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GATA-1 mutation alters the spermatogonial phase and steroidogenesis in adult mouse testis

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ABSTRACT

GATA-1 is a transcription factor from the GATA family, which features zinc fingers for DNA binding. This protein was initially identified as a crucial regulator of blood cell differentiation, but it is currently known that the *Gata-1* gene expression is not limited to this system. Although the testis is also a site of significant GATA-1 expression, its role in testicular cells remains considerably unexplored. In the present study, we evaluated the testicular morphophysiology of adult ΔdblGATA mice with a mutation in the GATA-1 protein. Regarding testicular histology, GATA-1 mutant mice exhibited few changes in the seminiferous tubules, particularly in germ cells. A high proportion of differentiated spermatogonia, an increased number of apoptotic pre-leptotene spermatocytes (Caspase-3-positive), and a high frequency of sperm head defects were observed in ΔdblGATA mice. The main differences were observed in the intertubular compartment, as ΔdblGATA mice showed several morphofunctional changes in Leydig cells. Reduced volume, increased number and down-regulation of steroidogenic enzymes were observed in ΔdblGATA Leydig cells. Moreover, the mutant animal showed lower serum testosterone concentration and high LH levels. These results are consistent with the phenotypic and biometric data of mutant mice, i.e., shorter anogenital index and reduced accessory sexual gland weight. In conclusion, our findings suggest that GATA-1 protein is an important factor for germ cell differentiation as well as for the steroidogenic activity in the testis.

1. Introduction

Proteins of the GATA family are transcription factors that recognize the DNA consensus sequence (T/A) GATA (A/G) (Evans et al., 1988; Wall et al., 1988; Tsai et al., 1989, 1991; Martin and Orkin, 1990). GATA-1 was the first described transcription factor featuring zinc finger domains for DNA binding (Tsai et al., 1989; Weiss and Orkin, 1995a; Kumar et al., 2018). After that, five other transcription factors with similar zinc finger domains were identified in vertebrates (named GATA-2 to GATA-6) (Weiss and Orkin, 1995a; Molkentin, 2000). It is well known that GATA-1 is expressed by erythroid and megakaryocytic blood lineages, playing a crucial role in cell maturation and differentiation (Morceau et al., 2004; Lally et al., 2019).

In the male genital system, GATA-1, GATA-4 and GATA-6

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expressions were identified in prostate and testes (Arceci et al., 1993; Ito et al., 1993; Grepin et al., 1994; Yomogida et al., 1994; Onodera et al., 1997; Feng et al., 1998; Viger et al., 1998; Ketola et al., 1999; Zhang et al., 2019). Testicular expression of GATA-1 is age-dependent in mice Sertoli cells (SC), i.e., its expression occurs at low levels in some SC nuclei at seven days post-partum and becomes more pronounced at nine days post-partum (Yomogida et al., 1994). Although GATA-1 is expressed in SCs in all seminiferous tubule (ST) cross-sections at 21 days of age, this pattern does not persist at 35 days of age when some cross-sections no longer express this factor (Yomogida et al., 1994). Interestingly, cryptorchid animals, which have a reduced amount of germ cells, express GATA-1 in all cross-sections. Thus, it is suggested that the presence of germ cells may suppress the expression of GATA-1 in SCs after the organ has fully matured (Yomogida et al., 1994). Zhang et al. (2002) also detected *Gata*-1 mRNA in Leydig cells (LCs) of 21-day--old rats. However, additional data is still missing to confirm whether GATA-1 is required for the normal function of LCs *in vivo* (Viger et al., 2004).

Although the gene inactivation technique is of great value for a better understanding of a target gene function, this methodology is not feasible for studying GATA transcription factors because knockout animals die during the embryonic phase due to heart failure and problems in hematopoietic precursor cell development (Pevny et al., 1991; Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998; Viger et al., 2004). The GATA-1 protein is formed after the testis and hematopoietic exon translation (Ito et al., 1993). The testis and erythroid GATA-1 cDNA differ in the 5′UTR sequence only, suggesting that the expression of GATA-1 protein is identical in both tissues (testis and bone-marrow) (Ito et al., 1993). Therefore, selective or partial inactivation becomes necessary to assess the role of GATA-1 protein in the testis (Viger et al., 2004). In this context, Yu et al. (2002) constructed a mouse model (ΔdblGATA), in which a targeted deletion of the palindromic GATA-1 site was performed, and GATA-1 gene deletion occurred in sequences from 691 to 671 bp upstream of the last nucleotide in the first hematopoietic exon (IE). The alteration of this exon compromises the function of GATA-1 protein, helping to elucidate the functional role of GATA-1 in different tissues. The ΔdblGATA mouse strain has been widely used for studies of allergy and asthma. In the bone marrow of this mouse strain, it is known that deleting a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage (*in vivo*) and impairment of basophil development. Although this animal model is beneficial for immunological studies, the consequences of GATA-1 mutation for testis function remain to be elucidated.

Despite being crucial for hematopoiesis and cardiac function, very few studies have explored the role of GATA-1 in testicular homeostasis so far (Ito et al., 1993; Yomogida et al., 1994; Onodera et al., 1997; Feng et al., 1998; Labbaye et al., 2002; Wakabayashi et al., 2003; Buaas et al., 2004; Jiang and Wang, 2004; Viger et al., 2004). Although GATA-1 expression has already been described in mice Sertoli cells and rat Leydig cells, its role in the testis is still not fully understood. In this context, this work aimed to study the involvement of GATA-1 protein in adult mice spermatogenesis in a more detailed way, investigating the testicular function of adult ΔdblGATA mice through detailed histomorphological, hormonal, genetic and *in vitro* analyses.

2. Material and methods

2.1. Animals

ΔdblGATA (BALB/c genetic background; Vieira et al. 2009) mice were obtained from the Immunopharmacology Laboratory at Federal University of Minas Gerais (UFMG), and BALB/c wild-type mice were obtained from Central Vivarium (CEBIO-UFMG, Brazil). All animals (n = 17 per group) were housed in a conventional specific pathogen-free facility at an average temperature of 22 °C, 70% average humidity, and 12h photoperiod. Water and pelleted food were available *ad libitum*. Sexually mature animals were euthanized at 90 days of age. The current study was performed according to the Brazilian Government's ethical and animal experiments regulations (Law 11794/2008). According to the Guidelines for Animal Use and Experimentation, all animal experiments were performed as set by the Animal Experimentation Ethics Committees from UFMG (CEUA/UFMG; #340/2019, Belo Horizonte, Brazil).

2.2. BrdU INJECTIONS

To estimate the duration of spermatogenesis, two animals from the ΔdblGATA group received intraperitoneal injections of BrdU (5-bromo2-deoxyuridine; 150 mg/kg BW) diluted in saline solution using a hypodermic needle. BrdU is a specific marker of cells that are synthesizing DNA at the moment of injection. Each animal received two BrdU injections at 17 days and 1 h before euthanasia.

2.3. Tissue preparation

All mice were euthanized by anesthetic overdose [ketamine (80 mg/kg BW) and xylazine (10 mg/kg BW); Sigma-Aldrich, St. Louis, MO, USA]. The testes, epididymis and seminal vesicles were collected and weighed. Since testicular density is close to 1 g/mL (França, 1992), testicular volume was considered equal to its weight. The gonadosomatic index (GSI, testes total mass divided by body weight) was estimated for all investigated animals. The anogenital index (AGI) was measured using a digital caliper, and the values obtained were corrected for the cube root of the bodyweight (Auharek et al., 2012). Testes samples were fixed in solutions of 4% glutaraldehyde, Bouin's, or methacarn solutions. Six testes per group were frozen at −80 °C for further use in qPCR analyses.

For morphometric analyses (n = 6), the samples fixed in glutaraldehyde were dehydrated in ethanol and routinely embedded in glycolmethacrylate (Historesin, Leica). Histological sections, 4 μm thick, were obtained using a glass knife with a rotatory microtome (Leica RM-2165) and stained with a toluidine blue solution in 1% sodium borate. For immunostaining (n = 6), 6 μm thick sections were obtained from samples fixed in Bouin's or methacarn solutions after dehydration in ethanol and embedding in paraplast® (Sigma-Aldrich).

2.4. Histomorphometric analysis

2.4.1. Seminiferous tubule diameter and epithelium height

The mean diameter and epithelium height of the ST were measured in twenty round ST cross-sections, independent of the stage of the seminiferous epithelium cycle (SEC). All histomorphometric analyses were performed using Image J v.1.45s software (Image Processing and Analysis, in Java).

2.4.2. vol densities of the testis parenchyma components

The testicular parenchyma components' volume densities (%) were calculated after counting 7800 points over testis parenchyma. For this, we analyzed fifteen fields (images) obtained randomly from a continuous slide horizontal scanning using a graticule of 520 points. The intersections in tunica propria, seminiferous epithelium, tubular lumen, LCs, spermatogonia, connective tissue cells, blood, and lymphatic vessels were scored.

2.4.3. Germ and Sertoli cell numbers

To estimate the efficiency of spermatogenesis progression, we counted the cells present at stage VII of the spermatogenic cycle (Russell et al., 1990a,b) in ten-round ST cross-sections for each animal at 100x magnification. These cell counts were corrected according to the method described by Abercrombie (1946) and modified by Amann (1962). Cell ratios/proportions were obtained from these corrected counts. Assuming no significant gem cell loss occurs during spermiogenesis, the

number of round spermatids counted at Stage VII of the spermatogenic cycle was considered the final spermatozoa population (Russell and Clermont, 1977; Hess and França, 2007).

The total number of SCs per testis and testis gram were determined from the SC nucleoli number per tubule cross-section and the total length of ST, according to Hochereau-de Reviers and Lincoln (1978). Following this and considering the net weight of testis, the number of SCs per gram of testis was defined. The differentiated spermatogonial cells were quantified according to the methodology described by Auharek and França (2010).

2.4.4. Stages of the seminiferous epithelium cycle

SEC stages were characterized based on the development of the acrosomic system and the morphology of the developing spermatid nucleus (Russell et al., 1990a,b; Hess and França, 2007). The relative stage frequencies were calculated by randomly evaluating 200 ST cross-sections per animal at 1000x magnification (Leal and França, 2008).

2.4.5. Duration of spermatogenesis and daily sperm production

The duration of the spermatogenic cycle was estimated based on the stage frequencies and the most advanced germ cell type labeled in the two time periods following the BrdU injections (Lara et al., 2016). The daily sperm production (DSP) per testis and testis gram were achieved based on the following formula developed by França (1992): DSP = (total number of SCs per testis) x (the ratio of round spermatids per SCs at stage VII) x (stage VII relative frequency [%])/(stage VII duration [days]).

