germ cells, and that females are born with a finite number of oocytes (Zuckerman, 1951), and therefore the primordial follicles represent the oocyte pool, has been debated (Telfer and Anderson, 2019).

Fundamental to the identification of germline progenitor cells is their ability to divide mitotically, as well as to express germline-related factors such as Vasa and Ckit, as well as pluripotency factors such as Oct4 and Nanog (Telfer and Anderson, 2019). The capacity to proliferate mitotically is necessary to maintain the progenitor cells pool, which is why it is of great importance to evaluate the cell proliferation of this cell lineage (Muskhelishvili *et al.*, 2003). At present, there are different methods to identify cell proliferation; among the most used, we find *in vivo* labelling of DNA with a synthetic nucleotide analogous to thymidine, Bromo-deoxy-Uridine (BrdU) (Gratzner, 1982). BrdU is readily incorporated by nucleotides during the synthesis (S) phase of the cell cycle and can be readily identified by immunofluorescence, using

an anti-BrdU antibody (Goldsworthy *et al.*, 1991). Another method is the use of cell proliferation markers, which are endogenous to the cells and their expression fluctuates throughout the different phases of the cell cycle. These are easy to use and can be applied in different cytological and histological preparations (Muskhelishvili *et al.*, 2003). Among the most common markers are: PCNA, Ki-67 and phospho-Histone H3 (pH3) (Whitfield *et al.*, 2006).

Proliferating cell nuclear antigen (PCNA) has been used in basic research as a tool to assess cell proliferation (Muskhelishvili *et al.*, 2003). PCNA was described by Miyachi *et al.* (1978) and was characterized as a nuclear protein synthesized in the G1 and S phases of the cell cycle that favours DNA synthesis, as it is a cofactor of DNA polymerase δ (Kurki *et al.*, 1986; Matsumoto *et al.*, 1987). Proliferating cells have been identified by means of immune-detection against PCNA (Foley *et al.*, 1991); however, the value of detecting proliferating cells has been questioned, as detectable levels of PCNA vary significantly depending on the fixatives used (Morris and Mathews, 1989; Hall *et al.*, 1990; Coltrera and Gown, 1991; Schwarting, 1993; Scholzen and Gerdes, 2000). The Ki-67 antigen is a protein of approximately 395 kDa, which is encoded by almost 30,000 base pairs within the genome (Schlüter *et al.*, 1993). It is a nuclear protein expressed at the interphase of the cell cycle (Gerdes *et al.*, 1984; Gerlach *et al.*, 1997). It was from pKi67 that Gerdes et al (1983) generated the first Ki-67 antibody by immunizing mice with the nuclei of a Hodgkin's lymphoma cell line L428 (Gerdes *et al.*, 1983). Its name derives from the city of origin (Kiel) and the clone number of the 96-well plate (Scholzen and Gerdes, 2000). Characterization of the Ki-67 antibody revealed, by detailed cell cycle analysis, that the antigen is present in the nuclei of cells in G1, S and G2 phases of the cell cycle, as well as during mitosis. Cells at rest or in G0 phase do not express this (Gerdes *et al.*, 1984). Ki-67 was also reported to be expressed in all proliferating cells, both normal and tumorous, suggesting that the presence of Ki-67 is a good marker for determining the growth fraction of a cell population (Alison, 1995). However, there is controversy regarding its expression pattern, as it has been reported that this can vary depending on the different cell types and according to fixation and immunodetection protocols (Littleton *et al.*, 1991). Histones play a central role in transcription regulation, DNA replication and chromosome stability. DNA accessibility is regulated through a complex set of histone post-translational modifications, also known as the histone code, and nucleosome remodelling. It has been documented that Histone 3 (H3), unlike other histones, is only phosphorylated during mitosis. H3 phosphorylation occurs at the Ser10 residue as part of the chromosome condensation mechanism (Hendzel *et al.*, 1997). This phosphorylation at Ser10 begins during prophase, is maximal during metaphase, decreases during anaphase and disappears during telophase (Gurley *et al.*, 1978; Paulson and Taylor, 1982).

In this work, we use the bat as a model since in reproductive terms it bears greater resemblance to that of the human, which is why it represents a better study model. Besides this, in the bat species *Artibeus jamaicensis*, the presence of oocyte progenitor cells that may be renewing the follicle pool in the adult ovary has been reported (Antonio-Rubio *et al.*, 2013). Therefore, the aim of this work was to analyze proliferative capacity in the adult ovary of *A. jamaicensis* bat, specifically of the adult cortical germ cells (ACGC), by employment of cell proliferation markers (BrdU, PCNA, Ki-67 and pH3), and correlating these with the protein expression of the transcription factor Oct4 and the germline Ddx4 as markers of germline progenitors cells.

Materials and methods

Animals

Adult females from the *Artibeus jamaicensis* species were used. The bats were collected in the municipalities of Yautepec and Tepoztlán, which are located in the State of Morelos, Mexico, supervised by the Undersecretary of Management for Environmental Protection and the General Directorate of Wildlife (SEMARNAT), who granted the following collection permits: SGPA/DGVS/12149/16 and SGPA/DGVS/00264/17. The town of Yautepec is located in the north of the State of Morelos at an altitude of 1,210 metres above sea level. It has a warm sub-humid climate with summer rains and low deciduous forest vegetation. Correspondingly, Tepoztlán is located in the north of the state with semi-warm, humid and temperate climates with rains in summer and early autumn. The species *Artibeus jamaicensis* was identified using the field key described by Medellin *et al.* (2008) for this species in Mexico. Ten sexually mature (adult) females were used, identified according to the complete ossification of the growth plates of the epiphysis of the fourth phalangeal metacarpal joint. The bats were placed in cloth sacks to be transported to the Instituto de Investigaciones Biomédicas (IIB), UNAM.

Because up until now, no proliferation markers have been used in bat ovaries, we used the contrasting markers for the ovaries and intestines of mice from the B6B5/EGFP strain, which carry the green fluorescent protein, as positive controls.

Conservation status

Jamaican Fruit-eating Bat *Artibeus jamaicensis* has been assessed for The IUCN Red List of Threatened Species in 2016. *Artibeus jamaicensis* is listed as Least Concern (Miller *et al.*, 2016; IUCN, 2016).

