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Characterization of Oct4-GFP transgenic mice as a model to study the effect of environmental estrogens on the maturation of male germ cells by using flow cytometry

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ABSTRACT

Oct4 is involved in regulation of pluripotency during normal development and is down-regulated during formation of postnatal reservoir of germ cells. We propose thatOct4/GFP transgenic mouse, which mimics the endogenous expression pattern of Oct4, could be used as a mammalian model to study the effects of environmental estrogens on the development of male germ cells. Oct4/GFP maturation profile was assessed during postnatal days -PND- 3, 5, 7, 10, 14 and 80, using flow cytometry. Then, we exposed pregnant mothers to 17α-ethinylestradiol (EE2) from day post coitum (dpc) 5 to PND7. Percentage of Oct4/GFP-expressing cells and levels of expression of Oct4/GPF were increased in PND7 after EE2 exposure. These observations were confirmed by analysis of GFP and endogenous Oct4 protein in the seminiferous tubules and by a reduction in epididymal sperm count in adult mice. We introduced Oct4/GFP mouse together with flow cytometry as a tool to evaluate changes in male germ cells development.

1. Introduction

Spermatogenesis is a stem cell–dependent process supported by self-renewal and differentiation of spermatogonial stem cells (SSCs). During fetal testicular development, germ cells undergo different maturation processes to become SSCs at birth. Primordial germ cells first appear in the mouse embryo on embryonic day (E) 6.5, and then, from E6.5–E10.5, proliferate and migrate towards the genital ridges, where they become enclosed by the differentiating Sertoli cells. Testicular cords are then formed in males. The germ cells present within the testicular cords mature into gonocytes, which proliferate for a few days followed by a mitotic arrest at the G1/G0 phase. During the first week after birth, gonocytes resume proliferation and relocate from their original central position in the seminiferous tubules toward the basement membrane and differentiate into spermatogonia between postnatal day (PND) 3 and PND6. These nascent spermatogonial cells have primitive morphological features similar to type A spermatogonia in adults [1–2]. SSCs are a subpopulation of the reservoir of undifferentiated spermatogonia residing in the basal layer of the seminiferous epithelium [3]. Concurrently, epigenetic changes and differential expression of pluripotency genes occur during development of male germ cells. Among the core genes associated with pluripotency is Oct4, a member of the class V Pic-1 Oct1,2 Unc-86 (POU) transcription factors. Oct4 is expressed in proliferating gonocytes during the embryonic stage and in undifferentiated spermatogoniaafter birth [4,5]. As spermatogenesis starts, Oct4 expression continues in undifferentiated type A spermatogonia and is down-regulated as germ cells begin spermatogenic maturation (4). Oct4 plays an important role in the establishment and maintenance of the stem cell pluripotency; a precise level of Oct4 is required to sustain stem cell self-renewal and Oct4 also governs distinct fates of stem cells [6,7]. In addition, Oct4 is required to maintain SSCs in an undifferentiated state and therefore to maintain their self-renewal in both cultured and transplanted testes [8,9].

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Estrogens exert a direct physiological effect on germ cells in the developing testis both at the prenatal and postnatal stages [10,11]. It has been shown that 17β-estradiol induces the proliferation of neonatal testicular gonocytes and spermatogonia *in vitro*, suggesting that it may act as the physiological regulator of gonocyte development *in vivo* [12,13]. Sinkevicius and colleagues showed that estrogen-dependent ER α signaling is required for germ cell viability, most likely through support of Sertoli cell function. They also provided strong evidence suggesting that neonatal estrogen signaling is required for the development of normal adult fertility rates [14]. Disruption of the hormonal microenvironment during gonad differentiation could result in developmental deficiencies and health problems later in life, including cryptorchidism, hypospadias, decreased spermatogenesis, hypogonadism and testicular germ cell cancer [15–17]. *In vivo* exposure to increased 17β-estradiol concentrations causes premature sperm capacitation in the epididymis with a potential negative impact on the sperm reproductive fitness in the female reproductive tract [18]. Several studies link male reproductive disorders to the disruption of the early testis development by endocrine-disrupting chemicals (EDCs) [15,19]. Germ cell sloughing, disruption of the blood–testis barrier and germ cell apoptosis have been reported after environmental exposure to exogenous estrogenic compounds during early childhood development [20,21]. In addition, transgenerational epigenetic effects on male reproduction may increase the potential impact of environmental toxins [21].

As mentioned above, Oct4 localizes in proliferating gonocytes and after birth in undifferentiated A spermatogonia [4,9]. It is thus used as a marker of SSCs (9). Based on this property, an Oct4-GFP transgenic mice that mimics the endogenous expression pattern of Oct4 in the prenatal and early postnatal period was developed, in which Oct4-expressing germ cells can be identified at various developmental stages [22,23].

In the last years, flow cytometry has demonstrated to be a powerful and versatile tool to study diverse biological problems. A major advantage of this technique is its ability to provide quantitative relevant information even from rare events, making it possible to analyze statistically small cell populations. Herein, we characterize the expression pattern of Oct4-GFP in this transgenic mouse through the postnatal and adult stages of male germ cells maturation, using flow cytometry as the main analysis tool. We identified and optimized various parameters to evaluate male germ cells development by Oct4/GFP expression. In addition, we assessed whether this transgenic mouse model would be suitable to identify the changes caused by exposure to ethinylestradiol (EE2) on Oct4/GFP expression associated to reservoir formation of male germ cells. Our results showed that the transgenic Oct4/GFP mouse together with flow cytometry is a suitable tool to evaluate changes in male germ cells development and they could be used to identify early life exposures to EDCs.

2. Material and methods

2.1. Animals

C57BL/6 Oct4/GFP transgenic mice were a kind gift from Dr. Konstantinos Anastassiadis, Biotec Technische Universität Dresden, Germany [24]. Animals were bred under specific pathogen-free conditions at the animal facility of the Transgenic and Experimental Animal Unit of Institut Pasteur de Montevideo, Uruguay (IPMon). Mice were housed in individual ventilated cages with positive pressure, at 20 ± 1 °C, relative humidity of 40–60% and a 14/10 light–dark cycle. Animals were fed with standard mouse diet *ad libitum* and had free access to water during the experimental protocol. All animal procedures were approved by the Animal Care Committee of the IPMon and in accordance with national law N° 18.611 and international guidelines of the Federation of Laboratory Animal Science Associations (FELASA) regarding laboratory animal protocols.