2.4.6. Leydig cell parameters

LC volume was obtained using the nuclear volume as well as the proportion between the nucleus and cytoplasm. For this purpose, 30 nuclei were measured per animal, and LC nucleus volume was obtained using the sphere formula ($4/3\pi R^3$, in which R = nuclear diameter/2). The proportion between the nucleus and cytoplasm was calculated after scoring 1000 points over LCs for each animal. The number of LCs was estimated from the LC size and the total volume occupied by these cells in the testis parenchyma (Costa et al., 2018). Although this methodology is helpful for a comparative evaluation between groups, it has some limitations in the case of Leydig cell shrinkage (Mendis-Handagama and Ewing, 1990; Mendis-Handagama, 1992; Wreford, 1995).

2.5. Immunostaining and pixel intensity quantification

The immunoperoxidase method was used for BrdU, CASPASE-3, 3βHSD1, GFRα-1, GATA-1 and 17βHSD3. For this purpose, serial sections were deparaffinized, rehydrated, and the antigens were exposed by heating in buffered sodium citrate (pH 6.0) at 96 °C for 5 min (this step was dismissed for Caspase-3 staining). Subsequently, the endogenous peroxidase activity was blocked by incubating the sections in a 3% hydrogen peroxide solution (Sigma, St. Louis, MO, USA) for 10 min. Then, the sections were treated with Ultra V Block (Thermo Scientific, Fremont, CA, USA) for 5 min to block non-specific antibody binding sites. Samples were incubated overnight at 4 °C with primary antibodies: anti-BrdU (1:200 dilution; sc-32323, Santa Cruz), anti-Caspase-3 (1:100 dilution; c8487, Sigma), anti-3βHSD1 (1:100 dilution; sc-30820, Santa Cruz), anti-GFRα-1 (1:50 dilution; sc-271546, Santa Cruz), anti-GATA-1 (1:200 dilution; ab-2818294, Thermofisher) and 17βHSD3 (1:50 dilution; ab126228), Abcam).

Reactions were visualized using biotin-conjugated secondary antibodies (for GFRA1 and 3βHSD1; 1:100 dilution, Abcam, ab6740, lot number GR27132-6), (for BrdU, 1:200 dilution, Imuny, IC1M02, lot number 16160), (for CASPASE-3, GATA-1 and 17βHSD3, 1:100 dilution, Abcam, ab93697, lot number GR313750-13) in combination with Elite ABC Kit (Vector Laboratories, CA, USA) and streptavidin (Thermo Scientific, TS-125-HR). Detection of the signal was performed by reaction with 3,3-diaminobenzidine (DAB, Sigma Aldrich). Negative controls had only the primary antibodies omitted. All the stained samples were analyzed using an Olympus microscope (BX60). The integrated pixel intensity of stained cells was calculated as the ratio between a stained region of interest inside a cell and the background, as previously described (Oliveira et al., 2020).

The immunofluorescence method was performed for CASPASE-3 (1:100 dilution; c8487, Sigma), GATA-1 (1:200 dilution, SC-362262, Santa Cruz) and GATA-4 (1:100 dilution; sc-25310, Santa Cruz) and visualized using a secondary antibody conjugated to Alexa Fluor 488 (1:400 dilution, IC-1M08; Thermo Fischer Scientific) in a Zeiss fluorescence microscope (ApoTome). The immunofluorescence was also performed in cultured Leydig cells (in coverslips). Briefly, the cells were fixed in 4% formaldehyde in PBS and treated with 1% of Sodium Dodecyl Sulfate (SDS) solution. The cells were incubated with a 1% BSA in PBS to block unspecific antibody binding. The primary antibody 17βHSD3 (1:50 dilution; ab62221, Abcam) was added to the fixed cells (2 h) at room temperature in a humidified chamber overnight at 4 °C. After washing the dishes, the fluorescent conjugated secondary antibody (CruzFluor™ 488, 1:50 dilution; sc362262, SantaCruz) was supplemented at room temperature for 30–60 min. The DNA was stained with DAPI, and the cells were fixed again using 4% formaldehyde. Finally, we added an anti-fading medium (ProLong®, Invitrogen) in the coverslip to mount the slides.

2.6. GENE expression analysis

2.6.1. RNA extraction

Testicular tissue samples were kept in RNAlater Solution (Invitrogen) at −80 °C until the moment of total RNA extraction. After maceration on dry ice, 1 ml of Trizol® (Invitrogen) was added, quickly homogenizing the small testicular tissue fragments. Next, 200 μL of chloroform (Sigma) was added to each sample and incubated for 3 min at room temperature. The homogenate was centrifuged at 12000g for 15 min at 4 °C, and the colorless supernatant containing the RNA was carefully removed and transferred. After that, 500 μL of isopropanol (Sigma) was added to the supernatant and incubated at 4 °C for 20 min to obtain precipitated RNA. The RNA pellet was washed twice with chilled 75% ethanol (Sigma), centrifuged at 7500g for 2 min, and resuspended in DEPC water. RNA samples were treated with DNAse (DNA-free™ DNA Removal kit, ThermoFisher Scientific) to avoid gDNA contamination. The integrity of the RNA was analyzed using agarose gel electrophoresis, and the RNA concentration in the samples was determined by fluorometry in Qbit® (Invitrogen).

2.6.2. cDNA synthesis and Real-Time PCR

2 μL of total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). Primer sequences were standardized for concentration and annealing temperature via StepOnePlus Real-Time PCR System (Applied Biosystems®). All qPCR reactions were conducted using Maxima SYBR Green qPCR MasterMix with cDNA 1:10 and primers 600 nM (Primer sequences can be found in Supplementary Table 1). The PCR protocol corresponds to 5 min at 95 °C and 45 cycles with 1min at 60 °C and 10s 95 °C, each cycle. The fluorescence was detected at the end of each extension phase, and the melting curve was analyzed. The fold change of each sample was determined using *Gapdh* as an endogenous gene by the comparative method of ΔΔCt ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). *Gapdh* was selected as the housekeeping gene based on the melting curves and analysis of its expression in different samples in which there was no Ct variation equal to or greater than 1.

2.7. Cell culture

The cell culture was obtained according to the protocol described by Oh et al. (2017). Briefly, testes (n = 5 per group) were decapsulated in

RPMI 1640 medium and digested in 0.5 mg/ml collagenase solution (type IV, Sigma-Aldrich) at 37 °C for 15 min. After, the supernatant containing interstitial cells was collected and filtered (70 μm nylon cell strainer). Discontinuous Percoll density gradients (36% and 60% in PBS) were used to purify Leydig cells. Cells in the 36–60% interface were aspirated carefully and submitted to the *in vitro* culture using RPMI 1640 medium supplemented with 10% FBS for 24 h. The cells were subsequently maintained in a serum-free medium for 12 h to promote starvation before the human chorionic gonadotropin (hCG; 0.5 IU/ml; Sigma-Aldrich) stimulus. After one, two and 3 h of the hCG stimulus, the supernatants were collected to estimate the testosterone levels.

2.8. Hormonal analysis

2.8.1. Plasma testosterone levels

In order to perform the hormonal analysis, blood samples were collected via cardiac puncture when mice were still anesthetized before euthanasia. The plasma was separated by centrifugation at 2000 rpm for 10 min and stored at −20 °C. Plasma testosterone concentration was measured by radioimmunoassay (RIA) using a commercial kit (Tecsa®, Belo Horizonte, MG, Brazil). Although RIA may cross-react with other steroids (Auchus, 2014), the samples were processed in the automated Cobas 8000 (Roche Diagnostics Inc., Indianapolis, IN, USA) platform for direct assessment of testosterone through electrochemiluminescence assay. The testosterone coefficients of variation (CV) intra- and inter-assay were 1.1% and 1.5%, respectively.

2.8.2. LH levels

LH levels were measured by ultrasensitive ELISAs, adapted from previously described methods for LH assays (Steyn et al., 2013; Aquino et al., 2017; Silva et al., 2020). The capture antibodies utilized were monoclonal anti-bovine LH-b subunit (518B7, University of California) at 1:2500. The detection antibodies utilized were rabbit anti-rLH (AFP240580Rb,NIDDK-NHPP) at 1:40000. The secondary antibody utilized was horseradish peroxidase-conjugated goat anti-rabbit IgG (P044801-2, Dako Pathology Solutions, Santa Clara, CA) at 1:2000 in both assays.

A 96-well plate (9018,Corning, Kennebunk, ME) was covered with 50 mL of capture antibody diluted in PBS overnight at 4 °C. The capture antibody was decanted, and wells were incubated with 200 mL of blocking buffer for 2 h at room temperature (RT). A standard curve was generated via a twofold serial dilution of the reference preparation. The wells were incubated with 50 mL of standards or samples for 24 h at RT. Afterward, wells were incubated with 50 mL of detection antibody for 24 h at 4 °C. The wells were incubated with 50 mL of secondary antibody for 1.5 h at RT. After that, wells were incubated with 100 mL of 2 mg/mL o-phenylenediamine dihydrochloride (P1526, Sigma-Aldrich) diluted in citrate–phosphate buffer (pH 5.0) containing 0.02% hydrogen peroxide for 45 min at RT. 50 mL of 3 M HCl was used to stop the reaction. The absorbance was determined at 490 nm with a microplate reader, and the wavelength of 650 nm was used for background correction. For LH ELISA, the lower detection limit was 0.07 ng/mL, and the interassay and intrassay coefficients of variation were 10.4 and 3.3%, respectively (Steyn et al., 2013; Aquino et al., 2017; Silva et al., 2020).

2.8.3. Testosterone levels in cell culture media

The collected medium was analyzed using the Kit Testosterone Test system - EIA protocol (Monobind inc., USA). For this, 10 μL of the calibrators, controls and samples were pipetted in each microwell of the plate. Following this, 50 μL of working reagent (enzyme conjugate) were added. The plate was gently shaken for 30 s, and then 50 μL of Biotin Testosterone reagent were supplemented and incubated for 60 min at room temperature. After this step, the contents of the entire plate were discarded and washed with 300 μL of washing solution. One hundred microliters of the substrate working solution were added to all microwells and incubated for 15 min at room temperature. Finally, 50 μL of the stop solution were added, and the plate was directed to the ELISA reader. The reading of each microcavity was taken at 450 nm, using the reference wavelength of 620–630 nm to minimize cavity imperfections. The absorbance of each duplicate represented the corresponding testosterone concentration (ng/mL).

2.9. Sperm analyses

For the sperm analyses, epididymides were collected, weighed and manually dissected on a Petri dish containing commercial Dulbecco's modified eagle's medium (#12500-062; DMEM/F12 - Gibco, Grand Island, NY, USA). Samples were analyzed under a light microscope at 35.5 °C (Vieira, 2019; Figueiredo et al., 2021). The sperm vitality and motility were analyzed as described by Vieira (2019). The sperm morphology was investigated through sperm smears, stained with Hematoxylin-Eosin (HE), by assessing 200 spermatozoa regarding the head, midpiece and tail morphology. The sperm defects were identified, counted and classified (Vieira, 2019; Figueiredo et al., 2021).