Sacrifice and sample procurement

All of the experimental procedures were conducted following the ethical standards for animal experiments as directed by the IIB from the UNAM and in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Animals were euthanized by applying an overdose of sodium pentobarbital anaesthetic (0.7 mL/20 g; SEDAL-VET, Lyfsa Laboratories, Tulancingo, Hidalgo, México) administered intra-peritoneally, to subsequently dissect the ovaries for processing and analysis.

Incorporation of 5-Bromo-2´-Deoxyuridine (BrdU)

A dose of 100mg/kg weight of BrdU (Roche) diluted in 200 µl of phosphate buffer (PBS) plus 200 µl of Dimethyl sulfoxide (EMS) was injected intraperitoneally. The animal was exposed to BrdU for two hours. After this period, it was sacrificed in order to obtain the ovaries and intestine. The tissues were embedded in optimal mounting medium for cold sections (OCT, Tissue-teck) and 20 µm sections were sliced in series.

The 5-Bromo-2'-deoxy-uridine Labeling Detection Kit I (Roche, 11 296 736 001) was used to identify the expression of BrdU. For this, the slides were submerged in a bath with washing buffer for 5 min at room temperature. They were

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transferred to a 70% acid alcohol solution for 30 min at -20ºC, washed and incubated with the primary anti-BrdU antibody (Roche) at a 1:60 dilution in incubation buffer for one hour at 37 ºC. Subsequently, they were washed and the secondary rhodamine anti-mouse antibody (TRICT) was added at a dilution of 1:100, for one hour at room temperature. Finally, these were mounted in an aqueous medium and observed under a laser confocal microscope (LSM Pascal, Zeiss Argon-Krypton and Helium-Neon), employing the BP 546/12 filter (TRITC-Rhodamine).

Immunodetection of PCNA, Ki-67 and pH3

Adult bat ovaries were fixed immediately after collection with 4% Paraformaldehyde (Sigma) for 15 min, followed by a 5 min wash in 1X PBS and then placed in 30% sucrose overnight at 4 °C. The tissues were included in Beem dishes with Tissu-tek, orienting the samples for cross sections and freezing them in cold hexane. Serial sections of 14 µm thickness were sliced using a cryostat, and then placed on Superfrost-plus (EMS) slides, and placed under vacuum for 1 hour.

A 1X PBS wash was performed for 10 min, and the sections were permeabilized with Triton X-100 for 10 min. Subsequently, these were washed with PBS and each slide was incubated for 2 h with 1% albumin in PBS. They were then incubated with the primary antibodies: PCNA (Anaspec 55421), Ki-67 (Biocare Medical CRM325A) and pH3 (Millipore 06-570), at a 1:100 dilution in 1% albumin in PBS overnight at 4 ºC. Four washes were then carried out with PBS and blocked with 1% albumin for 15 min. Cy3 anti-rabbit secondary antibody (Life Technologies A10520) was placed at a 1:100 dilution in 1% albumin in PBS for one hour at room temperature. Four washes were performed with 1X PBS and finally the sections were mounted in aqueous solution (Dako). They were observed under the laser confocal microscope (LSM Pascal, Zeiss. Argon-Krypton and Helium-Neon). Filter BP 546/12 (TRITC-Rhodamine) was used.

Double BrdU-PCNA and BrdU-Ki-67 labelling

Once the protocol for the detection of PCNA and Ki-67 markers was completed as described above, we proceeded with the established methodology to simultaneously detect BrdU incorporation. In this case, the secondary antibody used to visualize PCNA and Ki-67 was a Goat anti-Rabbit IgG (H+L Cross-Adsorbed Secondary Antibody, Cyanine5 (1:100, Life technologies A10523). In this way, once the secondary antibody washes were completed, the sections were placed in a solution of Pepsin (4mg/mL) in 0.01N HCl in 1X PBS for 20 min, followed by a bath with 2N HCl in PBS for 30 min at room temperature. Subsequently, they were placed in Sodium Borate Buffer (Na2B4O7•10H2O) 0.1M at pH 8.5 for 10 min at room temperature and washed with 1X PBS. This was then blocked with 1% albumin in PBS for 1 hour at room temperature and incubated with the primary anti-BrdU antibody diluted 1:60 (Roche), for 24 h at 4 °C. The anti-mouse rhodamine secondary antibody (TRICT) was then incubated at a dilution of 1:100 for one hour at room temperature, to finally mount the sections with permanent medium for fluorescence (Dako). Observations were made in the microscopy unit of the Biomedical Research Institute, under a confocal laser microscope (LSM Pascal, Zeiss. Argon-Krypton and Helium-Neon), using the BP 546/12 (TRITC-Rhodamine) and LP 650 filters (Cy5).

Double Ddx4-BrdU and Oct4-BrdU immunolabeling

In order to detect the presence of proliferating cells corresponding to germline progenitor cells, a double detection was performed using BrdU and antibodies against Ddx4 and Oct4; germline-specific markers. For this, we followed the same protocol described for the simultaneous detection of BrdU and proliferation markers. Thus, immunofluorescence was first performed to reveal the primary anti-Ddx4 antibody at a dilution of 1:200 (Abcam ab13840) and anti-Oct4 antibody at a dilution of 1:250 (Abcam ab19857), to subsequently apply the methodology for the detection of BrdU as described above. Observations were made in the microscopy unit of the Biomedical Research Institute, under a confocal laser microscope (LSM Pascal, Zeiss. Argon-Krypton and Helium-Neon), using the BP 546/12 (TRITC-Rhodamine) and LP 650 filters (Cy5).