2.2. Genomic DNA isolation and genotyping of Oct4/GFP transgene

Mouse tails were immersed in 0.5 mL lysis buffer (10 mMTris, 100 mMNaCl, 0.5% SDS and 0.5 mg/mL Proteinase K) and incubated overnight at 37 °C. After incubation, proteins were precipitated with the addition of 0.3 mL of 5 MNaCl and incubated for 10 min on ice. Samples were centrifuged 10 min at 9000 × *g* and the supernatant was transferred to a new tube containing 0.8 mL of glacial ethanol at room temperature (RT). Samples were centrifuged for 10 min at 14,000 × *g* and the DNA pellet was recovered and washed twice with 0.5 mL of 70% ethanol. Finally, the DNA pellet was dried at RT and resuspended in 0.03 mL of MilliQ water. Oct4/GFP mice were genotyped by polymerase chain reaction (PCR), using specific primers: 5′-CTT CTT CAA GGA CGA CGG CAA CTA-3′ (forward) and 5′-ATC GCG CTT CTC GTT GGG GTC TTT-3′ (reverse) for eGFP gene detection. Mammalian enabled (Mena) was used as housekeeping gene: 5′-CAG CAG ACT GAG GGG ACA G-3′ (forward) and 5′-GGCAGGACTT-GAATCTGGAG-3′ (reverse). The PCR conditions for both eGFP and Mena were as follows: 4 min at 94 °C; 35 cycles (45 sec 94 °C; 1 min 55 °C; 1 min 72 °C), a final extension of 10 min at 72 °C and 10 min at 4 °C.

2.3. Cell preparation

To establish the postnatal Oct4/GFP expression profile, testes were collected from animals at 3 ($n = 5$), 5 ($n = 4$), 7 ($n = 8$), 10 ($n = 8$), 14 ($n = 6$) days of age and at the adult stage (80 days old). Male mice were killed by decapitation (pups) or carbon dioxide inhalation (adults). Both testes for the pups or one testis for the adult animal were isolated (the tunica albuginea removed) and placed into a 12-well culture plate with 0.5 mL PBS-EDTA supplemented with 2% bovine serum albumin (BSA). The genotype was determined by fluorescence microscopy (Olympus IX81) of the whole testis, and further confirmed by PCR analysis as described previously. Decapsulated testes from pups were centrifuged at 300 × *g* for 5 min, the supernatant was discarded and the pellet was resuspended in 1 mL collagenase Type II (1 mg/mL, C6885, Sigma, St Louis, MO, USA) and incubated at 32°–35 °C for 15 min. The cell suspension was agitated every 5 min to dissociate the seminiferous tubules and to release interstitial cells. After incubation, the cell suspension was centrifuged for 5 min at 300 × *g* at RT and the supernatant was discarded. The remaining cells were washed with 1 mL PBS-EDTA-2% BSA. After centrifugation for 5 min at RT, the supernatant was discarded and cells were resuspended in 0.8 mL of 0.05% trypsin-EDTA and incubated for 10 min at 32–35 °C, agitating every 5 min. Enzyme activity was blocked by addition of 0.2 mL fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA). After centrifugation, cells were resuspended in 1 mL of Dulbecco's Modified Eagle Medium (DMEM), (Invitrogen, CA, USA) supplemented with 10 % FBS and further dissociated by pipetting up and down several times. To obtain the cell suspension from adult testes, the collagenase treatment was avoided, proceeding directly to the incubation with trypsin.

The testicular cell suspension was fixed by incubation with 4% paraformaldehyde for 0.5–2 h, centrifuged, resuspended in PBS-EDTA-2% BSA and filtered through a 50 μm pore size Nylon mesh. The resultant single cell suspension was used for flow cytometry analysis.

2.4. Flow cytometry analysis

Flow cytometry analysis was performed in a CyAn™ADP analyzer (Beckman Coulter Inc., IN, USA) equipped with 488 nm and 635 nm lasers. Data was acquired with Summit v4.3 (Beckman Coulter Inc., IN, USA) and postacquisition analysis was performed using FlowJo vX.0.7 (Tree Star Inc, Ashland, OR, USA) software. For each sample, the following parameters were studied: forward scatter *versus* side scatter to define the acquisition gates for intact cells, forward scatter *versus* Pulse Width dot plot for doublet discrimination and side scatter *versus* FL1 channel (530/40 band pass) dot plot for GFP detection. A minimum of 10,000 single intact cells were collected for analysis in each sample.

To study Oct4 expression by GFP fluorescence, three parameters were defined using side scatter *versus* FL1 channel dot plot and FL1 channel histogram: (1) the percentage of Oct4/GFP positive cells, as an indicator of the number of immature cells in the gonads; (2) the increase in the mean fluorescence intensity (MFIi), as an indicator of the maturation of the cells; (3) the percentage of Oct4/GFP positive cells with bright ($Oct4^+$ bright) and dim ($Oct4^+$ dim) fluorescence intensity.

The MFIi was defined as the difference between the Mean Fluorescence Intensity (MFI) of the GFPpositive and GFP negative cell fraction divided by the MFI of the negative fraction of each sample, as follows:

$$\text{MFLi} = \frac{(\text{MFLI GFP}^+ - \text{MFLI GFP})}{\text{MFLI GFP}^-}$$

Distinct positive populations expressing different amounts of GFP were detected, thus, the percentage of $Oct4^+$ dim and $Oct4^+$ bright cells were calculated considering the log decades in the FL1 fluorescence channel (from 10^1–10^2 and 10^2 –10^3, for dim and bright, respectively).

