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Original Research Article

IGF1 regulation of *BOULE* and *CDC25A* transcripts via a testosterone-independent pathway in spermatogenesis of adult mice



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ABSTRACT

The Deleted in AZoospermia (DAZ) gene family plays an essential role in spermatogenesis and fertility in mammals. This gene family contains two autosomal genes, *BOULE* and *DAZL* (DAZ-Like), and the DAZ gene cluster in the Y chromosome. *CDC25A* (a cell cycle regulator) has been proposed as a putative substrate for the RNA-binding proteins of DAZ family. However, mechanisms regulating DAZ gene expression have been poorly investigated. We analyzed immunohistochemical localization of *DAZL*, *BOULE* and *CDC25A*, as well as the involvement of testosterone (T) and insulin-like growth factor 1 (IGF1) in the modulation of mRNA expression for *DAZL*, *BOULE* and *CDC25A* in the adult mouse testes. It was found that *DAZL* was mostly immunolocalized in spermatogonia, while *BOULE* and *CDC25A* were detected in spermatocytes and round spermatids. Three-color immunofluorescence showed that *DAZL*-positive cells also expressed proliferating cell nuclear antigen (PCNA). *In vitro* incubation of the testes showed that neither T nor IGF1 affected *DAZL* mRNA expression. However, either T or IGF1 increased *BOULE* mRNA expression. Antiandrogen flutamide abolished the T-induced increase in *BOULE* mRNA, but had no effect on the IGF1 induced increase in the mouse testes. Extracellular-signal-regulated kinase 1/2 (ERK1/2) inhibitor, U0126, prevented IGF1-induction of *BOULE* mRNA. It was found that IGF1 increased *CDC25A* mRNA expression and that U0126 – but not flutamide – abolished the IGF1-induced *CDC25A* mRNA expression. These results showed that IGF1 regulated the expression of *BOULE* and *CDC25A* mRNAs via ERK1/2 signaling and in T-independent pathway during spermatogenesis in the adult mouse testes.

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1. Introduction

Spermatogenesis is a highly regulated process which requires normal function of many genes. Dysfunction of genetic factors leads to abnormal spermatogenesis and may cause male infertility [1–3]. In the last years, it has been shown that genes of the Deleted in AZoospermia (DAZ) family play an essential role during spermatogenesis and are required for preserving fertility in mammals [4–6]. An impaired expression of DAZ gene family members has been associated with a variety of testicular failures and disrupted spermatogenesis in humans [2,3,7]. This gene family contains two autosomal genes, *BOULE* and *DAZL* (DAZ-Like), and the DAZ gene cluster in the Y chromosome [2,8–10]. *BOULE* has been identified as the ancestor of the DAZ family, and its orthologue genes have been found in *Caenorhabditis elegans*, *Drosophila*, mice and humans [11,12]. *DAZL* orthologues are found only in vertebrates, whereas DAZ gene arises from an ancestral *DAZL* gene and is present only in the Old World monkeys and great apes [13,14].

In all species analyzed to date, DAZ members are mostly expressed in the male germ line and they are essential for development, maturation and differentiation of germ cells [2,9,10,15]. The generation and characterization of knockout mice in the last years highlighted the importance of *DAZL* and *BOULE* genes in spermatogenesis. In mice with a homozygous disruption of *DAZL* gene, gametogenesis results in a low number and arrested germ cells unable to progress beyond the leptotene stage of meiotic prophase I [4]. On the other hand, *BOULE* null mice show a complete spermatogenic arrest at the round spermatid stage, prior to elongation [5].

The proteins encoded by DAZ gene family contain a RNA-binding domain at their amino terminus, and have a functional role in mRNA stability and the translational regulation of their target RNA [8,16–18]. The mechanisms regulating the expression of DAZ gene family during spermatogenesis in mammals have been poorly investigated. Members of the CDC25 cell cycle regulators have been recognized as potential substrate candidates for DAZ family. Among these regulators, CDC25A, which is abundantly expressed in the testes and functions in the G1/S transition and M-phase exit, might play a role in the mitotic or meiotic regulation of spermatogenesis [18–21].

The proliferation and differentiation of male germ cells are finely regulated by androgen signaling via androgen receptors (ARs) localized in the somatic cells of the testis (Leydig, Sertoli and peritubular cells) [22,23]. In addition, growth factors and cytokines secreted by somatic cells and germ cells contribute to create an appropriate environment for the renewal and differentiation of spermatogonia [24,25]. Among these factors, only insulin-like growth factor 1 (IGF1), transforming growth factor (TGF) and activin are able to interact with cellular components of the seminiferous tubules [26]. In particular, IGF1 has been found to modulate many testicular functions. Leydig, Sertoli, peritubular and germ cells are capable to produce IGF1, and its specific receptor (IGF1R) was found both in germ cells and somatic testicular cells [25–27]. Several studies have shown that IGF1 regulates germ cell proliferation and survival, testosterone (T) production by the Leydig cells,

and stimulates the activity of the Sertoli cells [25,28,29]. The physiological effects of IGF1 are mediated via the activation of its specific receptors; mainly, the transmembrane tyrosine kinase receptor IGF1R that stimulates several downstream signaling mechanisms, including the mitogen-activated protein kinase (MAPK) pathway [30]. In the present study, we investigated the cellular distribution of *DAZL* and *BOULE* proteins as well as the cell cycle regulator CDC25A in the testes of adult mice. Moreover, we addressed the involvement of T and IGF1 in the modulation of mRNA expression of *DAZL*, *BOULE* and CDC25A.