Western Blot

Frozen ovaries of *A. mexicanum* were homogenized in lysis buffer (50 mM Tris-HCl 50 mM, pH 7.4, containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecylsulfate) in the presence of a protease inhibitor mixture and centrifuged at 16,000 g for 20 min at 4 °C. The supernatants were collected and stored at -80 °C until use. Total protein content in the supernatants was analyzed by Pierce's bicinchoninic acid protein assay (Thermo Sci, IL, USA). Fifty micrograms of protein from ovarian homogenizes, diluted in loading buffer (Laemmli 2× containing 1% β-mercaptoethanol), were separated by electrophoresis on 12% SDS-PAGE homemade gels at 150 V for 60 min and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) using a semi-dry blot system (Bio-Rad) at 25 V for 50 min. The membranes were blocked in PBS/2% non-fat dry milk overnight at 4 °C. Subsequently, they were incubated overnight at 4 °C with the primary antibodies: PCNA (1:1500 Anaspec 55421), Ki-67 (1:1000; Biocare Medical CRM325A), pH3 (1:1000 Millipore 06-570), Oct4 (1:1000; Abcam ab19857) and Ddx4 (1:500; Abcam ab13840). After washing with PBS/0.2% Tween, the membranes were incubated with rabbit HRP IgG-conjugated secondary antibody (Invitrogen A16104; 1:2500) at room temperature for 1.5 h. Immunoreactive bands were detected by chemiluminescence using the Super Signal West Dura Extended Duration Substrate kit (Thermo Scientific USA) according to the manufacturer's protocol. Subsequently, the membrane was stripped with wash solution (Tris-HCl 1M pH 6.8; SDS 1%; β-Mercaptoetanol) and incubated with rabbit anti-β-actin (1:1500, Sigma-Aldrich A2066) primary antibody and the same conditions were imposed as those previously described.

Results

Proliferation markers BrdU, PCNA, Ki-67 and pH3 were analyzed since the expression points within the cell cycle occur at different stages (Figure 1), which allowed us to identify and validate cell proliferation in the ovary. We observed that in the adult ovary of the *Artibeus jamaicensis* bat there are cells related to these markers, detecting a positive marker mainly in the follicular cells. We could also observe that some cells located in the cortical region of the ovary are positive for these factors, which may correspond to adult cortical germ cells (ACGC).

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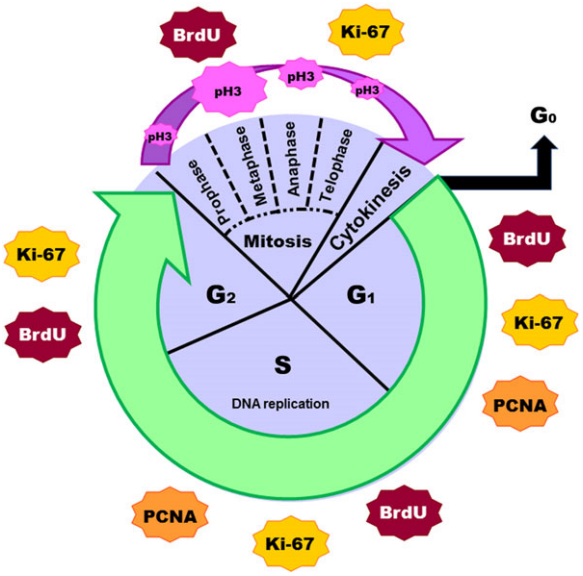

Figure 1. Expression points of the cell proliferation markers Ki-67, PCNA, pH3 and BrdU during the cell cycle. Ki-67 is detected in the nucleus during cell cycle interphase; it is present in G1, S, G2 and M phases and is absent in G0. PCNA is synthesized during early G1 and S phase of the cell cycle. Phosphorylation of histone 3 (pH3) at Ser10, Ser28 and Thr11 is closely related to chromatin condensation during mitosis and BrdU incorporation takes place during S phase.

Labelling with BrdU

As there are no reports on the incorporation of BrdU in bat organs, the in situ cell proliferation technique was standardized in mouse ovaries. The intestine was used as an internal control for both bat and mouse tissues, because it has an epithelium whose cell cycle is short, which allows continuous regeneration to compensate for the epithelial wear to which it is subjected. In the mouse case, BrdU expression was detected in the small intestinal epithelial cells (Figure 2A) and in the granulosa cells that make up the mouse B6B5/EGFP ovary (Figures 2B and C), revealing the correct administration of BrdU and proper application of the protocol. In adult females of the *Artibeus jamaicensis* bat, we also managed to incorporate BrdU into the cells of the organism. In the same way as in the mouse, the intestine was used as a control tissue due to its high cell proliferation rate, where BrdU expression was identified in intestinal epithelial cells (Figure 2D). In the case of the ovary, the incorporation of BrdU was observed in granulosa cells and in some cells located in the interstitium surrounding the follicles, which appear to correspond to myoid cells (Figures 2E and F).

Immunodetection of cell proliferation markers (PCNA, Ki-67 and pH3)

We chose to fix the tissues with 4% Paraformaldehyde because formaldehyde is known to react with arginine and lysine residues, which are very abundant in histones (Thavarajah *et al.*, 2012; Gwynn, 2001). Well-conserved histones can maintain an adequate DNA structure along with its associated molecules, which we believe may enable correct detection of BrdU, PCNA, Ki-67 and pH3. Under these experimental conditions of tissue collection, fixation method and immunodetection, it was possible to identify the expression of three non-invasive cell proliferation markers (PCNA, Ki-67 and pH3) in the adult ovary of the *Artibeus jamaicensis* bat (Figure 3).

The expression of PCNA was mainly observed in the nuclei of the granulosa cells that surround the oocytes. Immunoreactivity was also identified in a group of cells located in the cortical region of the ovary and in some oocytes. We did not observe variation in level of expression; this was homogeneous in all the ovaries analyzed (Figures 3A, D and G). Ki-67 appeared to be granular in the nuclei of cells. Similar to PCNA, there was positivity in the nucleus of follicular cells and among a group of cortical cells, no markers were ever observed in oocytes (Figures 3B, E and H). The expression of pH3 appeared to be restricted to a smaller number of cells in contrast to other markers. Immunoreactivity was detected in some granulosa cells of follicles at different stages of development. Markers were also observed in a group of cells located in the cortical region; we did not observe positivity in oocytes (Figure 3C, F and I). No positive markers were observed for any of the proliferation labels of the negative controls.