2.5. Oral administration of EE2

To assess the effect of estrogens in the development of male germ cells during the perinatal period, female pregnant mice were exposed to EE2 (Sigma, St Louis, MO, USA). Changes in cell maturation were evaluated in the offspring by the Oct4 flow cytometric expression profile, using the same parameters described previously. Six- to eight-week-old Oct4/GFP mice were mated. Females with a copulation plug (dpc 0.5) were randomly separated in two groups: EE2 5 μg/kg of body weight,and Ethanol (0.1 %) as the vehicle control group. The dose of 5ug/kg.day has been referred as the appropriate dose that must be assayed to demonstrate the sensitivity to estrogenic action of the endpoint of interest in an animal model when chemicals are administered orally [25]. Both compounds were administered daily by oral gavage from dpc5.5 to PND7, in a single dose using a maximum volume of 0.2 mL. The male offspring of the treated females ($n = 10$ for EE2, and $n = 11$ for the vehicle control group) were sacrificed on PND7 and the testicular cell suspensions were analyzed by flow cytometry.

2.6. Immunohistochemistry

In control and EE2-treated males, testes from PND7 male mice ($n = 11$ and $n = 10$, respectively) were assigned to GFP and Oct4 protein detection by immunohistochemical staining. Testes were isolated and fixed in 4% PFA for 2 h at RT and embedded in paraffin. At least three sections (5 μm thickness) at different depths from each testis were immunostained as previously described [26]. After deparaffinization, microwave pretreatment (antigen retrieval) was performed. GFP (A10260, Invitrogen, CA, USA) and Oct4 (sc5279, Santa CruzBiotechnology, CA, USA) antibodies were incubated overnight at 4 °C (GFP: 0.2 mg/mL; Oct4: 0.04 mg/mL). Biotinylated anti-rabbit IgG and anti-mouse IgG (Sigma–Aldrich Argentina S.A., Buenos Aires, Argentina) were used as secondary antibodies, respectively. Reactions were developed by the streptavidin–biotin peroxidase labeling method, which uses diaminobenzidine (DAB) (Sigma–AldrichArgentina) as a chromogen substrate. Samples were counterstained with Mayer hematoxylin (Biopur, Rosario, Argentina) and mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina). For negative controls, the primary antibody was replaced with non-immune mouse or rabbit serum (Sigma).

2.7. Sperm count, viability and motility of adult male mice treated perinatally with EE2

Adult male mice (PND 130) from control ($n = 4$) and EE2 ($n = 4$) groups were sacrificed by cervical dislocation. The left cauda epididymis were removed, cut three times with dissecting scissors and put in a 1.5 mL eppendorf with 1 mL of Phosphate-Buffered Saline (PBS). The tissues were incubated for 10 min at 37 °C to release the sperm.

Sperm count and viability were carried out using flow cytometry. Viability was evaluated with propidium iodide (PI) staining. This compound is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Sperm count was assayed at the same time, using fluorescence beads (CountBright™ Absolute Counting Beads; Life).

Sperm suspension was diluted 1:10 and stained with PI (final concentration 2 ug/mL) and mixed with 3000 fluorescence beads in a final volume of 300uL of PBS. For each duplicate sample, 1000 beads were counted using a gate on an SSC *versus* FL1 dot plot. The total sperm count per epididymis was calculated using the formula:

$$\text{cells per epididymis} = 100 \times \text{average count sperm}$$

Sperm motility was assessed within thirty minutes after epididymis removal to limit the deleterious effects of dehydration, pH or changes in temperature on motility. Two hundred whole spermatozoon (head and tail) were analyzed in duplicate per animal using an optic microscopy at 400 × magnification. Spermatozoa were classified according to three types of motility: Progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed. Non-progressive motility (NP): all other patterns of motility with an absence of progression. Immotility (IM): no movement.

2.8. Statistical analysis

Data was analyzed using the GraphPad Prism© software version 5.0 (San Diego, USA) and results are presented as the mean ± SEM. One-way ANOVA was conducted to analyze differences in the percentage of Oct4/GFP positive cells, $Oct4^+$ dim and $Oct4^+$bright fractions, MFIi and sex ratio at different ages. Bonferroni's multiple comparison test was performed when appropriate. The non-parametric Mann Whitney test was used to analyze the effects of EE2 treatment using the above-mentioned parameters. Levels of significance were set at $p \leq 0.05$.

3. Results

In the present study, we propose the Oct4/GFP transgenic mouse as a new mammalian model to study the effects of perinatal exposure to environmental estrogens on male germ cell

development. Three to five mothers were used for each age male newborn or treatment. The litter size was between 5 and 6 puppies. The average male/female ratio was 47.9 ± 10.5 % and 52.1 ± 10.5 % males and females, respectively. There were no significant differences between groups (ANOVA, $p = 0.17$). Flow cytometry was selected as the main analyzing tool. In order to characterize the germinal maturation profile in this mouse model, we optimized and assessed the following parameters during pre-pubertal (PND3 to PND14) and adult age: 1) the percentage of Oct4/GFP-expressing cells; 2) the MFIi of Oct4/GFP-positive cells and 3) the percentage of Oct4$^+$ bright and dim cells. Fig. 1 shows an example of how those parameters were established.

Moreover, the model was challenged by exposing pregnant females to EE2 from dpc 5.5 to PND7. The alteration of germ cell maturation by estrogenic exposure during perinatal period was evaluated by the Oct4/GFP flow cytometry profiles and later in life, in adult male offspring, by analyzing sperm parameters, as described in section 3.4.

3.1. Proportion of Oct4/GFPpositive cells during gonad maturation

The proportion of immature germ cells at the pre-pubertal stage was studied from mouse specimens at PND3, 5, 7, 10, 14 and adulthood (Fig. 2). Fig. 3 shows that the proportion of Oct4/GFP positive cells was 3.98 ± 0.25% at PND3 and then showed a significant increase during the maturation of the gonad from 5.22 ± 0.42% at PND5 to 14.66 ± 0.59% at PND7 (ANOVA: $p < 0.0001$). The proportion of Oct4/GFPpositive cells then decreased at PND10, showing two subpopulations within the same group of animals: some displayed slower gonad maturation, evidenced by a high proportion of Oct4/GFPpositive cells (14.75 ± 1.06%), while others displayed a decrease (2.60 ± 1.00%). The same behavior was still observed at PND14, with a subgroup of mice maintaining a high percentage of Oct4/GFPpositive cells (10.9 ± 0.14%) and the other subgroup having a low proportion of these cells (2.95 ± 1.52%). By PND80 (adult mice), the proportion of these cells remained below 1% (data not shown).