2. Materials and methods

2.1. Animals

Ten-week old Balb/C male mice ($n = 50$) were obtained from the breeding facilities at the Department of Radiobiology from CNEA (National Nuclear Energy Commission, Argentina) and housed in our experimental animal room at Universidad Maimónides, Buenos Aires, Argentina. They were kept under controlled temperature ($23 \pm 2^\circ\text{C}$) and light conditions (12 h dark/light cycle); food and water were supplied *ad libitum*. All experimental protocols were reviewed and authorized by the Institutional Committee on the Use and Care of Experimental Animals. Handling, and euthanasia of animals were performed in accordance with the Canadian Council on Animal Care (CCAC) Guide for the Care and Use of Laboratory Animals [31]. Male mice were euthanized by asphyxiation with CO_2 . Testes were removed and immediately placed in culture or fixed in 4% paraformaldehyde (PFA) and embedded in paraffin blocks for immunohistochemistry or kept at -80°C for molecular analyses.

2.2. Immunohistochemistry

Paraffin-embedded samples from 10-week old Balb/C male mice ($n = 5$) were deparaffinized, serially sectioned at $5\ \mu\text{m}$ and processed for immunohistochemistry [32]. The samples were incubated for 24 h at 4°C with primary antibodies i.e., rabbit anti-*DAZL* (1:100; Abcam, Cambridge, UK), anti-*BOULE* (1:50, Santa Cruz Biotechnology Inc., Dallas, TX, USA) or anti-CDC25A (1:100; Santa Cruz Biotechnology Inc) followed by incubation with appropriate biotinylated secondary antibodies (Vector Laboratories, Peterborough, UK) and developed for peroxidase activity with 0.05% w/v 3,3'-diaminobenzidine and 0.1% v/v H_2O_2 in Tris-HCl. Negative controls were processed simultaneously by omitting the primary antibody or pre-absorbing the primary antibody with specific synthetic peptides.

2.3. Three-color immunofluorescence

Testicular fresh samples from 10-week old Balb/C male mice ($n = 5$) were used for immunofluorescence studies. Mounted paraffin testis sections ($5\ \mu\text{m}$) were processed using standard techniques [32]. Sections were blocked for 1 h with 1% normal donkey serum in phosphate buffered saline (PBS), washed with PBS and then incubated overnight at 4°C with rabbit anti-*DAZL*

Table 1 – Oligonucleotide primers used for real time PCR.

Target (accession number)	Sequence of primer (5'–3')	Amplified product (bp)
DAZL (NM_010021)	F: AATGTTTCAGTTCATGATGCTGCT R: TGTATGCTTCGGTCCACAGACT	75
BOULE (NM_029267)	F: TATAAGGATAAGAACTCAACATTGGT R: GAAGTTACTTCTGGAGTATGAAAATA	169
CDC25A (NM_007658)	F: AGAACCTATTGTGCCTACTG R: TACTCATTGCCGAGCCTATC	123
GAPDH (NM_008084)	F: CCAGAACATCATCCCTGCAT R: GTTCAGCTCTGGGATGACCTT	67

primary antibody (1:100; Abcam). After five rinses in PBS, the sections were incubated for 1 h at room temperature with donkey anti-rabbit IgG (1:700) coupled with Alexa-Fluor 555 (Invitrogen, Carlsbad, CA, USA). After further washing in PBS, the sections were blocked for 1 h with 1.5% normal goat serum and incubated overnight at 4 °C with rabbit anti-proliferating cell nuclear antigen (PCNA) primary antibody (1:200; Abcam). After five rinses in PBS, the sections were incubated in darkness for 1 h at room temperature with fluorescein-tagged goat anti-rabbit IgG (1:300; Vector Laboratories). After further

washing in PBS, tissue sections were incubated overnight at 4 °C with rabbit anti-BOULE antibody (1:50; Santa Cruz Biotechnology), washed in PBS and incubated for 1 h with goat anti-rabbit IgG (H + L) Alexa fluor 350 (Invitrogen). Slides were mounted with DAKO fluorescence mounting medium (Dako, Carpinteria, CA, USA) and analyzed by using a Nikon C1 D-Eclipse Confocal microscope coupled to Ti Eclipse Fluorescence system. Negative controls were processed simultaneously by omitting the primary antibodies or pre-absorbing the primary antibody with synthetic peptides.