Double BrdU-PCNA and BrdU-Ki-67 labelling

In order to individually assess the specificity of the expression pattern found in the ovary of the three proliferation markers, we decided to carry out double labelling using the expression of BrdU as a model, as the controls and information reported for this marker, indicate its specificity. With double immunolabeling, we observed that not all cells co-localize with BrdU, which suggests that they are undergoing different phases of the cell cycle (Figure 4). However, double immunolabeling analysis revealed that BrdU strongly co-localizes with Ki-67, but not with PCNA. We observed a large number of follicular cells, positive to PCNA but negative to BrdU (Figures 4A-C), and contrarily, we observed a slight difference with Ki-67, as most of the cells positive to BrdU co-localize with Ki-67 (Figures 4D-F).

Double immunodetection of Ddx4-BrdU and Oct4-BrdU in ACGC

In order to determine whether ACGC are proliferating, it was essential to observe the co-localization of BrdU with a germline-specific marker. For this reason, double labelling was carried out to identify the protein expression of the Ddx4 and Oct4 genes, characteristic of primordial germ cells, in BrdU-positive cells. Ddx4 expression was identified in oocytes and in ACGC. For its part, BrdU was observed in some granulosa cells (Figure 5). Co-localization of Ddx4-BrdU was observed to be restricted to a few cells located in the cortical region. Likewise, we worked with a slide that was subject to the same treatment, except that it was only exposed to secondary antibodies (negative control) and where the expression of Ddx4 or BrdU was not identified, which suggests that the visible mark is specific for Ddx4 (germ cells) and BrdU (proliferation). *Oct4* gene protein expression was observed in the nuclei of cells located in the ovarian cortical region, which appear to correspond to ACGC and some primordial follicles. Interestingly, as with Ddx4, some Oct4-positive cells are mitotically active as evidenced by the incorporation of BrdU in their nuclei (Figure 6).

Validation of protein expression

PCNA, Ki-67, pH3, Ddx4 and Oct4 proteins were detected by Western blot analysis of adult bat ovary homogenizes (Figure 7), which is in agreement with the data obtained by immunofluorescence. The detected proteins presented a molecular mass, previously estimated, for each of the proteins: PCNA (38 kDa), Ki-67 (42 kDa), pH3 (13 kDa), Ddx4 (76 kDa), Oct4 (38 kDa) and

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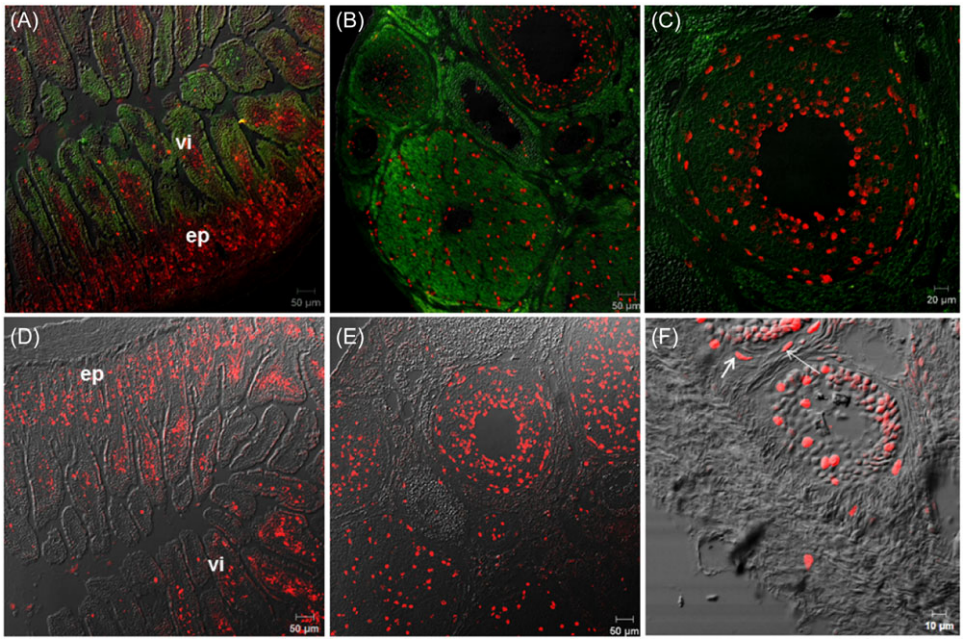

Figure 2. Detection of BrdU expression in the intestine and ovary of the B6B5/EGFP transgenic mouse carrying green fluorescent protein (green), and of the *A. jamaicensis* bat. (A) Localization of BrdU-positive cells (red) in the intestinal epithelium (ep) and within the intestinal villi (vi) of the mouse. (B) Detection of BrdU (red) in the granulosa cells of follicles at different stages of development that make up the transgenic mouse ovary. (C) Amplification of a mouse ovarian follicle where granulosa cell proliferation is evident (red). (D) Simultaneous detection of BrdU (red) and Nomarski microscopy. BrdU is observed in the epithelial cells (ep) and villi (vi) of the intestine of *A. jamaicensis*. (E) Expression of BrdU in granulosa cells of follicles at different stages, evidenced by Nomarski microscopy. (F) Amplification of a follicle where, in addition to BrdU-positive granulosa cells, some cells located around the follicles in the interstitial region that could correspond to follicular cells is observed (arrows).