3.2. Oct4/GFP expression level during gonad maturation

The expression level of the Oct4 gene in the immature germ cell population was assessed by two selected parameters: the MFIi of Oct4/GFP and the percentage of Oct4$^+$ bright cells (which reflects the most undifferentiated spermatogonia cells). As shown in Fig. 4, the MFIi was highest at PND3 (34.23 ± 6.13) and then decreased significantly at PND5 (ANOVA: $p < 0.0001$), reaching 12.37 ± 1.09. These levels remained unchanged at PND7, 10 and 14 (8.23 ± 1.17; 10.77 ± 1.01 and 6.17 ± 0.47, respectively). When analyzing the proportion of Oct4$^+$ bright and dim cells (Fig. 5), we observed that at PND3 the highest proportion of Oct4/GFPpositive cells showed strong GFP expression, with 61.28 ± 7.53% for Oct4$^+$ bright cells and 35.90 ± 6.48 % for Oct4$^+$ dim cells. This pattern was reversed at PND5, and remained so until PND14 (between 12.25 and 22.73% for Oct4$^+$ bright cells).

3.3. Oct4/GFP expression profile after EE2 prenatal exposure

To validate the transgenic C57BL/6 Oct4/GFP mouse model to evaluate the effect of perinatal estrogen exposure during the development of germ cells, pregnant females were treated with EE2 or vehicle from dpc 5.5 to PND7. The offspring were euthanized at PND7 and testicular cell suspensions were subjected to flow cytometry analysis using the above-described parameters. As shown in Fig. 6, the percentage of Oct4/GFP expressing cells was significantly increased after EE2 exposure (Mann Whitney test $p < 0.0048$). Similarly, the MFIi for Oct4/GFP-positive cells was significantly incremented in the treated group (Mann Whitney test $p < 0.0011$) together with the proportion of Oct4$^+$ bright cells (Mann Whitney test $p < 0.0067$).

To confirm whether the changes observed in Oct4/GFP cytometry profiles occur in parallel with changes in protein expression levels of Oct4 and GFP in the seminiferous tubules, immunohistochemical analysis of both proteins was performed in testes of 7-day-old pups treated with vehicle or EE2. Fig. 7 shows that both GFP and endogenous Oct4 were expressed in the seminiferous epithelium. The distribution within the seminiferous tubules correlated with the morphology and pattern of tissue expression previously described for Oct4 in newborn male mice at the same age, suggesting that both appear to be confined to the type Aspermatogonia population [7]. Additionally, both GFP and Oct4 protein expression showed increased levels in seminiferous tubules from EE2-treated mice compared to vehicle-treated animals (Fig. 7); this finding correlates with the flow cytometry data indicating increased number of Oct4/GFP positive cells.

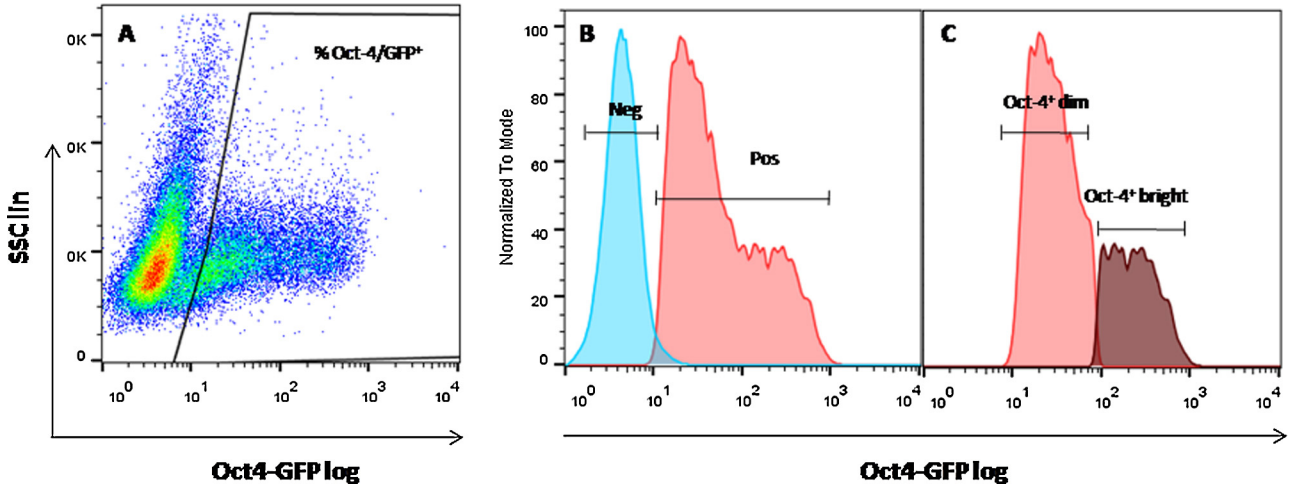

Fig. 1. Flow cytometry analysis of testicular cell suspension evaluating Oct4 expression: A) percentage of Oct4/GFP positive cells, B) Oct4/GFP histogram showing the fluorescent intensity of the GFP negative (light blue) and positive (pink) cell fraction and C) Oct4/GFP+ histogram illustrating the proportion of Oct4$^+$ dim (pink) and Oct4$^+$ bright (purple) cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