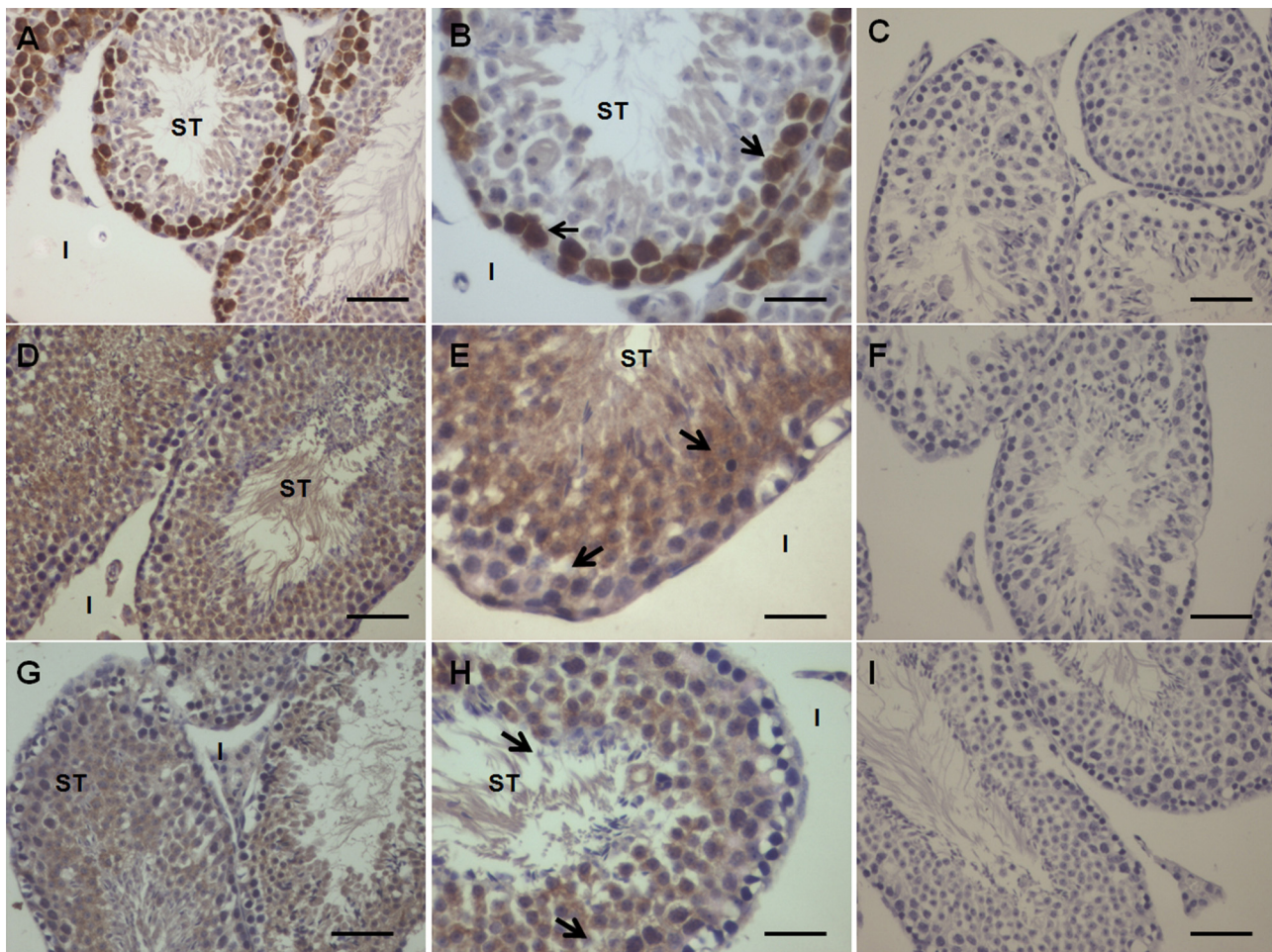


Fig. 1 – Immunolocalization of DAZL, BOULE and CDC25A in the adult mouse testes. (A and B) DAZL was mostly detected in spermatogonia (arrows) and some primary spermatocytes; (D and E) BOULE was localized in some spermatogonia, primary spermatocyte and round spermatids; (G and H) CDC25A was occasionally found in spermatogonia and was co-localized with Boule in germ cells from primary spermatocytes to round spermatids. Negative controls are shown for DAZL (C), BOULE (F) and CDC25A (I). Arrows indicate positively stained cells. I: interstitium; ST: seminiferous tubules; scale bar: 50 μ m.