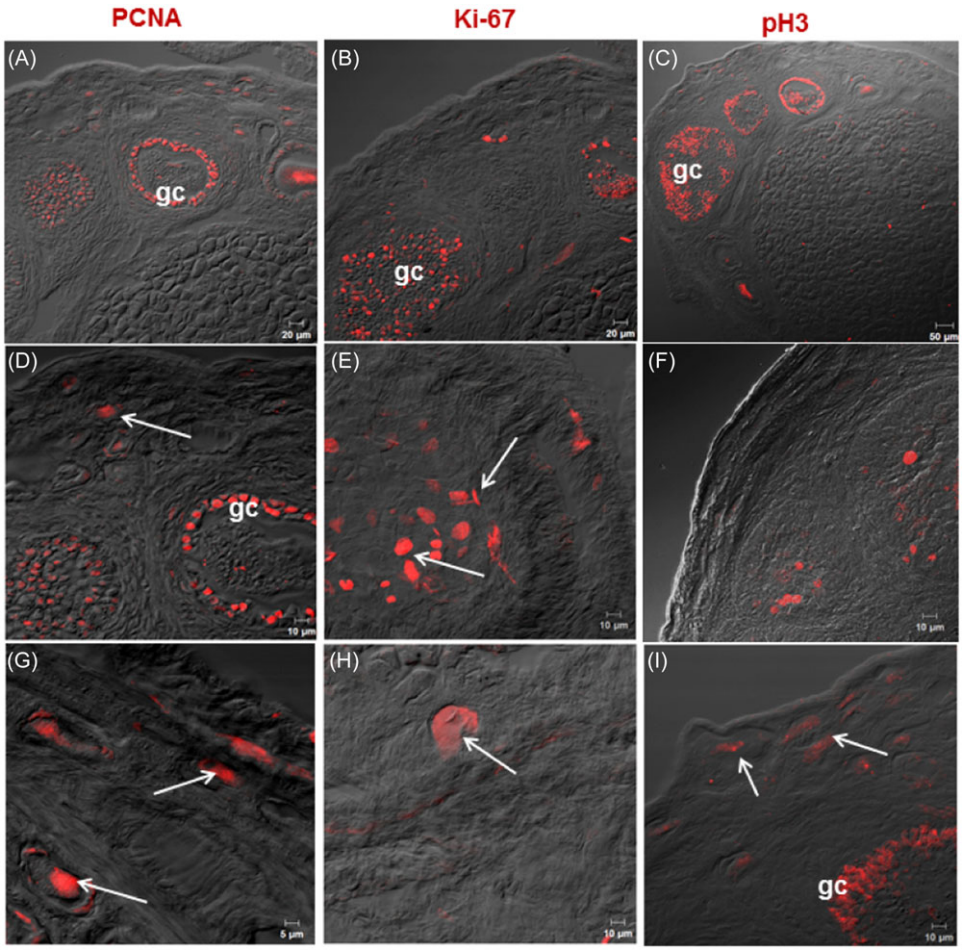

Figure 3. Immunodetection of PCNA, Ki-67 and pH3 in adult ovaries of the *Artibeus jamaicensis* bat. (A, D, G) PCNA expression is shown in granulosa cells (gc) and adult cortical germ cells (ACGC; arrows). (B, E, H) The expression of Ki-67 is visible in some granulosa cells (gc) and in ACGC located in the cortical region (arrows), and does not appear to be surrounded by somatic cells. (C, F, I) The expression of pH3 in the ovary is shown in granulosa cells (gc) that form follicles, and ACGC (arrows) positive to pH3 was visible.

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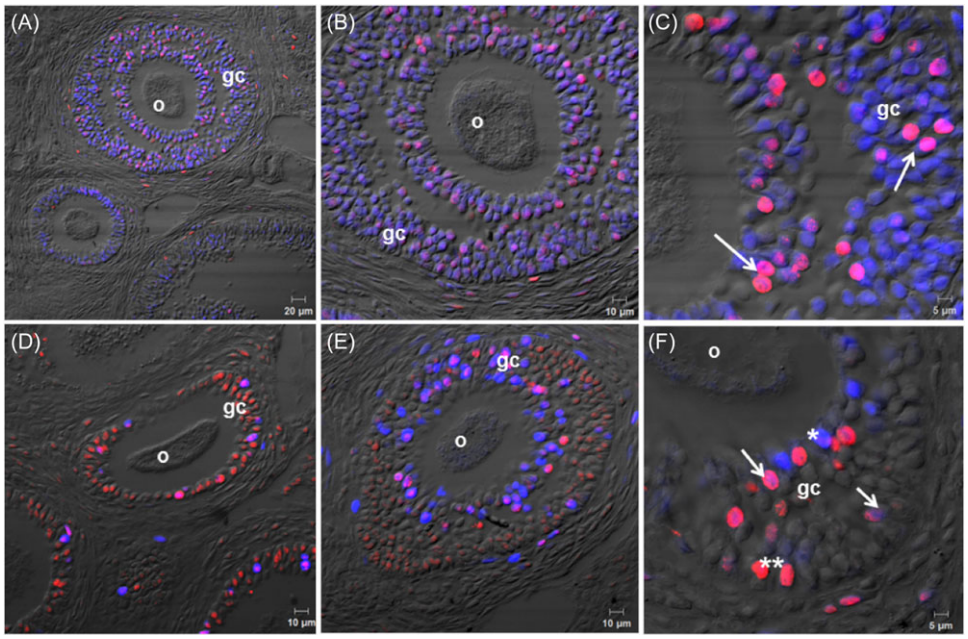

Figure 4. Double BrdU-PCNA and BrdU-Ki-67 labelling in adult ovaries of the *Artibeus jamaicensis* bat. (A) Both BrdU (red) and PCNA (blue) were located mainly in the granulosa cells (gc) that make up the follicles at different stages of folliculogenesis. In the oocytes (o), no sign of any of the markers was detected. (B) An ovarian follicle made up of an oocyte (o) and granulosa cells (gc) is shown at (A) magnification, where the majority of these granulosa cells are positive to PCNA and a smaller number are positive to BrdU. (C) At higher resolution, co-localization of BrdU (red) and PCNA (blue) (arrows) was identified in some granulosa cells (gc). (D) The expression of BrdU (red) and Ki-67 (blue) is visible in some granulosa cells (gc) of follicles at different stages of folliculogenesis. (E) At greater magnification, only some granulosa cells are positive for BrdU and Ki-67, whereas oocytes are negative. (F) A follicle with granulosa cells positive to Ki-67 (*) and BrdU (**) is shown. At higher resolution, it was possible to detect co-localization of BrdU and Ki-67 in some granulosa cells (arrows).