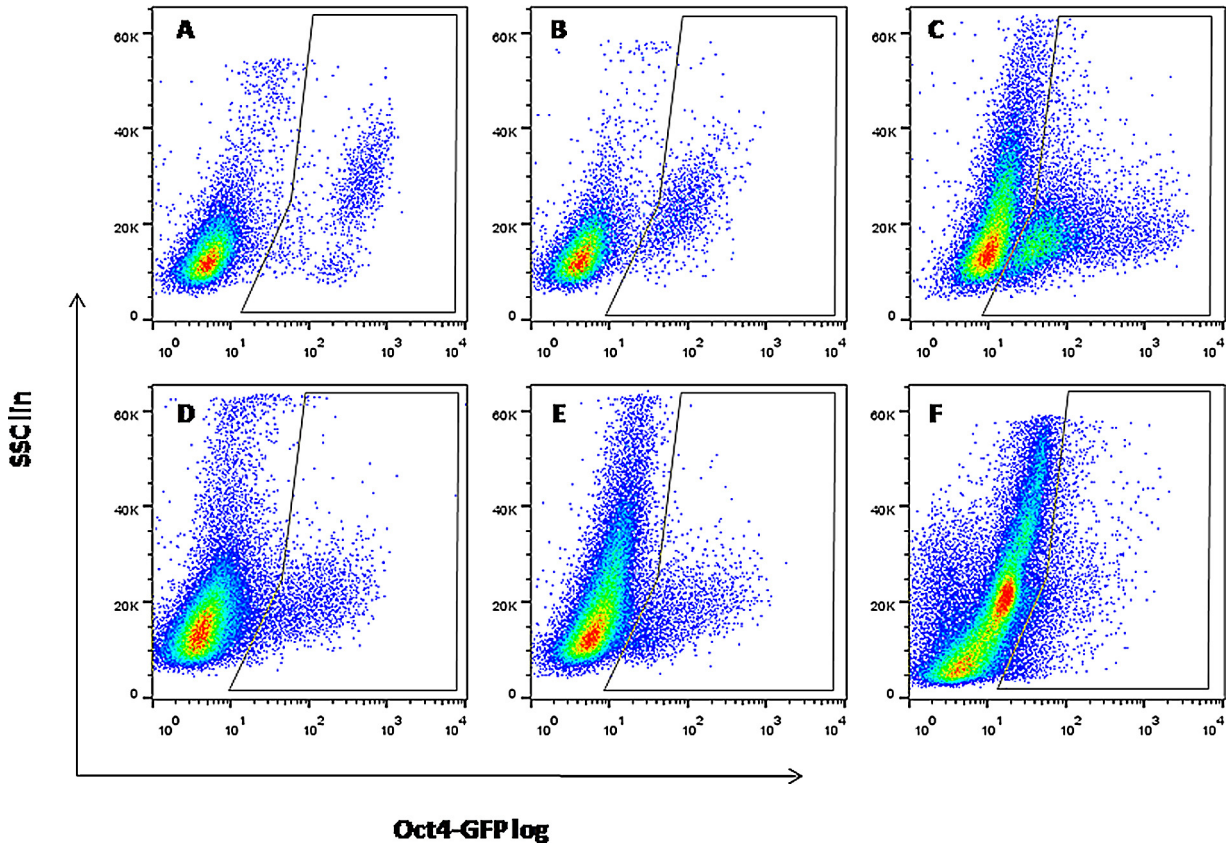

Fig. 2. Representative pseudocolordot plots of Oct4/GFP positive cells profile throughout prepubertal stages (A, B, C, D and E: PND3, 5, 7, 10 and 14, respectively) and adulthood (F: PND80) in male mice. Red dots indicate high density while the blue ones denote low density. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Sperm count, viability and motility in adult male mice after EE2 perinatal exposure

In order to evaluate if the perinatal treatment with EE2 induced adverse effects in adulthood, sperm count, viability and motility were analyzed in adult male mice.

Although there was no differences in sperm motility and viability, the number of spermatozoa per epididymis (Fig. 8) was significantly reduced in animals treated with EE2 (expressed in millions of spermatozoa per epididymis, Control = 7.87 ± 0.83; EE2 = 4.40 ± 0.23).

4. Discussion / Conclusion

Understanding the effects of environmental estrogens on germ cell differentiation is an area of growing interest in the field of endocrine disruption [27]. Herein, we propose that transgenic Oct4-GFP mouse could be a good mammalian model to study the effects of perinatal exposure to environmental estrogens on the development of male germ cells. We demonstrated the utility of this animal model to quantitatively assess early in life the alterations in germ cell differentiation caused by exposure to a low dose of EE2, a synthetic estrogen which is also considered an

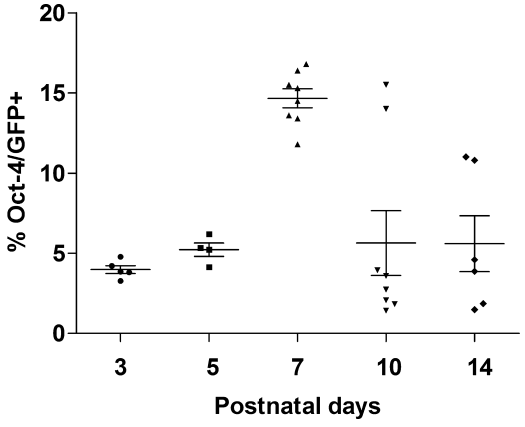

Fig. 3. Percentage of Oct4/GFP positive cells during prepubertal stages of the male gonad. A significant increase of these cells was observed at PND7 (Anova: $p < 0.0001$).

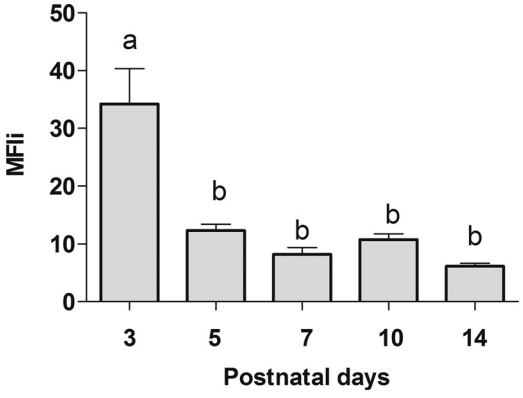

Fig. 4. MFIi of Oct4/GFP positive cells during prepubertal stages of the male gonad. Significant differences were observed through the maturation process (Anova: $p < 0.0001$). The highest increment was observed at PND3 with a significant decrease at PND5 (Bonferroni test $p < 0.05$) maintaining these levels throughout PND7, 10 and 14. Letter a indicates statistical difference from b.