2.10. Statistical analysis

All data were tested for normality and homoscedasticity of the variances following Kolmogorov-Smirnov (Dallal-Wilkinson-Lilliefor) and Bartlett tests. Analyses were conducted using the graphics and statistics program PRISM v5.0 (GraphPad Software, Inc.). Data were assessed by unpaired Student's *t* test and were represented as the mean ± SEM (standard error of the mean). The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Biometry and histomorphometric data

Biometric data collected from the investigated groups are depicted in Table 1. While both body and testis weights were significantly smaller in ΔdblGATA, the gonadosomatic index (Table 1) was higher. Seminal vesicle weight and anogenital index were also smaller in ΔdblGATA. Morphometric parameters of testicular parenchyma are shown in Table 2. There were no significant differences regarding tubular diameter, seminiferous epithelium height, volume density of the tubular compartment components, and the length of the ST between the evaluated groups ($p > 0.05$). Concerning the intertubular compartment, the proportion of LCs and connective tissue was higher in ΔdblGATA mice, whereas blood vessels and lymphatic space proportions were smaller in this group ($p < 0.05$).

3.2. GATA-1 protein and expression in ΔdblGATA animal

Specific and robust immunolabelings for GATA-1 protein in Sertoli cells were observed through immunohistochemistry in control mice (Fig. 1A and E, 1E′, 1E''). The testis of ΔdblGATA mouse also showed this protein expression in well-demarcated Sertoli cells, as shown in Fig. 1B, F, 1F'and 1F''. Pixel analysis showed a difference in the expression (intensity) of GATA-1 proteins between the groups, being higher in the control when compared to the ΔdblGATA group (Fig. 1C).

Table 1
Biometric data from control and ΔdblGATA mice (mean ± SEM).

Parameter	Control (n = 12)	ΔdblGATA (n = 12)
Body weight (g)	28.4 ± 0.7	22.9 ± 0.5*
Testis weight (mg)	104.1 ± 1.5	89.2 ± 1.2*
Gonadosomatic Index (%)	0.74 ± 0.02	0.78 ± 0.01*
Epididymis weight (mg)	35.9 ± 2.8	32.3 ± 1.8
Seminal Vesicle weight (mg)	259 ± 19	187 ± 11*
Anogenital Index (mm)	5.0 ± 0.11	4.4 ± 0.13*

n = number of mice. * Statistically significant differences ($p < 0.05$).

Table 2
Histomorphometric testicular data from control and ΔdblGATA mice (mean ± SEM).

Parameter	Control (n = 6)	ΔdblGATA (n = 6)
Tubular diameter (μm)	220 ± 2.6	218 ± 3.6
Seminiferous epithelium height (μm)	76.9 ± 1.1	74.3 ± 1.2
Volume Density (%)		
Tubular Compartment	93.1 ± 0.5	93.1 ± 0.4
Tunica propria	3.6 ± 0.2	3.7 ± 0.3
Seminiferous epithelium	80.4 ± 0.7	80.4 ± 0.9
Lumen	9.1 ± 0.7	9.0 ± 0.9
Intertubular Compartment	6.9 ± 0.4	6.9 ± 0.4
Leydig Cell	4.2 ± 0.4	5.1 ± 0.3*
Blood Vessels	2.0 ± 0.3	0.9 ± 0.09*
Lymphatic Space	0.6 ± 0.1	0.3 ± 0.1*
Connective Tissue	0.3 ± 0.05	0.6 ± 0.1*
Tubular length/testis (m)	2.4 ± 0.1	2.2 ± 0.1
Tubular length/g/testis (m)	24.6 ± 0.6	25 ± 0.8

n = number of mice. *Statistically significant differences ($p < 0.05$).

Labeled Sertoli cells (GATA1+) were seen in stages of the seminiferous epithelium cycle near spermiation. Regarding the *Gata-1* gene relative expression, it was possible to observe a significant reduction (~75%) in ΔdblGATA animals (Fig. 1D).

3.3. Duration of spermatogenesis

The mean percentages of the stages of the SEC as well as the frequencies of the pre-meiotic (VII-XI), meiotic (Stage XII), and post-meiotic (Stages I-VI) phases are displayed in Fig. 1G. Statistical differences were observed concerning the frequencies of stages I, V, VI, VII, VIII, and XII ($p < 0.05$) (Fig. 1G).

The most advanced labeled germ cell types observed in the ΔdblGATA group in the different periods evaluated after BrdU injections are shown in Fig. 1H. Approximately 1 h after the BrdU pulse, the most advanced germ cells labeled were pre-leptotene spermatocytes located in the basal compartment at Stage VIII. Seventeen days after the BrdU injection, round spermatids were the most advanced germ cell labeled at stage VII/VIII.

Based on BrdU-labelings and stage frequencies (Fig. 1I), the mean duration of the SEC for ΔdblGATA mice was estimated as 8.67 ± 0.17 days. Since approximately 4.5 cycles are necessary for the spermatogenic process to be completed, the total duration of spermatogenesis in ΔdblGATA mice was estimated as 38.28 ± 0.76 days.

3.4. Sertoli and germ cell data

Concerning SCs, no differences were observed regarding their number per ST cross-sections at stage VII (Fig. 2A) or per testis gram (Fig. 2B) ($p > 0.05$). Additionally, the immunofluorescence for GATA-4 (Fig. 2C and D) showed a similar staining pattern of expression in the Sertoli cells in both investigated groups. Regarding germ cells, ΔdblGATA mice exhibited increased number and volume density of differentiated spermatogonia in the testicular parenchyma ($p < 0.05$; Fig. 2E and F). Aiming to investigate an undifferentiated spermatogonia molecular marker, we performed immunolabeling using the GFRA1 antibody. Single and paired GFRA1-positive spermatogonia in the control group were frequently observed in the ST cross-sections (2D dimension) (Fig. 2G). Differently, in ΔdblGATA animals, GFRA1-positive spermatogonia were primarily perceived as a single cell per ST cross-sections (Fig. 2H).

A higher incidence of spermatocyte apoptosis was noted in ΔdblGATA mice testis compared to the control, which was further confirmed by CASPASE-3 immunolabeling. Both immunoperoxidase (Fig. 2I and J) and immunofluorescence (Fig. 2K-L) assays revealed a high prevalence of Caspase-3-positive pre-leptotene spermatocytes in ΔdblGATA mice.

Considering the testicular cell ratios obtained (Fig. 2M–O), only the overall meiotic yield (number of round spermatids per differentiated spermatogonia) was statistically different, being lower in the ΔdblGATA mice ($p < 0.05$). Although it seems that SC efficiency (round spermatids per SC) and daily sperm production tended to be lower in ΔdblGATA, it was not statistically different in comparison to the control group (Fig. 2O–P).

3.5. Leydig cell data

ΔdblGATA mice exhibited a range of significant alterations in LC parameters (Fig. 3A–F). The transgenic mice presented smaller LC nuclear, cytoplasmatic, and, consequently, individual volumes ($p < 0.05$; Fig. 3C–E). However, the number of these cells per gram of testis was higher than the control group ($p < 0.05$; Fig. 3F). A reflex of potential changes in the steroidogenic pathway, lower anogenital index and seminal vesicle weight were observed in the transgenic group ($p < 0.05$, Fig. 3G and H). To evaluate this question, we conducted a 3βHSD1 immunostaining, which showed that this enzyme, while easily observed in the control group, was low labeled in LCs from ΔdblGATA mice. (Fig. 3I and J). Corroborating this observation, pixel intensity analysis of 3βHSD1 showed a reduced expression in ΔdblGATA mice (Fig. 3K). A similar immunostaining pattern was observed using anti-17βHSD3 (Fig. 3L–M). Since it is known that another protein of the GATA family (GATA-4) is involved in testosterone synthesis (Schrade et al., 2015), we performed immunolabeling to evaluate this protein in ΔdblGATA Leydig cells. Interestingly, GATA-4 protein in LC was noted in both groups (Fig. 3N–O), suggesting that the molecular downstream of GATA-1 may not be hampering the GATA-4 pathway.

Our genetic analyses (qPCR data) of steroidogenesis-related genes showed that *Ar, Star, Hsd3b1, Cyp17* and *Cyp19* presented a lower expression in ΔdblGATA mice (Fig. 3P). *Cyp11* and *Wt1* showed a similar relative expression between the control and transgenic groups. Only the *Insl3* gene showed a significant increase in the ΔdblGATA group. Confirming these findings, ΔdblGATA also presented elevated LH concentrations (Fig. 3Q) associated with reduced plasma testosterone levels (Fig. 3R).

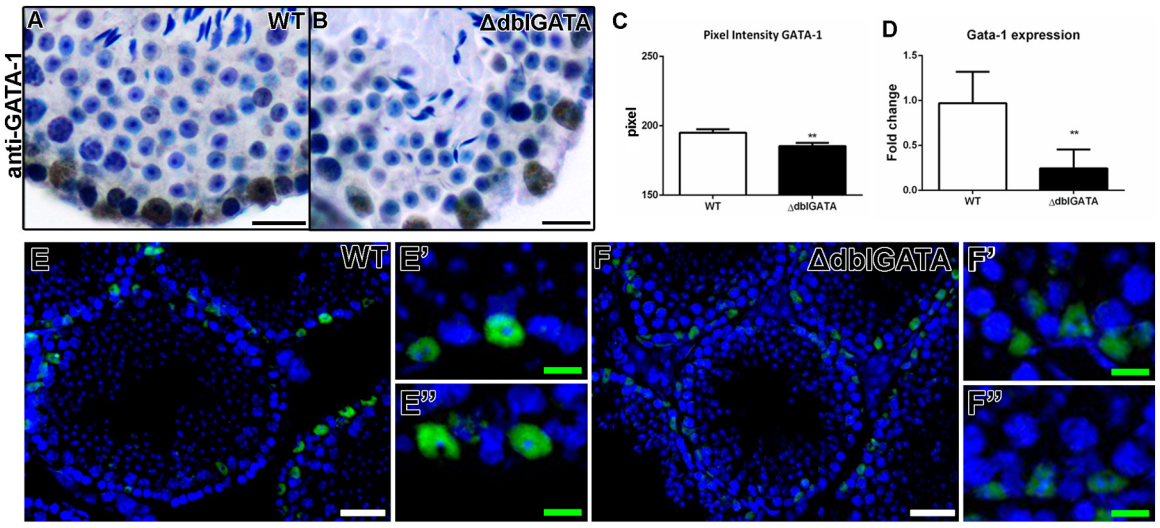
Leydig cells were successfully cultivated and immunolabeled with 17βHSD3 (Fig. 4A–C). After extracting their RNA and evaluating the relative gene expression, it was possible to observe three specific genes, *Hsd3b1, Hsd3b6* and *Insl3*. There was a decrease in *Hsd3b1* (Fig. 4D) and *Hsd3b6* (Fig. 4 E) expressions, while *Insl3* showed a higher expression (Fig. 4F). The cell culture medium was collected, and testosterone levels were measured by ELISA assay. The result showed lower testosterone levels in the ΔdblGATA Leydig cell culture (Fig. 4G).