2.4. In vitro incubation of testis

The right and left testes of male mice of 10 weeks of age ($n = 40$) were removed, carefully decapsulated and divided into two equal parts using needle tips and immersed in Medium 199 (Invitrogen) containing 0.1% bovine serum albumin (Sigma, St Louis, MO, USA). A total of five testes were used for each experimental treatment. To evaluate *DAZL*, *BOULE* and *CDC25A* gene expression, testes were incubated in 1 mL Medium 199 for 1, 3 or 6 h at 37 °C under a humid atmosphere of 5% CO₂/95% air (v/v) with T (1 μ M; Sigma) or IGF1 (100 ng/mL; Sigma). To analyze *BOULE* and *CDC25A* gene regulation by T or IGF1, the testes were pre-incubated overnight either in the presence or absence of flutamide (100 nM, Sigma), a nonsteroidal antiandrogen. The incubation with IGF1 and flutamide was performed to determine whether *BOULE* and *CDC25A* mRNA regulation was affected

by IGF1-induced T production. Testes were also incubated (1, 3 or 6 h) with IGF1 (100 ng/mL) and/or U0126 (10 μ M) (Sigma), the inhibitor of active and inactive forms of extracellular-signal-regulated kinase 1/2 (ERK1/2) [33]. After treatment, tissue samples were kept at –80 °C until RNA extraction, and incubation media were frozen at –20 °C until assayed for T quantification. Concentration of T was determined by RIA using the DAsource TESTO-RIA-CT kit (DAsource Immunoassays, Nivelles, Belgium). Testosterone concentration was expressed in ng/mL of incubation medium.

2.5. RNA isolation and real time-PCR

Total RNA from cultured testicular tissues was extracted with TRIzol (Invitrogen) and quantified using a spectrophotometer (Genequant pro, Amersham Biosciences, Little Chalfont, UK). Total RNA (3 μ g) was treated with DNase I (Invitrogen) and used