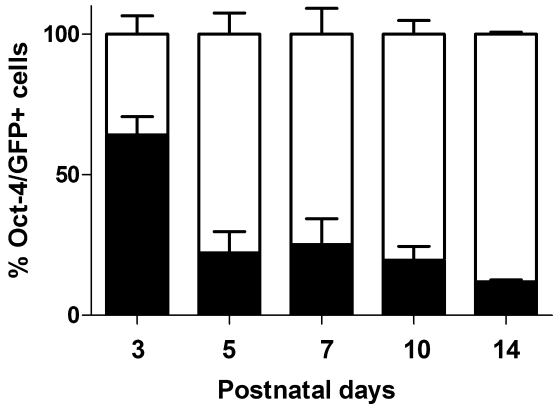

Fig. 5. Proportion of Oct4+ bright (black) and dim (white) cells during prepubertal stages of the male gonad maturation (total Oct4+ cells were considered 100%). Oct4 + bright cells were significant higher at PND3 (Anova $p < 0.0001$). This pattern was reversed at PND5, and remained so until PND14.

environmental estrogen [25,28], using flow cytometry as the main analytical tool.

Oct4 belongs to the Octamer class and, together with Pit and Unc proteins, define the POU family of transcription factors [29]. After gastrulation in the embryo, Oct4 expression is confined to the primordial germ cells [23]. In wild type mice, Oct4 is expressed in mitotically arrested prospermatogonia until birth, and then is detected in newborn male mice at PND1 and PND7 in undifferentiated spermatogonia [4]. Transgenic mice expressing a transgene consisting of GFP under the control of the Oct4 promoter in the germ cell line were generated by Yoshimizu and colleagues [30]. Expression of Oct4-GFP transgenes reflects that of the endogenous Oct4 gene throughout mouse fetal germline development and postnatal testis until PND7 [30]. As with wild type mice, at PND1, the expression of Oct4/GFP was detected in the center of the testicular tubules and the fluorescent-positive cells represented primitive spermatogonia, persisting until PND7 [30]. In the present study, we used Oct4/GFP transgenic mice expressing GFP under the control of the Oct4 promoter to analyze the male immature germ cell population development throughout the postnatal period. Testicular cells were collected and analyzed from PND3 until the adult stage and allowed us to establish the ontogeny of the germ line during this period (Fig. 2). Based on the cytometric profiles obtained (Fig. 1), we were able to define variables to estimate both the abundance of the population (percentage of Oct4/GFP-expressing cells) and the expression levels of Oct4/GFP (MFIi of Oct4/GFP and percentage of Oct4^{+} bright cells). According to our results, the highest percentage of positive cells was found on PND7, after which we observed two subpopulations of animals: a subgroup with a high percentage of Oct4/GFPpositive cells and another with a smaller proportion of this population (Fig. 3). In contrast, the levels of Oct4/GFP showed a different expression pattern, as we observed a peak on PND3, after which it decreased and remained low from PND5 onwards (Figs. 4 and 5). Previously, Shimizu et al. [31] observed that, as testes mature, the percentages of testicular cells expressing GFP in Oct4-EGFP transgenic mice gradually decrease from 18.7% at PND3 to 1.2% in adults. Our present results are different from those reported by Shimizu et al. when considering the percentage of cells expressing GFP (Figs. 2 and 3), but are in agreement when considering only the percentage of brighter cells (Fig. 5). Interestingly, these authors reported that in the testes of 7 and 10-day-old or adult males, the populations of GFP positive and GFP negative cells were not clearly divided, and some cells showed weak expression of GFP. Some of the different conditions used to obtain the testicular cell suspensions can help to explain the observed differences (pore size of the nylon mesh,

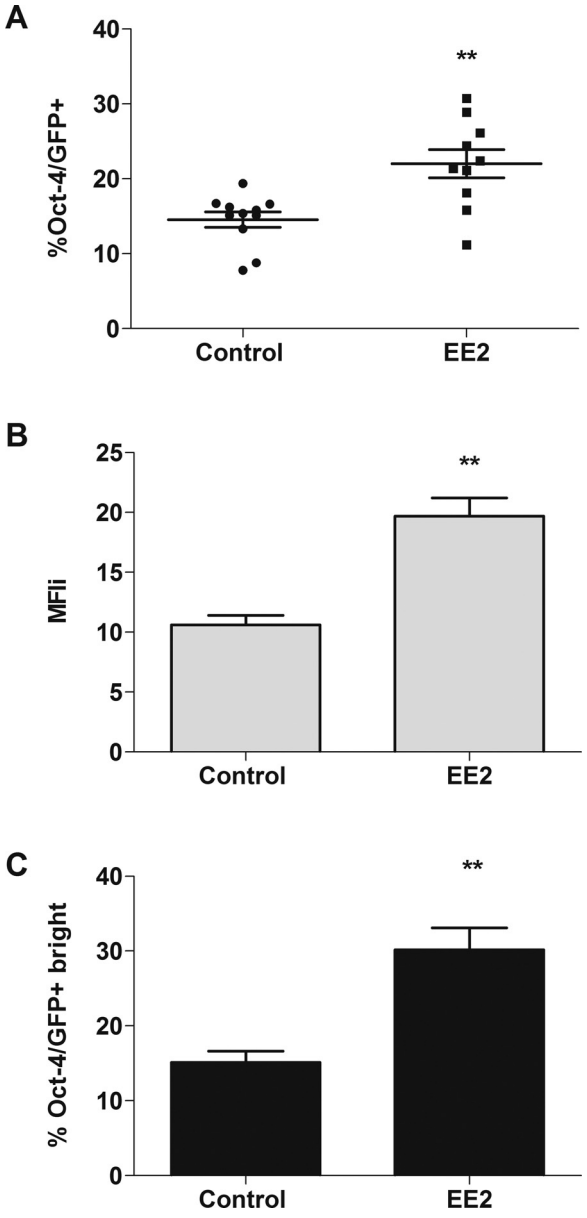

Fig. 6. Oct4/GFP expression profile after EE2 perinatal exposure: A) percentage of Oct4/GFP positive cells (Mann Whitney test $p < 0.0048$), B) MFIi of Oct4/GFP positive cells (Mann Whitney test $p < 0.0011$) and C) proportion of Oct4+ bright cells out of the Oct4/GFP positive cells (Mann Whitney test $p < 0.0067$).

fixation of cell suspension, etc). Probably one or more of these conditions allowed us to distinguish two subpopulations with different intensity of GFP. In our case, the inclusion of the fraction with lower intensity would explain the observed differences between these results and those previously published.