3.6. ΔdblGATA mice sperm

Sperm parameters are shown in Table 3. No statistical differences were observed regarding sperm vitality and motility between the investigated groups. However, in comparison to controls, ΔdblGATA mice showed an increased number of spermatozoa with amorphous heads ($p < 0.05$) (Fig. 4H-Q).

4. Discussion

For the first time, in the present study, detailed histomorphological, hormonal, and genetic analyses showed several testicular alterations resulting from the GATA-1 protein mutation in adult mice testis. It was also showed that even the ΔdblGATA animal has GATA-1 protein in Sertoli cells, as observed in control animals. However, this expression is weaker in mutant mice, suggesting that the mutation could decrease the production of this protein in the testis. Furthermore, because it is a mutant protein with a lower expression, the GATA-1 function could be altered, generating some testicular changes, as demonstrated in the present study.



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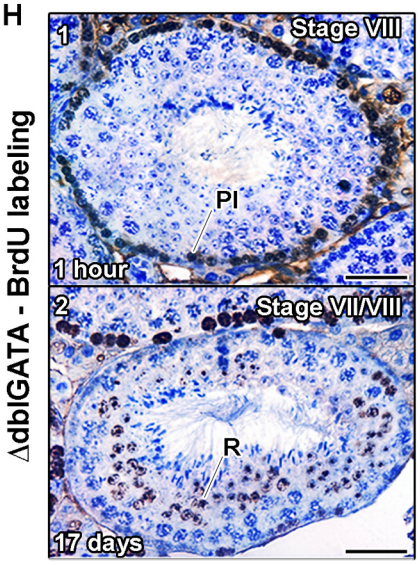
Stages of the SEC	WT	ΔdblGATA	
I	10.1 ± 0.8	3.5 ± 1.1	*
II-III	10.1 ± 0.7	13.85 ± 1.8	
IV	4.2 ± 0.3	4.99 ± 0.9	
V	8.1 ± 0.7	3.98 ± 0.4	*
VI	14.2 ± 1.2	8.62 ± 1.1	*
VII	19.5 ± 0.8	12.05 ± 1.6	*
VIII	5.4 ± 0.4	15.40 ± 2.1	*
IX	10 ± 0.7	13.05 ± 1.4	
X	7.7 ± 0.5	7.31 ± 1.4	
XI	8.2 ± 0.7	6.86 ± 0.9	
XII	2.3 ± 0.3	10.14 ± 1.9	*
Pre-meiotic (VII-XI)	50.9 ± 0.8	54.67 ± 2.1	
Post-meiotic (I-VI)	46.7 ± 1.2	35.19 ± 1.8	
Meiotic (XII)	2.3 ± 0.3	10.14 ± 1.9	



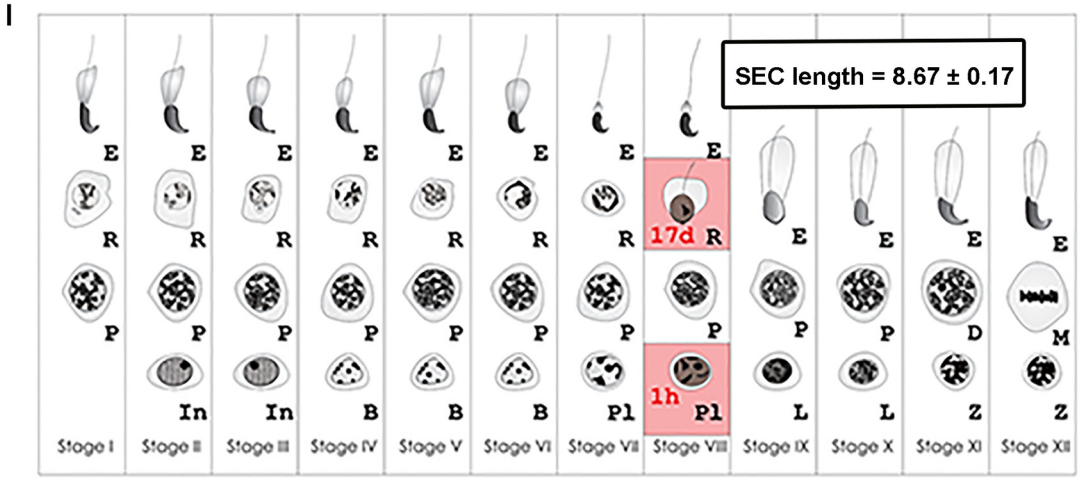


Fig. 1. GATA-1 expression and duration of the seminiferous epithelium cycle in ΔdblGATA mice. Immunolabeling for GATA-1 protein in Sertoli cells through immunoperoxidase (A–B). The intensity (in pixels) of GATA-1 proteins is lower in ΔdblGATA compared to control mice (C). Gata-1 expression in control and ΔdblGATA mice testes (D). GATA-1 protein immunofluorescence in Sertoli cells from controls (E-E'') and ΔdblGATA mice (F–F''). Relative frequency of stages of the seminiferous epithelium cycle (G). Immunoperoxidase (H) and schematic diagram (I) of labeled germ cells 1 h (H1) and 17 days (H2) after BrdU injections. In = intermediate spermatogonia; B = type B spermatogonia; Pl = pre-leptotene; L = leptotene; Z = zygote; P = pachytene; D = diplotene; M = Meiosis; R: round spermatids; E = elongated sperm; SEC = seminiferous epithelium cycle. White and black scale bars = 50 μm. Green bars = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

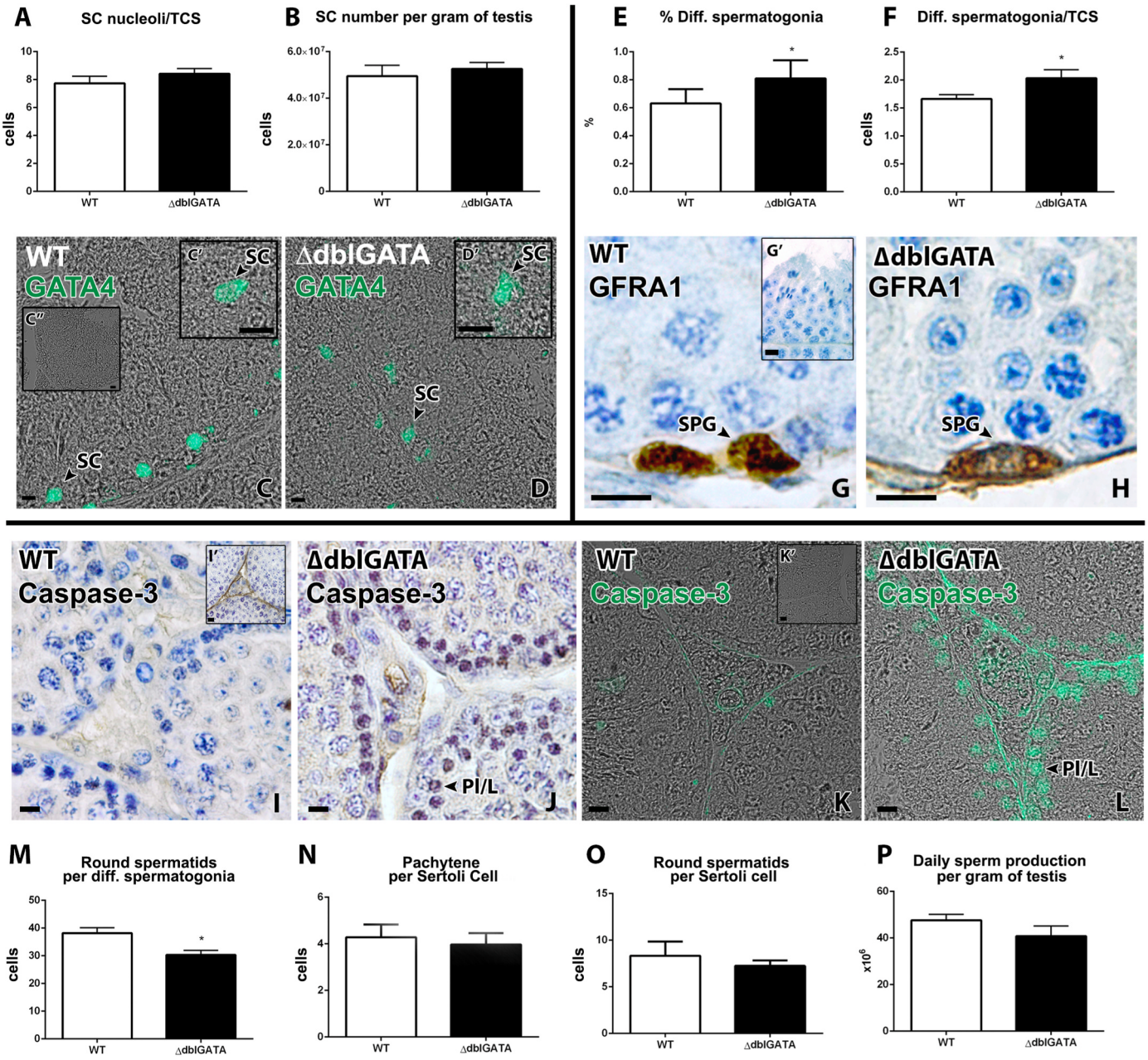


Fig. 2. Analysis of the tubular compartment in wild and ΔdblGATA mice. The number of Sertoli cells per cross-section of seminiferous tubules at stage VII of the seminiferous epithelium cycle (A). The total number of Sertoli cells per gram of testis (B). Immunofluorescences for GATA-4 in WT (C) and ΔdblGATA (D) mice. The number of differentiated spermatogonial cells per testis (%) (E) and per seminiferous tubules cross-sections at stage VII of the SEC (F). Immunoperoxidase for GFRA1 in WT (G) and ΔdblGATA (H) mice. Immunoperoxidase and immunofluorescence for CASPASE3 in WT (I and K) and ΔdblGATA (J and L) mice. Cellular ratios of different germ cell types per Sertoli cell (M-O). Daily sperm production per gram of testis (P). Scale bars = 10 μm.

Previously, Yomogida and collaborators (1994) showed that GATA-1-positive Sertoli cells were found exclusively in cross-sections of seminiferous tubules at stages VII, VIII and IX, whereas seminiferous tubules in other stages were negative for GATA-1-staining in adult animals. We observed the same pattern, with only stages close to spermiation presenting GATA1 positive Sertoli cells. Although daily sperm production was not altered, ΔdblGATA mice exhibited important tubular alterations. These mice have a significantly higher volume density of differentiated spermatogonia and an increased number of Caspase-3-positive pre-leptotene spermatocytes.