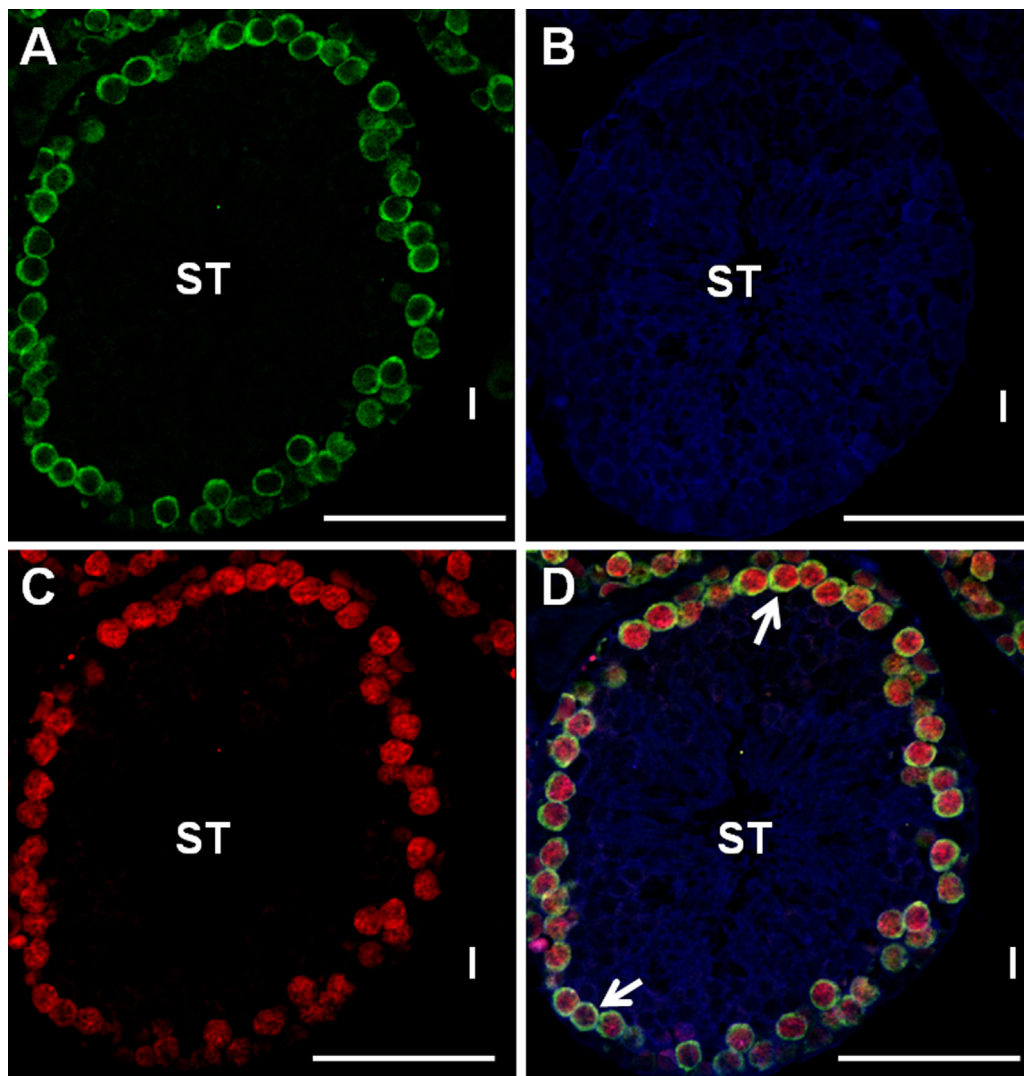


Fig. 2 – Three-color immunofluorescence of *DAZL*, *BOULE* and proliferating cell nuclear antigen (PCNA) in the adult mouse testes. (A) *DAZL* (cytoplasmic green staining); (B) *BOULE* (cytoplasmic blue staining); (C) PCNA (nuclear red staining); (D) merged immunofluorescence images of *DAZL*, *BOULE* and PCNA in adult male germ cells; note that the majority of *DAZL* positively stained spermatogonia were actively dividing (PCNA positive; upper arrow); some *DAZL*-stained spermatogonia also showed a faint signal for *BOULE* (lower arrow). I: interstitium; ST: seminiferous tubules; scale bar: 50 μ m.

for reverse transcription in a 20 μ L final volume containing M-MLV reverse transcriptase (200 U/ μ L, Promega, Madison, WI, USA) and random hexamers primers (Biodynamics, Buenos Aires, Argentina). Reverse transcribed cDNA was employed for quantitative Real-time polymerase chain reaction (RT-PCR) using SYBR Green PCR Master Mix and specific forward and reverse primers (Table 1) in a Stratagene MPX500 cycler (Stratagene, La Jolla, CA, USA). Primers were used at a concentration of 0.3 μ M in each reaction. The PCR conditions were as follows: step 1, 10 min at 95 °C; step 2, 30 s at 95 °C; step 3, 30 s at 60 °C, repeating from step 2 to step 3, 40 times. Melting curves were run to confirm the specificity of the product. Data from the RT-PCR were collected and analyzed by the complementary computer software (MxPro3005P v4.10 Build 389, Schema 85). Data are expressed as the ratio of target gene expression to reference gene (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) expression and presented as arbitrary units [34]. The amplified cDNA fragments were size-fractionated in 2% agarose gel and visualized by staining with Gel Red Nucleic Acid Stain (Biotium, Hayward, CA, USA).

2.6. Statistical analysis

The data were presented as the mean and standard error of mean (mean \pm SEM) and analyzed with the GraphPad Prism Software (version 5.0 for Windows, GraphPad Software, San Diego, CA, USA). One-way analysis of variance followed by the

Newman-Keuls *post hoc* test was used to analyze the obtained data. P-value of less than 0.05 was considered significant.

3. Results

3.1. Immunolocalization of DAZL, BOULE and CDC25A

DAZL protein was detectable in the cytoplasm of spermatogonia and some spermatocytes in adult mice with higher intensity in the cytoplasm of spermatogonia (Fig. 1A and B). BOULE protein was strongly expressed in the cytoplasm of spermatocytes and round spermatids, it was also detectable in some elongated spermatids, spermatozoa and a few spermatogonia (Fig. 1D and E). Immunostaining for CDC25A was strong in the cytoplasm of spermatocytes and persisted in spermatids (Fig. 1G and H). Neither interstitial cells nor Sertoli cells were found to be immunostained for DAZL, BOULE or CDC25A.

Three-color immunofluorescence for DAZL, BOULE and PCNA showed clearly that the DAZL positive germ cells located nearest the basal lamina (Fig. 2A) expressed also PCNA (Fig. 2C and D). The expression of BOULE was mainly found in the cytoplasm of spermatocytes and spermatids that were negative for PCNA (Fig. 2B and D). The cytoplasm of few DAZL/PCNA positive germ cells was also found to express BOULE protein (Fig. 2D).

3.2. Effects of T and IGF1 on DAZL, BOULE and CDC25A gene expression

In vitro incubation of the testes was used to identify DAZL, BOULE and CDC25A mRNA expression in the presence of T or IGF1. Neither T (Fig. 3A) nor IGF1 (Fig. 3B) affected DAZL mRNA expression. However, T induced a significant increase ($p < 0.05$) in BOULE mRNA expression after 3 h of incubation (Fig. 4A). This stimulatory effect was abolished by flutamide (Fig. 4B). IGF1 also induced a significant increase ($p < 0.05$) in BOULE mRNA expression after 1 and 3 h of incubation (Fig. 4C). Flutamide did not block the increase in BOULE mRNA expression induced by IGF1 after 3 h of incubation (Fig. 4D). On the contrary, U0126 (ERK1/2 inhibitor) abolished the BOULE mRNA increase caused by IGF1 after 3 h of incubation (Fig. 4D). The same results were found after 1 h of incubation (data not shown). In addition, it was found that IGF1 significantly increased ($p < 0.05$) basal medium concentration of T (1.30 \pm 0.20 ng/mL) after 1 h (2.40 \pm 0.17 ng/mL), 3 h (4.80 \pm 0.28 ng/mL) and 6 h (5.70 \pm 0.31 ng/mL) of incubation.