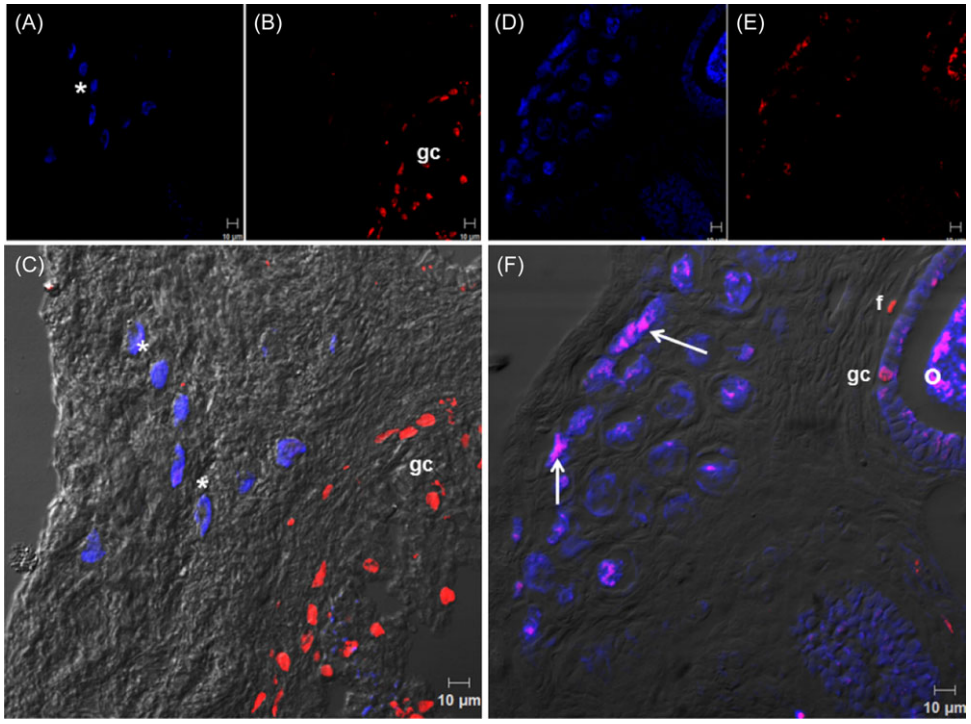

Figure 5. Double immunofluorescence for co-localization of Ddx4 protein and BrdU in the adult ovary of the *Artibeus jamaicensis* bat. (A) Ddx4 expression is visible in some cells located in the cortical region (*) of the ovary. (B) BrdU-positive granulosa cells (gc) are also visible. (C) Combination of Ddx4 and BrdU protein expression patterns with Nomarski´s optics, showing only Ddx4-positive cells in the cortical region (*), and BrdU-positive granulosa cells (cg). In other ovarian regions, Ddx4 expression (D) was detected in several cortical cells, as well as BrdU expression (E). (F) Combining Ddx4 and BrdU expression with Nomarski's optic, the presence of proliferating cortical germline cells is evident (arrows). A Ddx4-positive oocyte (o) is visible. Also evident is the presence of granulosa (gc) and follicular (f) cells that exclusively express BrdU.

β-actin (42 kDa). These results validate the specificity of the antibodies used and their affinity for the corresponding antigen.

Discussion

For many years, analyzes of cell proliferation focused on the study of cell division, which provided the most reliable data related to proliferative activity, predominantly based on observations at the level of conventional microscopy. Currently, the different techniques for studying and evaluating cell proliferation enable us to establish more precisely, the increase in the number of cells in tissues, resulting from the growth and multiplication of these cells. In particular, the use of proliferation markers for the identification of proliferative activity in progenitor and/or stem cells is of great importance for ensuring that it is this type of cell combined with other markers, thus indicating cells in self-renewal.

The analysis of cell proliferation markers in the mammalian ovary has served as an indication of the presence of precursor cells from the germ line, with the capacity to restore the follicle pool during adult stages. This will expand the accepted dogma related to reproductive biology, concerning the existence or not of a mechanism for follicular self-renewal that was thought only possible in invertebrates and some vertebrates, but not in adult mammals. In this regard, the adult ovary of phyllostomid bats has

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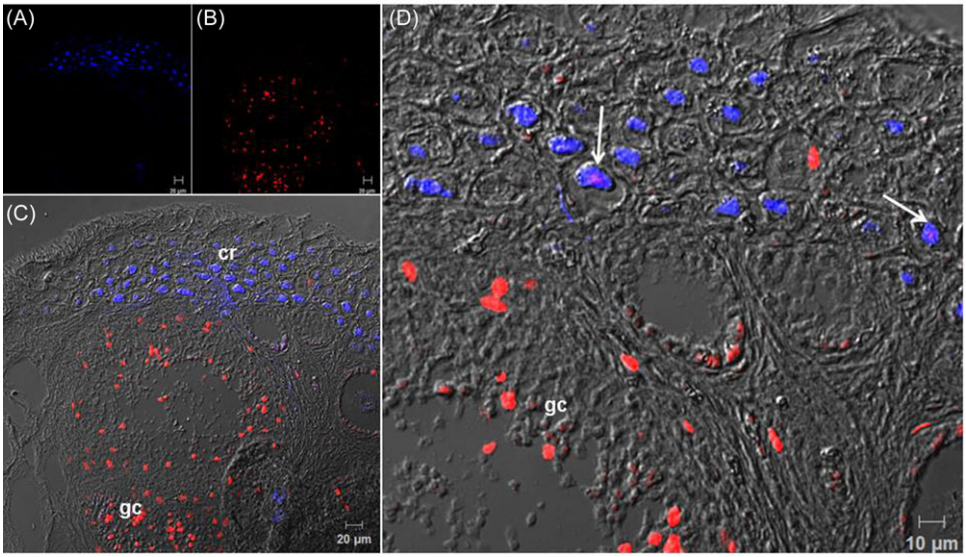

Figure 6. Double immunofluorescence for co-localization of Oct4 protein and BrdU in the adult ovary of the *Artibeus jamaicensis* bat. (A) Oct4 is observed in in cells located in the cortical region (blue). (B) BrdU-positive granulosa cells are visible in follicles (red-gc). (C) Simultaneous detection of Oct4 (blue) and BrdU (red), combined with Nomarky´s microscopy where is evident the cortical region (cr) with adult cortical germ cells positives to Oct4, medullary region containing some follicles with BrdU-positive granulosa cells (cg). (D) At higher amplification it is possible to detect Oct4-positive cortical cells that also express BrdU (arrows).