The main significant changes occurred in the intertubular compartment, where LCs were profoundly affected by the presence of a dysfunctional GATA-1 protein. Differences were observed in their number, volume, and protein expression. Besides the lower testosterone levels in the plasma and the culture of Leydig cells, five genes related to the androgenic activity (*Star, Hsd3b1, Cyp17, Cyp19,* and *Ar*) presented lower expression, suggesting a crucial role of GATA-1 in the steroidogenic pathway in adult mice. The qPCR from the Leydig cell culture revealed a significant decrease in *Hsd3b1* and *Hsd3b6* expressions, which is consistent with the lower testosterone levels. According to Yokoyama et al. (2019), the *Hsd3b6* is a classical marker for adult Leydig cells. Confirming genetic data, the 3βSHD1 showed weaker immunolabeling (pixel analysis) in the ΔdblGATA Leydig cells. The same immunostaining pattern was found for the 17βHSD3.

Only the *Insl3* gene expression was increased *in situ* and *in vitro*. Lakis et al. (2019) demonstrated that the increase in *Insl3* gene expression is

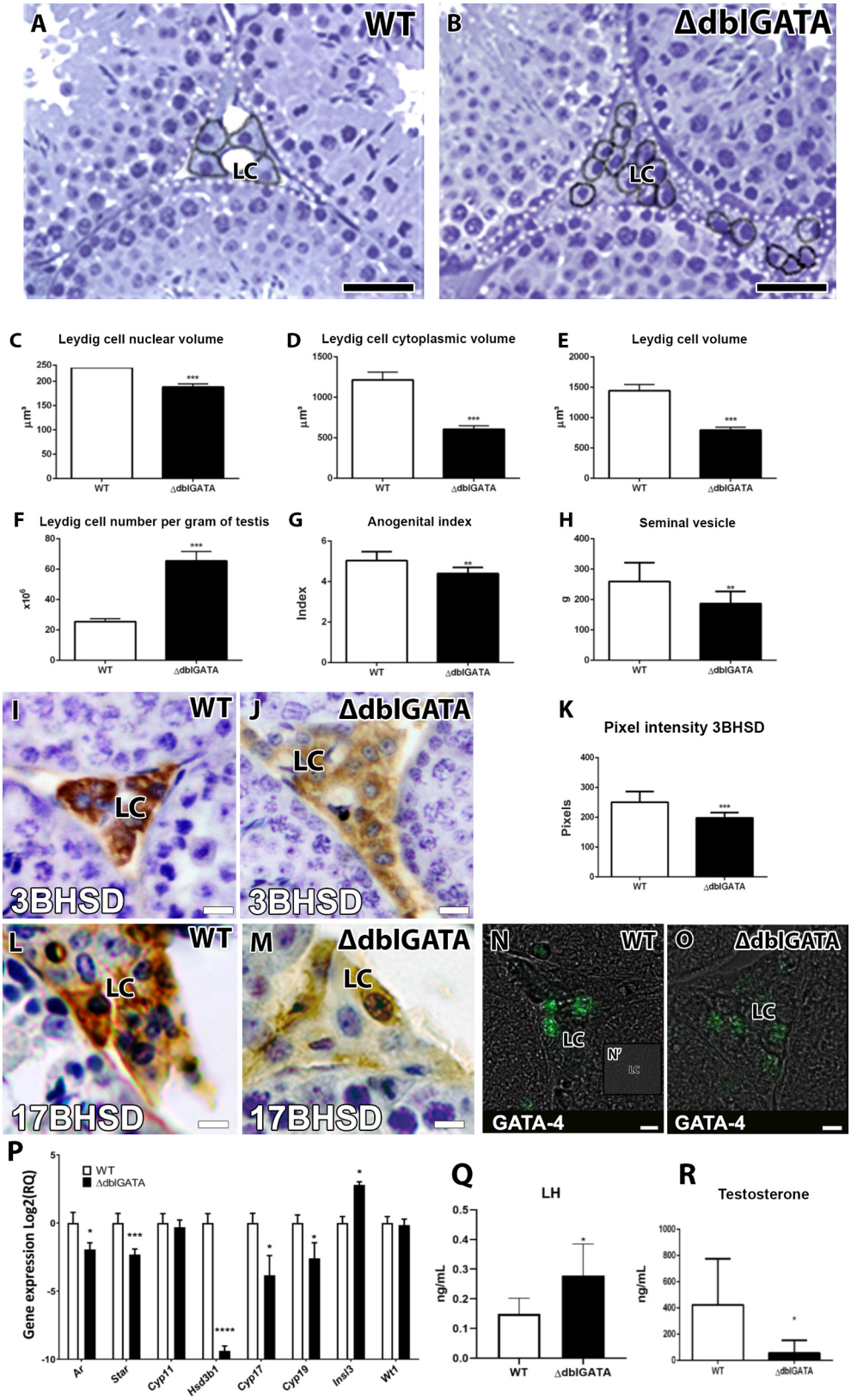


Fig. 3. Analysis of the intertubular compartment in wild-type and ΔdblGATA mice. Testicular histology showing the difference in Leydig cell volume between groups (A–B). Nuclear, cytoplasmic and total Leydig cell volume (C–E). The total number of Leydig cells per gram of testis (F). Anogenital index and seminal vesicle weight in both groups (G–H). Immunoperoxidase for 3βHSD1 in wild type (I) and ΔdblGATA mice (J). The pixel intensity of 3βHSD1 staining (K). Immunoperoxidase for 17βHSD3 in wild type (L) and ΔdblGATA mice (M). Immunofluorescence for GATA-4 in Leydig cells (N–O). qPCR technique, showing the relative expression of the following genes: *Ar, Star, Cyp11, Hsd3b1, Cyp17, Cyp19, Insl3 and Wt1* in the studied groups (P). LH (Q) and Serum testosterone (R) levels. Black scale bars = 50 μm. White scale bars = 10 μm.

directly related to the hyperplasia of Leydig cells, a pattern observed in ΔdblGATA mice. Moreover, in the mutant mice, the increase in Leydig cell number can probably be explained by the rise in LH levels (Teerds et al., 1989; Wakui et al., 2013; Zhu et al., 2021). Chen et al. (2014) showed that the crosstalk between Sertoli cells and Leydig cells has essential roles in Leydig cell steroidogenesis. Therefore, we can suggest that the steroidogenic pathway regulated by Wt1 continued preserved even with the mutation in the GATA-1 protein because no difference was observed for this gene expression. Further studies should focus on the crosstalk between LCs and hematopoietic cells in the testicular

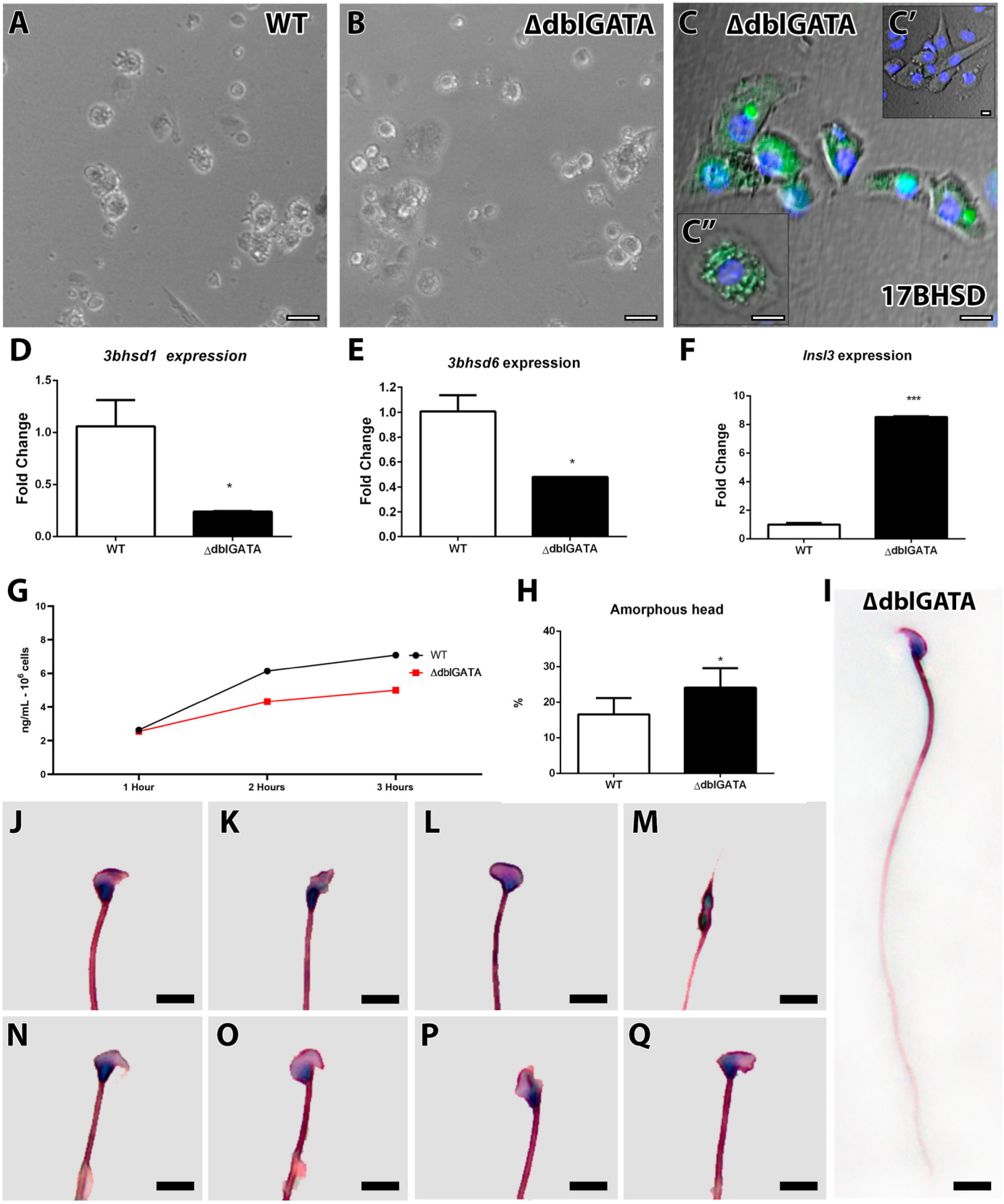


Fig. 4. Wild-type and ΔdblGATA mice Leydig cell culture and sperm morphology. Leydig cell culture images from wild-type and ΔdblGATA mice (A–B). 17BSHD3 immunofluorescence confirming the presence of Leydig cells in the culture system (C). Negative control (C′). Insert highlighting the immunolabeling in Leydig cell cytoplasm (C"). Altered gene expression in isolated Leydig cells (D–F). Levels of testosterone in the RPMI media in WT and ΔdblGATA Leydig cell culture (G). Frequency of amorphous head in WT and ΔdblGATA mice (H). Normal sperm morphology in ΔdblGATA mice (I). Sperm head defects in ΔdblGATA mice (J–Q). White scale bars = 10 μm. Black scale bars = 5 μm.