Testosterone did not affect the expression of CDC25A mRNA (Fig. 5A), whereas IGF1 stimulated ($p < 0.05$) CDC25A gene expression after 6 h of incubation (Fig. 5B). Flutamide did not block the increase in CDC25A mRNA expression induced by IGF1 after 6 h of incubation (Fig. 5C). On the contrary, U0126 (ERK1/2 inhibitor) abolished the CDC25A mRNA increase caused by IGF1 after 6 h of incubation (Fig. 5C).

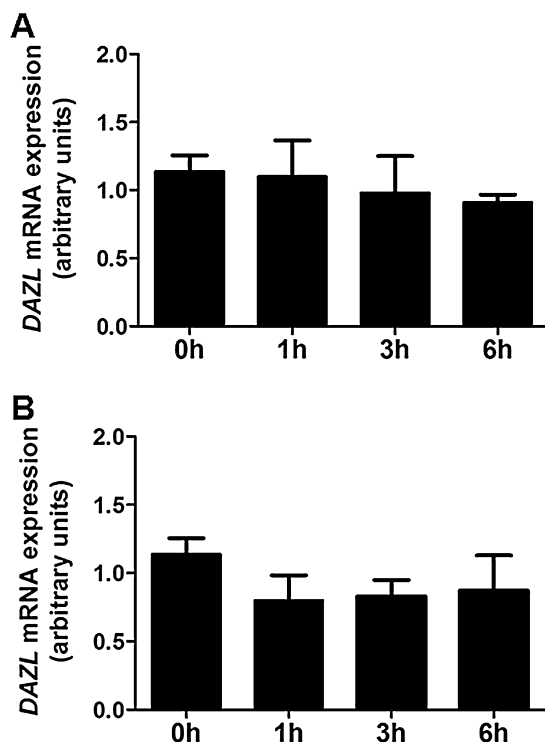


Fig. 3 – Effects of testosterone (T) and insulin-like growth factor 1 (IGF1) on relative expression of DAZL mRNA (mean \pm SEM) in the adult mouse testes. Testes were incubated with or without T (1 μ M) (A) or IGF1 (100 ng/mL) (B) for 1, 3 and 6 h ($n = 5$ independent experiments). The results are expressed in relation to the housekeeping gene GAPDH. No significant differences were found.

4. Discussion

In the present study, we demonstrated that IGF1 increased the expression of BOULE and CDC25A during spermatogenesis in