To determine whether reservoir formation of male germ cells in this animal model is a sensitive endpoint to the estrogenic action, we evaluated the effects of exposure to EE2, a synthetic estrogen characterized as an EDC [19]. Analyzing the expression pattern of Oct4/GFP along the postnatal stage, we selected PND7 to study the possible effects of EE2 on the differentiation of male germ cells. We selected PND7 because it was the time of development where we found the highest percentage of cells to analyze and because, at that point, the cells have completed the migration from the lumen of the seminiferous tubule to rest on the basement membrane as SSCs and spermatogonia [32,33]. Thus, we exposed the animals to

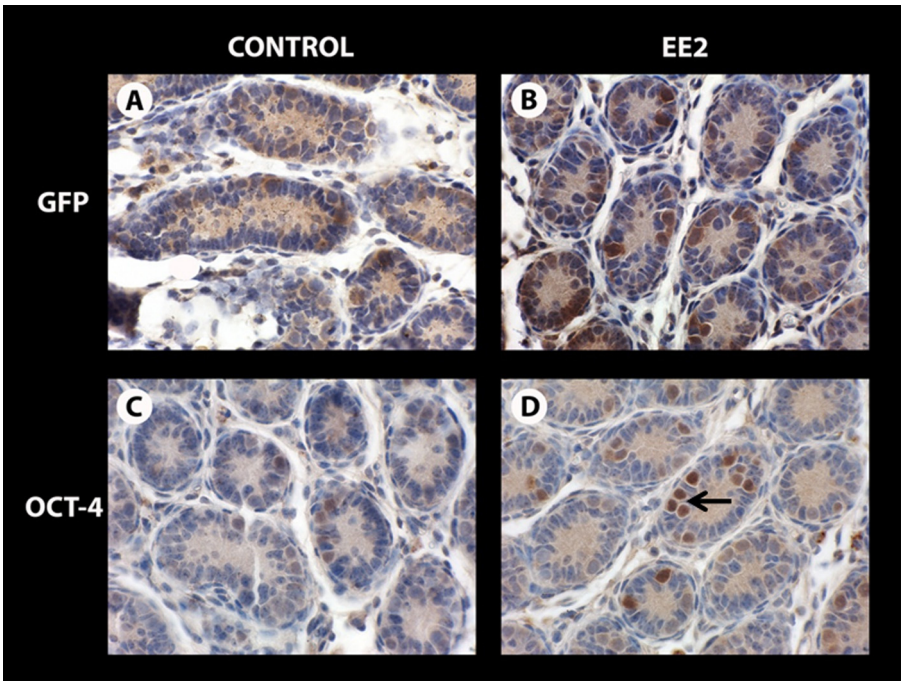

Fig. 7. Immunohistochemical analysis of GFP and Oct4 expression in testes after EE2 perinatal exposure (PND7), showing an increase in the GFP (A *versus* B) and Oct4 (C *versus* D) positivity of spermatogonia population in treated animals (indicated with the arrow). In control testes, the cells expressing Oct4 are seen to be distributed exclusively at the periphery of the seminiferous epithelium, whereas in multiple sections of EE2-treated animals Oct4-expressing cells were located closer to the lumen of the seminiferous tubule. Magnification 600×.