Table 3
Sperm parameters from control and ΔdblGATA mice (mean ± SEM).

Sperm Parameters	Control (n = 6)	ΔdblGATA (n = 6)
Sperm vitality (%)	29.3 ± 3.5	29.7 ± 4.0
Dead sperm	70.7 ± 3.5	70.3 ± 4.0
Sperm motility (%)	27.6 ± 4.5	30.9 ± 2.7
Progressive	8.2 ± 1.3	7.9 ± 2.0
Non-progressive	19.4 ± 3.8	23.0 ± 2.0
Immobile	72.4 ± 4.5	69.1 ± 2.7
Normal sperm (%)	20.3 ± 4.8	16.9 ± 7.0
Sperm defects (%)	79.7 ± 4.8	83.1 ± 7.0
Head defects	44.5 ± 3.5	49.9 ± 5.6
Amorphous head	16.6 ± 4.6	24.1 ± 5.5*
Midpiece defects	14.8 ± 3.5	15.7 ± 2.7
Tail defects	15.9 ± 0.9	12.9 ± 1.3
Without midpiece and tail	4.6 ± 1.6	4.7 ± 2.1

n = number of mice. *Statistically significant differences ($p < 0.05$).

interstitium to investigate signaling alterations.

GATA-1 expression is well described in SCs, and some *in vitro* studies have also demonstrated its expression in LCs (Zhang et al., 2002; Qamar et al., 2009). Indeed, our results suggest that GATA-1 is a critical factor for LCs physiology. ΔdblGATA mice exhibited impaired steroidogenesis, reflecting lower plasma testosterone levels, lower gonadosomatic index, lower anogenital index and seminal vesicle weight. Another morpho-functional feature that reflects the lower testosterone production is the reduced LC cytoplasmatic volume in ΔdblGATA mice, which can be associated with a reduced volume of smooth endoplasmic reticulum and lipid droplets accumulation (Johnson and Thompson, 1987). The increased LH levels in ΔdblGATA mice suggest that their Leydig cells are less functional than those from controls (indicating possible primary hypogonadism) (Carnegie, 2004; Liang et al., 2021).

Concerning other transcription factors of the GATA family, it is already known that GATA-4 and GATA-6 play an essential role in the steroidogenic pathway (Viger et al., 2004; Schrade et al., 2015; Penny et al., 2017). Evaluating the presence of GATA-4 protein in ΔdblGATA mice, our transgenic model mice showed this transcription factor in LC nuclei. These findings suggest that GATA-1 mutation does not impair the GATA-4 activity. Thus, we may assume that even with the alteration of GATA-1, some steroidogenesis stimuli are maintained by the GATA-4-triggered pathway (George et al., 2015). Other GATA-family factors, such as GATA-2, have also been described as regulating steroidogenesis genes in the human placenta (Lai et al., 2017).

The lack of androgenic support through the ablation of androgen receptors arrests the development of the third phase of spermatogenesis, impairing spermatid differentiation (De Gendt et al., 2004). Interestingly, we observed a significant number of sperm head defects in the ΔdblGATA group. This data suggests that lower testosterone production in these animals may be affecting spermiogenesis (Blanco-Rodriguez and Martinez-Garcia, 1996; Sofikitis et al., 2008). Although a significant reduction in plasmatic testosterone levels was detected in the present work, the spermatogenesis developed rather satisfactorily, and there was no significant difference in daily sperm production. This data suggests that intratesticular testosterone levels were sufficient to maintain the progression of spermatogenesis in ΔdblGATA mice. It is known that even low levels of intratesticular testosterone are still able to keep a healthy development of spermatogenesis (Cunningham and Huckins, 1979). Future studies should be conducted investigating the intratesticular levels in ΔdblGATA mice.

Lindeboom et al. (2003) developed a specific conditional knockout of the GATA-1 gene in SCs. Surprisingly, their GATA-1-mutant testes were both morphologic and functionally normal, indicating that the absence of this factor, specifically in SCs, does not interrupt spermatogenesis. Herein, despite an imbalance observed in the spermatogonial phase, we observed a somehow similar feature. The spermatogenesis progressed without serious adverse effects in ΔdblGATA mice. Additionally, the number of SCs, SC efficiency and GATA-4 labeling was similar between the investigated groups. Conversely, the sperm morphology defects could indicate an altered SC function related to the spermatid's differentiation (Syed and Hecht, 2002; Yan, 2009).

In ΔdblGATA mice, the increased number of differentiated spermatogonia suggests the involvement of GATA-1 specifically regulating the spermatogonial phase, whose signaling network is highly complex and deserves a cautionary discussion. Based on our cell counts and GFRA1 labeling pattern, it is suggested that altered GATA-1 promoted increased differentiation of the spermatogonial stem cells (SSCs). Although more investigations are necessary, the high *Insl3* expression in the ΔdblGATA mice may be stimulating the spermatogonial differentiation (Assis et al., 2016). It is also worth mentioning that GATA-1 interacts with PLZF in megakaryocyte development and erythroid cell production (Labbaye et al., 2002), meaning that there is a possibility that GATA-1 may be interacting with PLZF in the testis as well. Although we did not investigate this protein interaction, it is currently known that PLZF plays a crucial role in promoting SSCs self-renewal (Buaas et al., 2004). Furthermore, reduced androgen levels may be decreasing PLZF expression (Jiang and Wang, 2004). In this context, one can say that ΔdblGATA mice present a predisposition towards SSCs differentiation. However, as the stock of SSCs is preserved, other routes are maintaining their self-renewal. Future studies are necessary to investigate the number of differentiated and undifferentiated spermatogonial cells through whole-mount analyses and the WNT5A signaling in promoting SSCs self-renewal (factor increased in situations of low testosterone levels). (Tanaka et al., 2016).

Finally, the increased number of primary spermatocytes undergoing apoptosis may be related to a germ cell number control, possibly as a reflex of the extensive differentiation of SCCs leading to an excessive number of primary spermatocytes. It is known that SCs limit the number of germ cells that enter meiosis based on their support capacity (Lee et al., 1997; Murphy and Richburg, 2014). Another exciting aspect is that apoptosis of primary spermatocytes occurred mainly at stages VII and VIII, stages in which the expression of GATA-1 is accentuated in SCs (Ketola et al., 2002). Interestingly, these stages also presented significant differences regarding their frequency in the SEC in ΔdblGATA mice. Furthermore, it seems that GATA-1 does not influence the germ cell pace since no differences were observed concerning the duration of spermatogenesis (Oakberg, 1956; Costa et al., 2018; Oliveira et al., 2020).

5. Conclusion

Several adverse effects on the testicular steroidogenic activity were demonstrated for the first time due to reduced functional GATA-1 protein. Reduced Leydig cell size, decreased expression of steroidogenic genes, smaller seminal vesicle, reduced anogenital index, diminished testosterone levels, and high LH levels were herein observed in adult ΔdblGATA mice. Concerning the tubular compartment, the main finding was the increased number of differentiated spermatogonia, which seems to be counterbalanced by increased primary spermatocyte apoptosis. The sperm analyses indicated a high frequency of sperm head defects, suggesting alterations in spermiogenesis (phase highly dependent on androgens). In sum, our results show the critical role that GATA-1 plays in steroidogenesis and testis function.

6. Compliance with ethical standards

Conflict of interest

No conflict of interest was declared.

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Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals followed the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee on Animal Use from the Federal University of Minas Gerais - CEUA document #340/2019).

CRediT authors contribution statement

Matheus Felipe Fonseca Gonçalves: Methodology, Performed experiments, Formal analysis, Writing – review & editing. **Samyra Maria dos Santos Nassif Lacerda:** Methodology, Performed experiments, Formal analysis, Resources. **Nathália de Lima e Martins Lara:** Performed experiments, Formal analysis. **Carolina Felipe Alves de Oliveira:** Performed experiments, Formal analysis. **André Felipe Almeida Figueiredo:** Performed experiments, Formal analysis. **Marcos Rocha Gouvêa Brener:** Performed experiments, Formal analysis. **Marina Alcântara Cavalcante:** Performed experiments, Formal analysis. **Anderson Kenedy Santos:** Performed experiments, Formal analysis. **Gabriel Henrique Campolina-Silva:** Performed experiments, Formal analysis. **Vivian Vasconcelos Costa:** Methodology, Resources. **Ana Clara Campideli Santana:** Performed experiments, Formal analysis. **Roberta Araújo Lopes:** Performed experiments, Formal analysis. **Raphael Escorsim Szawka:** Performed experiments, Formal analysis. **Guilherme Mattos Jardim Costa:** Methodology, Performed experiments, Formal analysis, Resources, Writing – review & editing, All authors reviewed and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2021.111519.

References

Abercrombie, M., 1946. Estimation of nuclear population from microtome sections. Anat. Rec. 94, 239–247. https://doi.org/10.1002/ar.1090940210.

Amann, R.P., 1962. Reproductive capacity of dairy bulls. III. The effect of ejaculation frequency, unilateral vasectomy, and age on spermatogenesis. Am. J. Anat. 110, 49–67. https://doi.org/10.1002/aja.1001100106.

Aquino, N.S.S., Araujo-Lopes, R., Henriques, P.C., Lopes, F.E.F., Gusmao, D.O., Coimbra, C.C., Franci, C.R., Reis, A.M., Szawka, R.E., 2017. α-Estrogen and progesterone receptors modulate kisspeptin effects on prolactin: role in estradiol-induced prolactin surge in female rats. Endocrinology 158, 1812–1826. https://doi.org/10.1210/en.2016-1855.

Arceci, R.J., King, A.A., Simon, M.C., Orkin, S.H., Wilson, D.B., 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol. Cell Biol. 13, 2235–2246. https://doi.org/10.1128/MCB.13.4.2235.

Assis, L.H.C., Crespo, D, Morais, R.D.V.S., França, L.R., Bogerd, J., Schulz, R.W., 2016. INSL3 stimulates spermatogonial differentiation in testis of adult zebrafish (Danio rerio). Cell Tissue Res 363, 579–588. https://doi.org/10.1007/s00441-015-2213-9.

Auchus, R.J., 2014. Steroid assays and endocrinology: best practices for basic scientists. Endocrinology 155, 2049–2051. https://doi.org/10.1210/en.2014-7534.

Auharek, A.S., França, L.R., 2010. Postnatal testis development, Sertoli cell proliferation and number of different spermatogonial types in C57BL/6J mice made transiently hypo- and hyperthyroidic during the neonatal period. J. Anat. 216, 577–588. https://doi.org/10.1111/j.1469-7580.2010.01219.x.