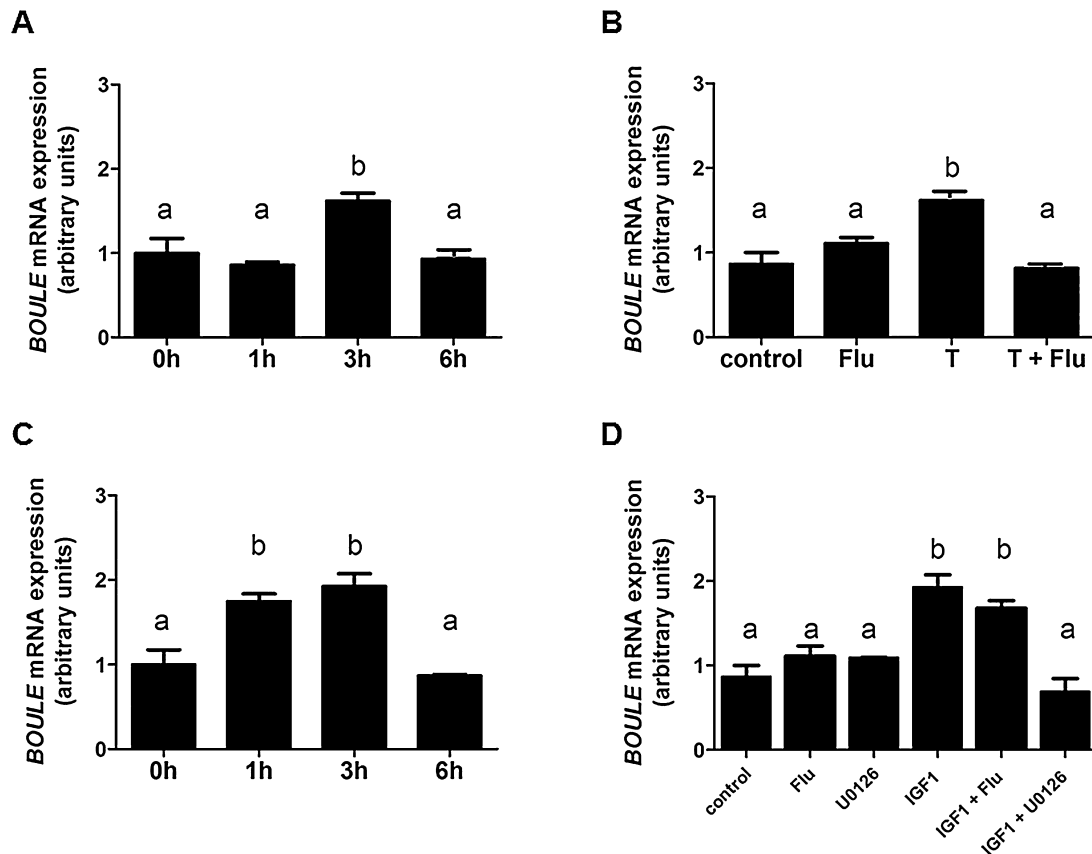


Fig. 4 – Effects of testosterone (T) and insulin-like growth factor 1 (IGF1) on relative expression of BOULE mRNA (mean \pm SEM) in the adult mouse testes. (A) The effect of T (1 μ M) after 1, 3 and 6 h of incubation; (B) effects of T (1 μ M) and flutamide (Flu; 100 nM) after 3 h of incubation; (C) the effect of IGF1 (100 ng/mL) after 1, 3 and 6 h of incubation; and (D) effects of IGF1 (100 ng/mL), flutamide (100 nM) and/or U0126 (10 μ M) after 3 h of incubation. The results are expressed in relation to the housekeeping gene GAPDH; $n = 5$ independent experiments. Different letters indicate significant differences ($p < 0.05$).

adult mice. This regulation was mediated via the activation of ERK1/2 signaling and did not depend on testosterone. In addition, we analyzed immunolocalization of proteins encoded by DAZ gene family members, DAZL and BOULE, as well as CDC25A, a cell cycle regulator recognized as potential substrate candidate for DAZ family proteins. We found that DAZL expression occurred mainly in the cytoplasm of actively dividing spermatogonia. BOULE was mainly expressed in spermatids and CDC25A expression was detected in spermatocytes and persisted in spermatids.

In nearly all animal species, the DAZ gene family has a unique pattern of expression restricted to germ cells [6]. In mice and humans, DAZL is initially expressed in embryonic stem cells and primordial germ cells [35,36]. DAZL protein was detected in the cytoplasm of spermatogonia and spermatocytes in humans, rats and mice [4,37–40]. Similarly, in the current study, DAZL expression was demonstrated in the cytoplasm of spermatogonia and, occasionally, in spermatocytes. In contrast to report of Reijo et al. [40], we did not observe nuclear localization of DAZL. This difference could result from using different antibodies.

In most animals, BOULE is expressed in the testes at later stages of male germ cell development than DAZL [6,9]. In humans and mice, BOULE protein may be found in the

cytoplasm of pachytene spermatocytes and round spermatids [6,12]. Similarly, in the present study, BOULE was demonstrated in spermatocytes and spermatids of the adult mouse testes.

Germ cells containing DAZL were also found to express PCNA, indicating that they were mitotically active. A few of these germ cells were also positive for BOULE; thus, it is likely that the germ cells co-expressing DAZL and BOULE are close to enter meiotic division. In mice with homozygous disruption of DAZL gene, it was shown that germ cells still express PCNA suggesting that DAZL is not essential for mitotic activity of spermatogonia after birth [4]. On the other hand, BOULE knockout mice were able to complete meiosis, but spermatids did not progress beyond step six of spermiogenesis [5], revealing that BOULE is needed for modulation of spermiogenesis.

CDC25A is a phosphatase necessary for the G1/S transition in the cell cycle involved in meiosis of male germ cells [20,41,42]. BOULE and DAZL proteins are known to interact with CDC25A [5,12]. The immunolocalization of CDC25A was similar to that of BOULE. CDC25A was expressed in spermatocytes and spermatids in mature mice. In adult rat testes, CDC25A was reported in spermatogonia, spermatocytes and spermatids [41]. This immunolocalization pattern of CDC25A suggests a role for this enzyme in the completion of meiosis during spermatogenesis in rodents.