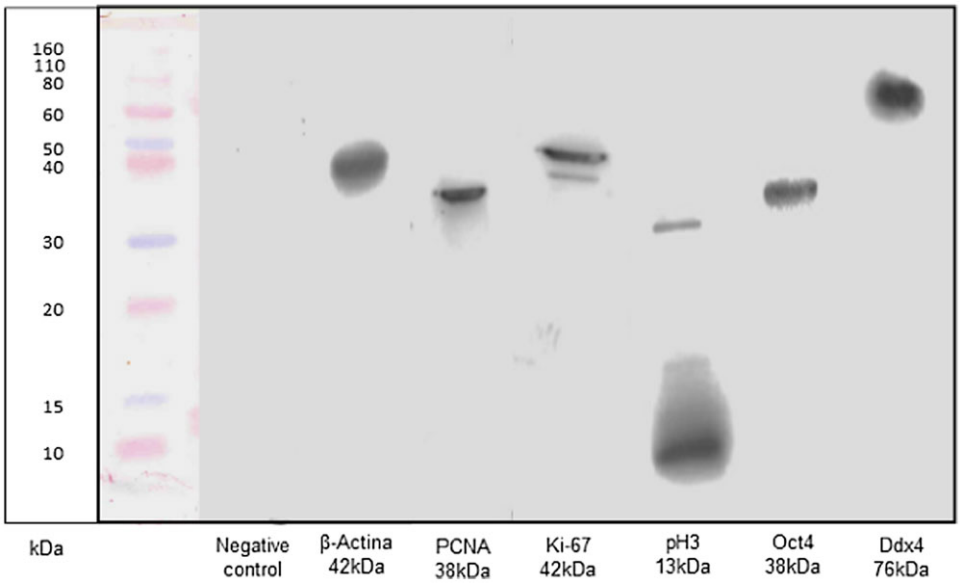

Figure 7. Western blot analysis was used to validate protein expression of the proliferation markers PCNA, Ki-67 and pH3, as well as the germline markers Ddx4 and Oct4, and β-actin as a loading control in *A. jamaicensis* ovaries. Bands that corresponded to the weight reported for each of the proteins PCNA (38 kDa), Ki-67 (42 kDa), pH3 (13 kDa), Ddx4 (76 kDa), Oct4 (43 kDa) and β-actin (43 kDa) were identified. The primary antibody was omitted in the case of the negative control. Bands were obtained individually and the figure was created by joining the different slides obtained from each exposure, to facilitate comparison.

turned out to be a great study model, as a cortical region has been observed, where cells with characteristics of progenitor cells of the germ line are located, known as adult cortical germ cells (ACGC). They also manifest great proliferative activity at different stages of their development, especially with regard to the granulosa cells that make up the follicles. In this way, in the bat ovary, specific antibodies can be evaluated that make it possible to distinguish between cells undergoing active division and those that are quiescent; while also revealing their specificity when viewed together.

For the evaluation of proliferative activity in the adult ovaries of *A. jamaicensis*, tests were carried out for the detection by Immunofluorescence of four markers reported to indicate cell proliferation: BrdU (Gould and Gross, 2002), Ki-67 (Ladstein *et al.*, 2010), PCNA (Foley *et al.*, 1991) and pH3 (Hendzel *et al.*, 1997). These markers were used because each of these presents points of expression within the cell cycle at different moments, coinciding for some of these. This would therefore ensure that we identify proliferating cells, regardless of the phase of the cycle during which they are found. In the present study, a dose of 100 mg/kg of 5-bromo-2'-deoxyuridine (BrdU) was administered intraperitoneally to live animals, as exposure to high doses has been observed to affect body and brain morphology (Kolb *et al.*, 1999). Specific investigations indicate the need for higher doses of BrdU (up to 300 µg/g) to label most dividing cells (Gould and Gross, 2002). In our study, after single injection with BrdU and after 120 min, a large number of BrdU-positive cells were identified in the chiropteran ovary. Therefore, we can suggest that this labelling was specific, and we can recommend the application of a single dose of BrdU.

The most widely used endogenous proliferation marker is the Proliferating Cellular Nuclear Antigen (PCNA). It has been reported that the half-life of PCNA is approximately 20 h, and its expression begins to increase at the end of the G1 phase and at the beginning of the S phase, decreasing throughout the G2 phase and during mitosis (Kurki *et al.*, 1986; Kurki *et al.*, 1988). It has been described that PCNA is present in some proliferating cells, even G0, although its expression is very low (Bravo and Macdonald,

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1987). In this study, the anti-PCNA antibody (Anaspec 55421) was used, which has been widely used in cell proliferation studies. It has been reported that the quality of the marker depends on the fixation method used (Gwynn, 2001), so 4% paraformaldehyde (PFA) was used to fix the tissues, as previous reports have shown that fixation with PFA results in cells with intense markers (Valero *et al*., 2005). PCNA expression was mostly observed in granulosa cells; however, it was also detected in some oocytes, which is not feasible, as these are mostly detained during the diplotene of prophase I of meiosis. The reason for this may refer to the fact that PCNA is known to participate in DNA repair pathways (Matsumoto *et al*., 1987; Zhang *et al*., 1999). Cells normally undergo DNA repair processes necessary to correct damage, which are mediated by enzymes that ensure genome stability (Memo, 2006). Because repair involves DNA synthesis processes, and PCNA is a marker that is expressed in this phase of the cell cycle, non-specific labelling may be due to this repair function. The results obtained in animals marked with BrdU-PCNA enabled us to study the relationship between the DNA replication zones in fixed ovaries. Based on the degree of expression found in the follicular cells of mouse ovaries and *A. jamaicensis*, we can state that there are a greater number of PCNA-positive cells compared to those marked with BrdU. We did not perceive a homogeneous distribution, and very few cells co-localized, i.e. not all PCNA-positive cells turned out to be BrdU positive. These findings do not corroborate previous results that show a typical distribution of the S phase for both markers (Bravo and Macdonald, 1987; Somanathan *et al*., 2001), so we cannot ensure that the marker is an active replication of PCNA during the S phase of the cell cycle. This means PCNA cannot be considered to represent a specific marker of mitotic cell division, as due to its DNA repair function, it may be expressed at other moments during the life of the cell and not only during mitosis.