Auharek, A.S., Lara, N.L.M., Avelar, G.F., Sharpe, R.M., França, L.R., 2012. Effects of inducible nitric oxide synthase (iNOS) deficiency in mice on Sertoli cell proliferation and perinatal testis development. Int. J. Androl. 35, 741–751. https://doi.org/10.1111/j.1365-2605.2012.01264.x.

Blanco-Rodriguez, J., Martinez-Garcia, C., 1996. Induction of apoptotic cell death in the seminiferous tubule of the adult rat testis: assessment of the germ cell types that exhibit the ability to enter apoptosis after hormone suppression by oestradiol treatment. Int. J. Androl. 19, 237–247. https://doi.org/10.1111/j.1365-2605.1996.tb00468.x.

Buaas, F.W., Kirsh, A.L., Sharma, M., Mclean, D.J., Morris, J.L., Griswold, M.D., De Rooij, D.G., Braun, R.E., 2004. Plzf is required in adult male germ cells for stem cell self-renewal. Nat. Genet. 36, 647–652. https://doi.org/10.1038/ng1366.

Carnegie, C., 2004. Diagnosis of hypogonadism: clinical assessments and laboratory tests. Rev. Urol. 6, 3–8.

Chen, M., Wang, X., Wang, Y., Zhang, L., Xu, B., Lv, L., Cui, X., Li, W., Gao, F., 2014. Wt1 is involved in Leydig cell steroid hormone biosynthesis by regulating paracrine factor expression in mice. Biol. Reprod. 90, 1–9. https://doi.org/10.1095/biolreprod.113.114702.

Costa, G.M.J., Lacerda, S.M.S.N., Figueiredo, A.F.A., Leal, M.C., Rezende-Neto, J.V., França, L.R., 2018. Higher environmental temperatures promote acceleration of spermatogenesis in vivo in mice (Mus musculus). J. Therm. Biol. 77, 14–23. https://doi.org/10.1016/j.jtherbio.2018.07.010.

Cunningham, G.R., Huckins, C., 1979. Persistence of complete spermatogenesis in the presence of low intratesticular concentrations of testosterone. Endocrinology 105, 177–186. https://doi.org/10.1210/endo-105-1-177.

De Gendt, K., Swinnen, J.V., Saunders, P.T.K., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lécureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M., Verhoeven, G., 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc. Natl. Acad. Sci. Unit. States Am. 101, 1327–1332. https://doi.org/10.1073/pnas.0308114100.

Evans, T., Reitman, M., Felsenfeld, G., 1988. An erythroid-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. Proc. Natl. Acad. Sci. U.S.A. 85, 5976–5980. https://doi.org/10.1073/pnas.85.16.5976.

Figueiredo, A.F.A., Wnuk, N.T., Vieira, C.P., Gonçalves, M.F.F., Brener, M.R.G., Diniz, A. B., Antunes, M.M., Castro-Oliveira, H.M., Menezes, G.B., Costa, G.M.J., 2021. Activation of C–C motif chemokine receptor 2 modulates testicular macrophages number, steroidogenesis, and spermatogenesis progression. Cell Tissue Res. 386, 173–190. https://doi.org/10.1007/s00441-021-03504-w.

Feng, Z.-M., Wu, A.Z., Chen, C.-L.C., 1998. Testicular GATA-1factorup-regulates the promoter activity of rat inhibin α-subunit gene in MA-10 Leydig tumor cells. Mol. Endocrinol. 12, 378–390. https://doi.org/10.1210/mend.12.3.0079.

França, L.R., 1992. Daily sperm production in Piau boars estimated from Sertoli cell population and Sertoli cell index. In: Proceedings of the 12th International Congress on Animal Reproduction and Artificial Insemination, 1992. ICAR, The Hague, Netherlands. The Hague, pp. 1716–1718.

George, R.M., Hahn, K.L., Rawls, A., Viger, R.S., Wilson-Rawls, J., 2015. Notch signaling represses GATA4-induced expression of genes involved in steroid biosynthesis. Reproduction 150, 383–394. https://doi.org/10.1530/REP-15-0226.

Grepin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T., Nemer, M., 1994. A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. Mol. Cell Biol. 14, 3115–3129. https://doi.org/10.1128/MCB.14.5.3115.

Hess, R.A., França, L.R., 2007. Spermatogenesis. Cycle of the seminiferous epithelium. In: Cheng, C.Y. (Ed.), Molecular Mechanisms in Spermatogenesis. Landes Bioscience, pp. 1–15.

Hochereau-De Reviers, M., Lincoln, G.A., 1978. Seasonal variation in the histology of the testis of the red deer, Cervus elaphus. Reproduction 585, 2437–2444. https://doi.org/10.1530/jrf.0.0540209.

Ito, E., Toki, T., Ishihara, H., Ohtani, H., Gu, L., Yokoyama, M., Engel, J.D., Yamamoto, M., 1993. Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis. Nature 362, 466–468. https://doi.org/10.1038/362466a0.

Jiang, F., Wang, Z., 2004. Identification and characterization of PLZF as a prostatic androgen-responsive gene. Prostate 59, 426–435. https://doi.org/10.1002/pros.20000.

Johnson, L., Thompson, D.L., 1987. Effect of seasonal changes in Leydig cell number on the volume of smooth endoplasmic reticulum in Leydig cells and intratesticular testosterone content in stallions. J. Reprod. Fertil. 81, 227–232. https://doi.org/10.1530/jrf.0.0810227.

Ketola, I., Rahman, N., Toppari, J., Bielinska, M., Porter-Tinge, S.B., Tapanainen, J.S., Huhtaniemi, I.T., Wilson, D.B., Heikinheimo, M., 1999. Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. Endocrinology 140, 1470–1480. https://doi.org/10.1210/endo.140.3.6587.

Ketola, I., Anttonen, M., Vaskivuo, T., Tapanainen, J.S., Toppari, J., Heikinheimo, M., 2002. Developmental expression and spermatogenic stage specificity of transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2 in the mouse testis. Eur. J. Endocrinol. 147, 397–406. https://doi.org/10.1530/eje.0.1470397.

Kumar, S., Kang, H., Park, E., Park, H.S., Lee, K., 2018. The expression of CKLFSF2B is regulated by GATA1 and CREB in the Leydig cells, which modulates testicular steroidogenesis. Biochim. Biophys. Acta. Gene. Regul. Mech. 1861, 1063–1075. https://doi.org/10.1016/j.bbagrm.2018.10.002.

Kuo, C.T., Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek, M.S., Soudais, C., Leiden, J.M., 1997. GATA-4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 11, 1048–1060. https://doi.org/10.1101/gad.11.8.1048.

Labbaye, C., Quaranta, M.T., Pagliuca, A., Militi, S., D Licht, J., Testa, U., Peschle, C., 2002. PLZF induces megakaryocytic development, activates Tpo receptor expression