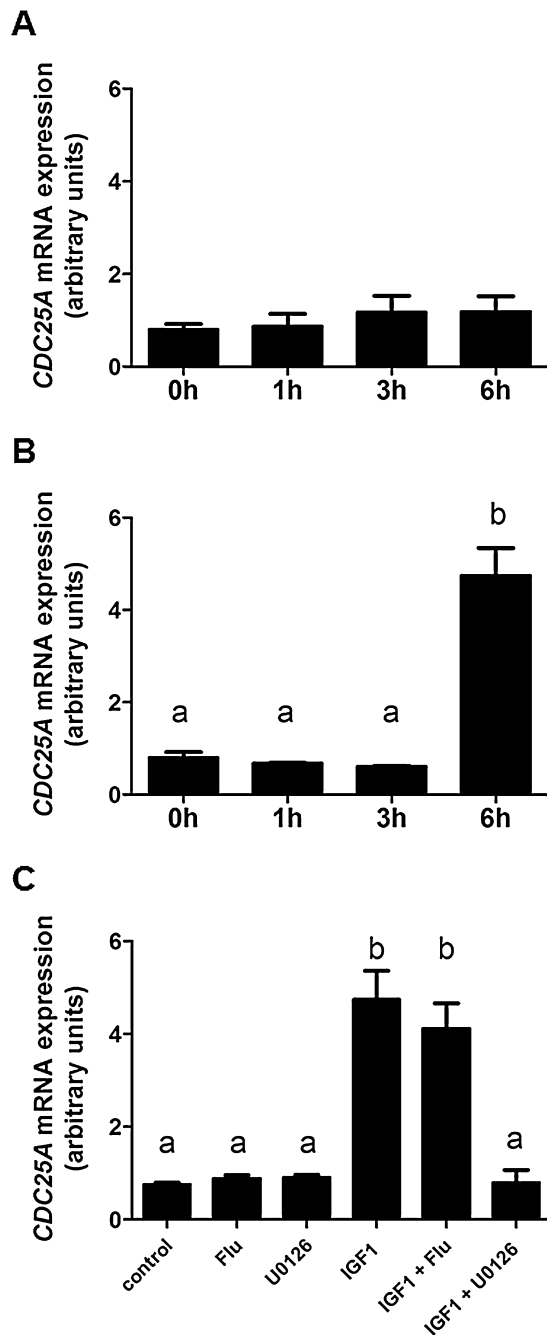


Fig. 5 – Effects of testosterone (T) and insulin-like growth factor 1 (IGF1) on CDC25A mRNA expression (mean \pm SEM) in the adult mouse testes. (A) The effect of T (1 μ M) after 1, 3 and 6 h of incubation; (B) the effect of IGF1 (100 ng/mL) after 1, 3 and 6 h of incubation; and (C) effects of IGF1 (100 ng/mL), flutamide (Flu; 100 nM) and/or U0126 (10 μ M) after 6 h of incubation. The results are expressed in relation to the housekeeping gene GAPDH; $n = 5$ independent experiments. Different letters indicate significant differences ($p < 0.05$).

The testicular mechanisms regulating DAZ family expression have been poorly investigated. Although testosterone is the main hormone regulating germ cell proliferation and differentiation, locally produced factors can also have a

paracrine role in the modulation of spermatogenesis [23–25]. In the present study, we described the involvement of T in the regulation of *BOULE* mRNA expression and a novel mechanism involving IGF1 in the regulation of the *BOULE* and *CDC25A* mRNA expression in the mouse testes.

Testosterone increased *BOULE* mRNA expression after 1 h of incubation but failed to affect the expression of *CDC25A* mRNA. The effect might be mediated via AR present in Leydig or Sertoli cells. This notion was confirmed by blocking the T-induced increase in *BOULE* mRNA with flutamide (androgen antagonist).

IGF1 increased the testicular expression of *BOULE* mRNA after 1/3 h and *CDC25A* mRNA after 6 h of incubation. Since IGF1 is able to induce T production by Leydig cells, we examined the possibility that T production mediated the effect of IGF1 on *BOULE* mRNA expression. *In vitro* incubation of adult testes with IGF1 in the presence of flutamide did not affect *BOULE* expression, indicating that IGF1-induced increase in *BOULE* was T-independent. In addition, we showed that ERK1/2 was involved in the induction of *BOULE* and *CDC25A* mRNA expression by IGF1. It was reported that IGF1 regulates germ cell proliferation and stimulates the activity of Sertoli cells [25,28]. Our results suggest that the effects of IGF1 and T on proliferation and differentiation of germ cells may partially depend on a mechanism involving up-regulation of *BOULE* and *CDC25A* gene expression.

In the present study, expression of *DAZL* mRNA was not modified by T or IGF1. This is in agreement with previous findings [39] that GnRH antagonist did not modify the staining pattern of *DAZL* protein in the rat testes. Therefore, mechanisms regulating *DAZL* gene expression remain unknown.

In conclusion, we demonstrated that in the testes of adult mice, testosterone increased *BOULE* gene expression and IGF1 increased *BOULE* and *CDC25A* gene expression via ERK1/2 signaling in T-independent manner. The mechanisms of these effects remain to be elucidated. The knowledge on the mechanism of action of the DAZ family members is of great importance for male reproductive health.

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