Ki-67 protein expression directly reflects a certain physiological state of the cell. Although the functional role of Ki-67 protein during cell proliferation is unknown, there is no doubt that Ki-67 protein expression and cell proliferation are closely related (Scholzen and Gerdes, 2000). Ki-67 has a short half-life, is not detectable during DNA repair processes and is strongly down-regulated/absent in quiescent cells (Takahashi and Caviness, 1993; Scholzen and Gerdes, 2000; Zacchetti *et al*., 2003). Quantification of Ki-67-positive cells coincides with BrdU labelling in proliferating cells, so it can be considered as a more reliable marker to identify cells re-entering the cell cycle (Tanapat *et al*., 1999; Kee *et al*., 2002). During interphase, the antigen can be detected dispersed within the nucleus, whereas during mitosis most of the protein is concentrated at the surface of the chromosomes (Endl and Gerdes, 2000). Counting of mitotic figures has been reported to be more sensitive using Ki-67, as it recognizes cells during all active phases of the cell cycle (Scholzen and Gerdes, 2000). Based on these arguments, it was extremely relevant to localize the protein expression of the Ki-67 gene in some cells located in the cortical region of the *A. jamaicensis* ovary. Thus, we can suggest that these cells are undergoing mitotic proliferation, one of the fundamental characteristics of a progenitor cell. Based on this expression pattern, it was suggested that Ki-67 might be a good candidate for assessing the proliferative status of cell populations, since it was observed that all proliferating cells analyzed were positive for Ki-67 staining (Gerdes *et al*., 1984). Although BrdU and Ki-67 are widely used as proliferation markers and often show similar expression patterns, they stain different groups of cells. As mentioned, BrdU marks cells during S phase; whereas Ki67 marks cells in G1, S, G2 and M phases (only G0 cells should be Ki-67 negative). Therefore, the use of only one marker may yield only partial information, whereas the use of both will guarantee and increase the reliability of the results obtained (Tanaka *et al*., 2011). In the present study, we observed that the majority of BrdU-positive cells co-localize with Ki-67, suggesting that the cells that capture the double label are indisputably in the S phase of the cell cycle; whereas the Ki-67-positive cells may be in G1 or G2. Therefore, cells negative for both BrdU and Ki-67 could be in a quiescent state.

Another marker such as 3-phosphorylated histone (pH3) has been shown to be a valid candidate for studying cell proliferation (Engstrom *et al*., 1985; Hendzel *et al*., 1997; Zhu *et al*., 2003; Zhu *et al*., 2005). pH3 is expressed during the initial stages of chromatin condensation in late G2 interphase to anaphase (Hendzel *et al*., 1997). With pH3, we have reproduced the results previously obtained in the ovary of *Artibeus jamicensis* (Antonio-Rubio *et al*., 2013), where pH3 expression was detected in the adult cortical germ cells (ACGC). These results are important as it has been documented that Histone 3 (H3), unlike other histones, is only phosphorylated during mitosis. This phosphorylation at Ser10 begins during prophase, is maximal during metaphase, decreases during anaphase, and disappears during telophase (Gurley *et al*., 1978; Paulson and Taylor, 1982). Due to these factors, pH3 is the best marker for the validation of cell proliferation, as its expression occurs exclusively during mitosis. Thus, to estimate the proliferative activity of cells in various tissues, as in this case in bat ovaries, this method of double labelling with BrdU and Ki-67 can yield more accurate data on cell proliferation rates. Furthermore, according to Tanaka *et al*, using this method; it would be possible to estimate kinetic parameters in tissue repair and tumour progression (Tanaka *et al*., 2011).

After the experimental analysis with the four markers of cell proliferation, we can argue that each one has advantages and disadvantages over another as a marker of mitotic proliferation. The general characteristics that an ideal cell proliferation marker must have are that it must exclusively label dividing cells, it must be a highly specific nuclear marker and it must mark all the active phases of the cell cycle. However, cell proliferation is a process that each individual cell undertakes and cannot be referred to as a precise state, but instead as a future event. This decision is taken by each cell during the G1 phase, so the marker should be positive, if the cell decides to divide, or negative if the cell decides against this. However, this is only an idealistic view, as experimental data show that the decision can be taken later in the cell cycle, postulating that cells can enter a dormant state even after DNA synthesis is complete (Darzynkiewicz and Traganos, 1982; Drewinko *et al*., 1984; Lazebnik *et al*., 1991; Wei *et al*., 1993). This leads us to the conclusion that cell proliferation markers can only be used to indicate the proliferation potential that a cell may have.

Based on our findings with the proliferation factors, and considering BrdU as a marker of the S phase of the cell cycle we evaluated the proliferative capacity of ACGC by identifying them with the marker Ddx4 and Oct4, which are gene that are expressed exclusively in germ cells of vertebrates (Fujiwara *et al*., 1994; Noce *et al*., 2001). Thus, we corroborated the presence of germline progenitor cells (GPCs) in the cortex of *A. jamaicensis* bat ovaries, some of which are in the S phase of the cell cycle, as evidenced by co-localization with BrdU. This finding supports the experiments performed by Johnson et al. (2004), where they observed that ovaries of young and adult mice possess mitotically active germ cells, whose function is to maintain the production of oocytes and

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follicles in the postnatal mammalian ovary, a mechanism known as neo-ovogenesis. BrdU immunolabeling in Ddx4-positive cells was comparable in intensity to that observed in both ovarian (follicular) and intestinal (epithelial) somatic cells and it should be emphasized that in none of the ovaries analyzed was BrdU-positive labelling observed in adjacent oocytes contained within the follicles. In humans, Oct4 is expressed at early stages of gonadal development and is negatively regulated as germ cells initiate meiosis in the ovaries (Kerr *et al*., 2008). Therefore, the detection of the markers Ddx4 and Oct4, as well as their co-localization with BrdU in bat ACGC suggests that these cells have not initiated the process of meiotic division, maintaining characteristics of pluripotent cells. These findings support the fact that in adult mammalian ovaries there are germline progenitor cells with proliferative capacity that renew the follicle pool (Johnson *et al*., 2004; Bukovsky *et al*., 2004).

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