and interacts with GATA1 protein. Oncogene 21, 6669–6679. https://doi.org/10.1038/sj.onc.1205884.
Lai, T.C., Li, H.F., Li, Y.S., Hung, P.Y., Shyu, M.K., Hu, M.C., 2017. Proximal GATA-binding sites are essential for human HSD3B1 gene transcription in the placenta. Sci. Rep. 7, 4271. https://doi.org/10.1038/s41598-017-04133-6.
Lakis, N.S., Lombardo, K.A., Mangray, S., Netto, G.J., Salles, D., Matoso, A., 2019. INSL3 expression in Leydig cell hyperplasia and Leydig cell tumors. Appl. Immunohistochem. Mol. Morphol. 27, 203–209. https://doi.org/10.1097/PAI.0000000000000567.
Lally, J., Boasman, K., Brown, L., Martinelli, V., Cappuccio, I., Sovani, V., Marinaccio, C., Crispino, J.D., Graham, C., Rinaldi, C., 2019. GATA-1: a potential novel biomarker for the differentiation of essential thrombocythemia and myelofibrosis. J. Thromb. Haemostasis 17, 896–900. https://doi.org/10.1111/jth.14433.
Lara, N.L.M., Santos, I.C., Costa, G.M.J., Cordeiro-Junior, D.A., Almeida, A.C.G., Madureira, A.P., Zanini, M.S., França, L.R., 2016. Duration of spermatogenesis and daily sperm production in the rodent Proechimys guyannensis. Zygote 24, 783–793. https://doi.org/10.1017/S0967199416000137.
Leal, M.C., França, L.R., 2008. Postnatal Sertoli and Leydig cell proliferation and the establishment of puberty and sexual maturity in Chinchilla lanigera (Rodentia, Chinchillidae). Reprod. Fertil. Dev. 20, 665–673. https://doi.org/10.1071/RD07134.
Lee, J., Richburg, J.H., Younkin, S.C., Boekelheide, K., 1997. The fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138, 2081–2088. https://doi.org/10.1210/endo.138.5.5110.
Liang, G., Song, Y., Liu, L., Zhou, K., Tian, J., Li, J., Shi, H., Zhu, Q., Wang, J., Zheng, J., Yu, X., Zhang, S., Li, Z., Liu, X., 2021. Association of hypogonadism symptoms and serum hormones in aging males. Andrologia 53, e14013. https://doi.org/10.1111/and.14013.
Lindeboom, F., Gillemans, N., Karis, A., Jaegle, M., Meijer, D., Grosveld, F., Philipsen, S., 2003. A tissue-specific knockout reveals that Gata1 is not essential for Sertoli cell function in the mouse. Nucleic Acids Res. 31, 5405–5412. https://doi.org/10.1093/nar/gkg723.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using RealTime quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
Martin, D.I., Orkin, S.H., 1990. Transcriptional activation and DNA binding by the erythroid factor GF-1/NF-El1/Eryf 1. Genes Dev. 4, 1886–1898. https://doi.org/10.1101/gad.4.11.1886.
Mendis-Handagama, S.M., Ewing, L.L., 1990. Sources of error in the estimation of Leydig cell numbers in control and atrophied mammalian testes. J. Microsc. 159, 73–82. https://doi.org/10.1111/j.1365-2818.1990.tb03020.x.
Mendis-Handagama, S.M., 1992. Estimation error of Leydig cell numbers in atrophied rat testes due to the assumption of spherical nuclei. J. Microsc. 168, 25–32. https://doi.org/10.1111/j.1365-2818.1992.tb03247.x.
Molkentin, J.D., Lin, Q., Duncan, S.A., Olson, E.N., 1997. Requirement of the transcription factor GATA-4 for heart tube formation and ventral morphogenesis. Genes Dev. 11, 1061–1072. https://doi.org/10.1101/gad.11.8.1061.
Molkentin, J.D., 2000. The zinc finger–containing transcription factors GATA-4, 25, and 26. Ubiquitously expressed regulators of tissue-specific gene expression. J. Biol. Chem. 275, 38949–38952. https://doi.org/10.1074/jbc.R000029200.
Morceau, F., Schnekenburger, M., Dicato, M., Diederich, M., 2004. GATA-1: friends, brothers, and coworkers. New York Acad. Sci. 1030, 537–554. https://doi.org/10.1196/annals.1329.064.
Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S., Parmacek, M.S., 1998. GATA-6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev. 12, 3579–3590. https://doi.org/10.1101/gad.12.22.3579.
Murphy, C.J., Richburg, J.H., 2014. Implications of Sertoli cell-induced germ cell apoptosis to testicular pathology. Spermatogenesis 4, e979110. https://doi.org/10.4161/21565562.2014.979110.
Oakberg, E.F., 1956. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am. J. Anat. 99, 507–516. https://doi.org/10.1002/aja.1000990307.
Oh, Y.S., Koh, I.K., Choi, B., Gye, M.C., 2017. ESR1 inhibits hCG-induced steroidogenesis and proliferation of progenitor Leydig cells in mice. Sci. Rep. 7, 43459. https://doi.org/10.1038/srep43459.
Oliveira, C.F.A., Lara, N.L.M., Lacerda, S.M.S.N., Resende, R.R., França, L.R., Avelar, G. F., 2020. Foxn1 and Prkdc genes are important for testis function: evidence from nude and scid adult mice. Cell Tissue Res. https://doi.org/10.1007/s00441-019-03165-w.
Onodera, K., Yomogida, K., Suwabe, N., Takahashi, S., Muraosa, Y., Hayashi, N., Ito, E., Gu, L., Rassoulzadegan, M., Engel, J.d., Yamamoto, M., 1997. Conserved structure, regulatory elements, and transcriptional regulation from the GATA-1 gene testis promoter. J. Biochem. 121, 251–263. https://doi.org/10.1093/oxfordjournals.jbchem.a021581.
Penny, G.M., Cochran, R.M., Pihlajoki, M., Kyrönlahti, A., Schrade, A., Häkkinen, M., Toppari, J., Heikinheimo, M., Wilson, D.B., 2017. Probing GATA factor function in mouse Leydig cells via testicular injection of adenoviral vectors. Reproduction 154, 455–467. https://doi.org/10.1530/REP-17-0311.
Pevny, L., Simon, M.C., Robertson, E., Klein, W.H., Tsai, S.F., D'agati, V., Orkin, S.H., Costantini, F., 1991. Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 349, 257–260. https://doi.org/10.1038/349257a0.
Qamar, I., Park, E., Gong, E., Lee, H.J., Lee, K., 2009. ARR19 (androgen receptor corepressor of 19 kDa), an antisteroidogenic factor, is regulated by GATA-1 in testicular Leydig cells. J. Biol. Chem. 284, 18021–18032. https://doi.org/10.1074/jbc.M900896200.
Russell, L.D., Clermont, Y., 1977. Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. Anat. Rec. 187, 347–366. https://doi.org/10.1002/ar.1091870307.
Russell, L.D., Ettlin, R.A., Sinha-Hikim, A.P., Clegg, E.D., 1990a. Histological and Histopathological Evaluation of the Testis. Cache Rivers Press, Clearwater.
Russell, L.D., Ren, H.P., Sinha-Hikin, I., Schulze, W., Sinha-Hikim, A.P., 1990b. A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. Am. J. Anat. 118, 21–30. https://doi.org/10.1002/aja.1001880104.
Schrade, A., Kyrönlahti, A., Akinrinad, O., Pihlajoki, M., Häkkinen, M., Fischer, S., Alastalo, T., Velagapudi, V., Toppari, J., Wilson, D.B., Heikinheimo, M., 2015. GATA4 is a key regulator of steroidogenesis and glycolysis in mouse Leydig cells. Endocrinology 156, 1860–1872. https://doi.org/10.1210/en.2014-1931.
Silva, J.F., Henriques, P.C., Campideli-Santana, A.C., Araujo-Lopes, R., Aquino, N.S.S., Hipolito, L.T.M., Lopes-Aguiar, C., Reis, A.M., Grattan, D.R., Szawka, R.E., 2020. Estradiol potentiates but is not essential for prolactin-induced suppression of luteinizing hormone pulses in female rats. Endocrinology 161, bqaa022. https://doi.org/10.1210/endocr/bqaa022.
Sofikitis, N., Giotitsas, N., Tsounapi, P., Baltogiannis, D., Giannakis, D., Pardalidis, N., 2008. Hormonal regulation of spermatogenesis and spermiogenesis. J. Steroid Biochem. Mol. Biol. 109, 323–330. https://doi.org/10.1016/j.jsbmb.2008.03.004.
Steyn, F.J., Wan, Y., Clarkson, J., Veldhuis, J.D., Herbison, A.E., Chen, C., 2013. Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice. Endocrinology 154, 4939–4945. https://doi.org/10.1210/en.2013-1502.
Syed, V., Hecht, N.B., 2002. Disruption of germ cell–Sertoli cell interactions leads to spermatogenic defects. Mol. Cell. Endocrinol. 186, 155–157. https://doi.org/10.1016/S0303-7207(01)00656-6.
Tanaka, T., Kanatsu-Shinohara, M., Lei, Z., Rao, C.Y., Shinohara, T., 2016. The luteinizing hormone-testosterone pathway regulates mouse spermatogonial stem cell self-renewal by suppressing WNT5A expression in Sertoli cells. Stem Cell Reports 7, 279–291. https://doi.org/10.1016/j.stemcr.2016.07.005.
Teerds, K.J., de Rooij, D.G., Rommerts, F.F.G., van den Hurk, R., Wensing, C.J.G., 1989. Proliferation and differentiation of possible Leydig cell precursors after destruction of the existing Leydig cells with ethane dimethyl sulphonate: the role of LH/human chorionic gonadotrophin. J. Endocrinol. 122, 689–696. https://doi.org/10.1677/joe.0.1220689.
Tsai, S.-F., Martin, D.I., Zon, L.I., D'andrea, A.D., Wong, G.G., Orkin, S.H., 1989. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature 339, 446–451. https://doi.org/10.1038/339446a0.
Tsai, S.-F., Strauss, E., Orkin, S.H., 1991. Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter. Genes Dev. 5, 919–931. https://doi.org/10.1101/gad.5.6.919.
Vieira, A.T., Fagundes, C.T., Alessandri, A.L., Castor, M.G.M., Guabiraba, R., Borges, V. O., Silveira, K.D., Vieira, E.L.M., Gonçalves, J.L., Silva, T.A., Deruaz, M., Proudfoot, A.E.I., Sousa, L.P., Teixeira, M.M., 2009. Treatment with a novel chemokine-binding protein or eosinophil lineage-ablation protects mice from experimental colitis. Am. J. Pathol. 175, 2382–2391. https://doi.org/10.2353/ajpath.2009.090093.
Vieira, C.P., 2019. Efeitos morfoestruturais da exposição pré-natal a glicocorticoide no testículo e avaliação dos parâmetros espermáticos de camundongos. Universidade Federal de Minas Gerais, Dissertação (Mestrado).
Viger, R.S., Mertineit, C., Trasler, J.M., Nemer, M., 1998. Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Müllerian inhibiting substance promoter. Development 125, 2665–2675.
Viger, R.S., Taniguchi, H., Robert, N.M., Tremblay, J.J., 2004. Role of the GATA family of transcription factors in andrology. J. Androl. 25, 441–452. https://doi.org/10.1002/j.1939-4640.2004.tb02813.x.
Wakabayashi, J., Yomogida, K., Nakajima, O., Yoh, K., Takahashi, S., Engel, J.D., Ohneda, K., Yamamoto, M., 2003. GATA-1 testis activation region is essential for Sertoli cell-specific expression of GATA-1 gene in transgenic mouse. Gene Cell. 8, 619–630. https://doi.org/10.1046/j.1365-2443.2003.00658.x.
Wakui, S., Takahashi, H., Mutou, T., Shirai, M., Jutabha, P., Anzai, N., Wempe, M.F., Kansaku, N., Hano, H., Inomata, T., Endou, H., 2013. Atypical Leydig cell hyperplasia in adult rats with low T and high LH induced by prenatal di(n-butyl) phthalate exposure. Toxicol. Pathol. 41, 480–486. https://doi.org/10.1177/0192623312457272.
Wall, L., Deboer, E., Grosveld, F., 1988. The human β-globin gene 3' enhancer contains multiple binding sites for an erythroid-specific protein. Genes Dev. 2, 1089–1100. https://doi.org/10.1101/gad.2.9.1089.
Weiss, M.J., Orkin, S.H., 1995. GATA transcription factors: key regulators of hematopoiesis. Exp. Hematol. 23, 99–107.
Wreford, N.G., 1995. Theory and practice of stereological techniques applied to the estimation of cell number and nuclear volume in the testis. Microsc. Res. Tech. 32, 423–436. https://doi.org/10.1002/jemt.1070320505.
Yan, W., 2009. Male infertility caused by spermiogenic defects: lessons from gene knockouts. Mol. Cell. Endocrinol. 306, 24–32. https://doi.org/10.1016/j.mce.2009.03.003.
Yokoyama, C., Chigi, Y., Baba, T., Ohshitanai, A., Harada, Y., Takahashi, F., Morohashi, K., 2019. Three populations of adult Leydig cells in mouse testes revealed by a novel mouse HSD3B1-specific rat monoclonal antibody. Biochem. Biophys. Res. Commun. 511, 916–920. https://doi.org/10.1016/j.bbrc.2019.02.100.

Yomogida, K., Ohtani, H., Harigae, H., Ito, E., Nishimune, Y., Engel, J.D., Yamamoto, M., 1994. Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. Development 120, 1759–1766. https://doi.org/10.1242/dev.01198.

Yu, C., Cantor, A.B., Yang, H., Browne, C., Wells, R.A., Fujiwara, Y., Orkin, S.H., 2002. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J. Exp. Med. 195, 1387–1395. https://doi.org/10.1084/jem.20020656.

Zhang, Z., Wu, A.Z., Feng, Z., Mruk, D., Cheng, C.Y., Chen, C.C., 2002. Gonadotropins, via cAMP, negatively regulate GATA-1 gene expression in testicular cells. Endocrinology 143, 829–836. https://doi.org/10.1210/endo.143.3.8688.

Zhang, H., Chen, F., Dong, H., Xie, M., Zhang, H., Chen, Y., Liu, H., Bai, X., Li, X., Chen, Z., 2019. Loss of Fbxw7 in Sertoli cells impairs testis development and causes infertility in mice. Biol. Reprod. 102, 963–974. https://doi.org/10.1093/biolre/ioz230.

Zhu, X., Hu, M., Ji, H., Huang, T., Ge, R., Wang, Y., 2021. Exposure to di-n-octyl phthalate during puberty induces hypergonadotropic hypogonadism caused by Leydig cell hyperplasia but reduced steroidogenic function in male rats. Ecotoxicol. Environ. Saf. 208, 111432. https://doi.org/10.1016/j.ecoenv.2020.111432.