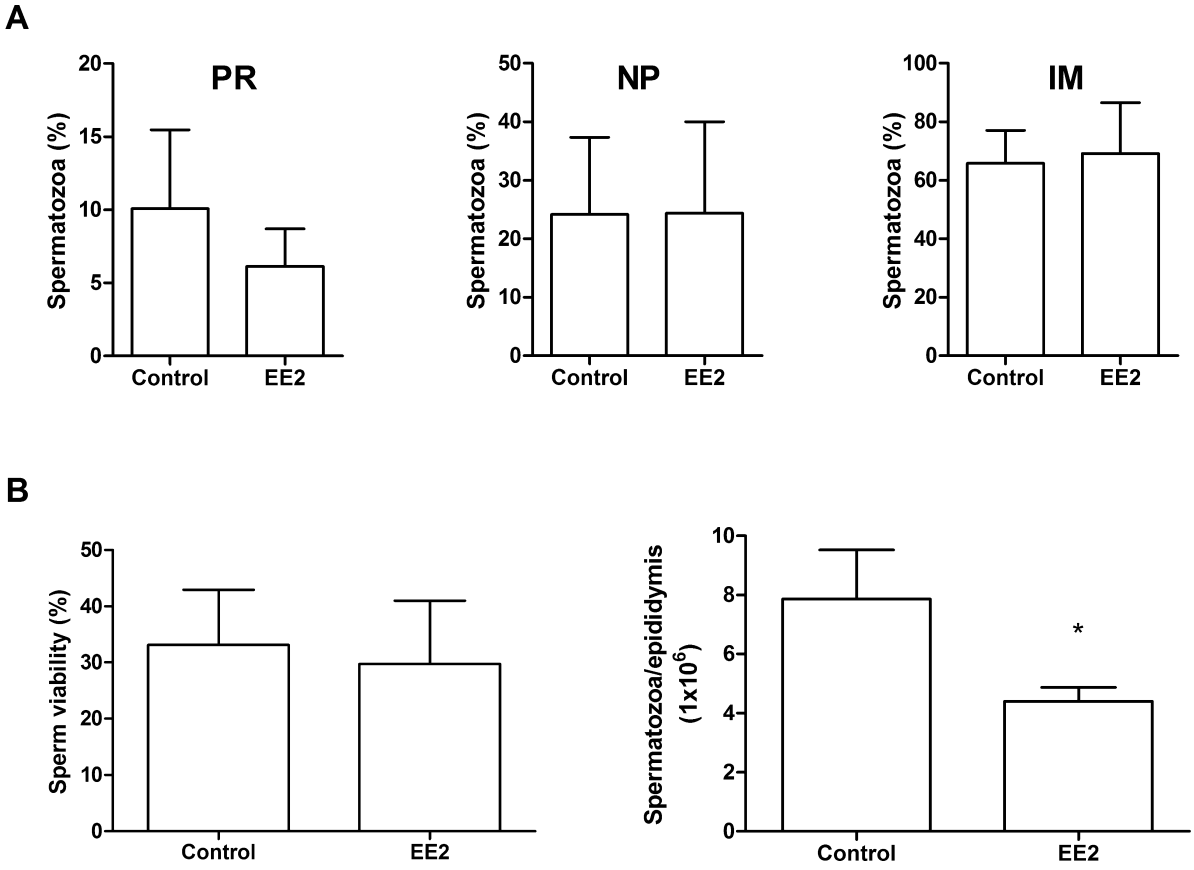

Fig. 8. Effect of perinatal exposure to EE2 in adult male mice. (A) Sperm motility: Progressive motility (PR); Non-progressive motility (NP); Immotility (IM);(B) Sperm viability; (C) Sperm count. Significant differences were observed in the number of spermatozoa/epididymis in adult male mice treated early in life with EE2 (Mann Whitney test $p < 0.03$).

EE2 from dpc 5.5 to PND7 in a dose which has been indicated as appropriate for a positive control of orally administered estrogenic EDCs [25,26]. We found that EE2 altered the differentiation pattern of germ cells on PND7 (Fig. 6), as evidenced by an increase in both the percentage of Oct4/GFP-expressing cells and the levels of expression of Oct4/GPF (increased MFIi and percentage of brighter cells). These findings indicate that formation of germ cells within the testis is perturbed by exposure to EE2 during a period that has been characterized as a window of sensitivity to germ cell cancer (GCC) formation. This relates to the initial maturation of gonocytes to prespermatogonia. In this period, subtle changes might lead to a blockade in maturation of gonocytes, which has been postulated as the earliest pathogenic step in GCC formation [34]. The presence of Oct4 protein has been related to pluripotent capacity of germ cell tumors [35] and has been identified as one of the main diagnostic markers on tissue sections for the earliest developmental stages of GCC pathogenesis [34]. In addition, many studies have shown that gonocyte proliferation/survival just prior to SSC development is hormone-dependent [33]. In fact, there is a growing body of evidence indicating that estrogens play an important role in the proliferation, differentiation and development of male germ cells [10] and, specifically, a role for estrogen in spermatogonial division [12,13,36,37]. Given that gonocytes and differentiating spermatogonia during the early neonatal period have been shown to contain ERβ, a direct action of estrogen on germ cells is entirely possible [38,39]. Interestingly, a link between estrogenic disruption and germ cell anomalies has been established, evidenced by findings of germ cell sloughing, disruption of blood–testis barrier and germ cell apoptosis provoked by exposure to some environmental xenoestrogens [20]. However, it is difficult to conclude whether this effect is direct, or via perturbation of the signals from the cellular microenvironment [10,33].

To confirm whether the increase in Oct4/GFP in response to EE2 observed in flow cytometry is reflected histologically, we analyzed the expression of GFP and endogenous Oct4 in seminiferous tubules by immunohistochemistry. We observed that expression of both proteins GFP and Oct4 was higher in the seminiferous tubules of the animals that were exposed to EE2 when compared with controls (Fig. 7). We also detected this increase in the levels of endogenous Oct4 by RT-PCR when the GFP-positive cells from the testes of control and EE2-treated pups were isolated by cell sorting (unpublished data). In 2000, Niwa and coworkers established that the amount of Oct4 is critical in the control of cell pluripotency, acting as a master regulator that controls lineage commitment [6]. Recently, to gain insight into the mechanisms by which some xenoestrogens could modulate stem cell function, Yang et al. demonstrated that exposure to the xenoestrogenbisphenol A (BPA) up-regulates the expression of the transcription factors required for maintaining stem cell pluripotency and self-renewal, including Oct4, in a dose dependent manner [40]. Based on these and other results, it has been suggested that BPA disrupts differentiation by promoting a stem cell phenotype [27]. Herein, we found increased Oct4 protein expression on PND7 by exposure to EE2. As mentioned earlier, Oct4 is part of the spectrum of diagnostic markers suitable for application in a clinical setting of human germ cell tumors [34]. Moreover, recent evidence suggests that upon specific phosphorylation, Oct4 protein interacts more dominantly with the pluripotency markers SOX2 and NANOG, and this would be related to an induction of pluripotency [41]. The changes simultaneously observed using flow cytometry and histology indicate that estrogen may be able to modify the stemness state in germ cells.

The changes we observed in early life by flow cytometry and histology in response to EE2 were confirmed by a reduction in epididymal sperm count in adult mice. These results agree with the data reported by others authors, in which sperm production is reduced in male rodent offspring both perinatally or prenatally exposed to EE2 [42,43].The World Health Organization (WHO) and the United Nations Environment Programme (UNEP) have reported in their last update about EDCs that semen quality has declined and sperm counts are in the subfertile range in several countries (16). However, the question of declining sperm counts continues to cause controversy, even today [44,45]. Present data add evidence to the hypothesis of EDCs as a cause of reduced sperm quality worldwide.

Our results support the use of the Oct4/GFP transgenic mouse as a tool to study the impact of environmental estrogens in the differentiation of male germ cells. Moreover, the model would be useful to detect alteration early in life, which can interfere with adult reproduction. As has been recently emphasized, our results suggest that estrogen may affect the spermatogonial stem cell pool of the developing testis [46]. On the other hand, flow cytometry has proved to be an appropriate tool to identify immature male germ cells populations in a fast and robust quantitative manner and with high statistical value. In addition, flow cytometry offers the possibility to perform cell sorting to work with pure populations of germ cells. Our results suggest that the transgenic Oct4/GFP mouse together with flow cytometry could be used to identify early life exposures to EDCs.

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