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# The Effect of D-Aspartate on Spermatogenesis in Mouse Testis<sup>1</sup>

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## ABSTRACT

Spermatogenesis is controlled by hormonal secretions from the hypothalamus and pituitary gland, by factors produced locally in the testis, and by direct interaction between germ cells and Sertoli cells in seminiferous tubules. Although the mammalian testis contains high levels of D-aspartate (D-Asp), and D-Asp is known to stimulate the secretion of testosterone in cultured Leydig cells, its role in testis is unclear. We describe here biochemical, immunohistochemical, and flow cytometric studies designed to elucidate developmental changes in testicular D-Asp levels and the direct effect of D-Asp on germ cells. We found that the concentration of D-Asp in mouse testis increased with growth and that fluctuations in D-Asp levels were controlled in part by its degradative enzyme, D-aspartate oxidase expressed in Sertoli cells. In vitro sperm production studies showed that mitosis in premeiotic germ cells was strongly inhibited by the addition of D-Asp to the culture medium. Moreover, immunohistochemical analysis demonstrated that D-Asp accumulated in the differentiated spermatids, indicating either transport of D-Asp to spermatids or its de novo synthesis in these cells. Such compartmentation seems to prevent premeiotic germ cells in mouse testis from being exposed to the excess amount of D-Asp. In concert, our results indicate that in mouse testis, levels of D-Asp are regulated in a spatiotemporal manner and that D-Asp functions as a modulator of spermatogenesis.

*D-aspartate, Sertoli cell, spermatogenesis, testis*

## INTRODUCTION

Sperm play an extremely important role in the continuation of the species through gene transmission and are continuously produced in the mammalian testis during life. Spermatogenesis is controlled primarily by the secretion of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone by

the hypothalamus-pituitary-testis axis. During this process, germ cells adhere to support cells in the seminiferous tubules, Sertoli cells, differentiating from the basement membrane toward the center of the lumen. Although defects in spermatogenesis are recognized as a major clinical problem, with the exception of FSH and hCG, there are few effective drugs available for the treatment of these conditions.

Although proteins in vivo are derived exclusively from amino acid L-enantiomers, the presence of free D-form amino acids in mammals, predominantly D-serine and D-aspartate (D-Asp), has been demonstrated previously [1–3]. Although D-serine is known to play an important neurophysiological role in humans [4], the physiological roles of D-Asp are unclear. That said, D-Asp is present in the pineal body, pituitary gland, and testis [5], and has been shown to stimulate the secretion of testosterone by cultured rat Leydig cells [6–8]. Although the biosynthesis and transport of D-Asp are yet to be characterized, it is known to be degraded by D-aspartate oxidase (Ddo) to oxaloacetic acid, hydrogen peroxide, and ammonium ion [9].

The effect of D-Asp on cultured Leydig cells implied an effect on spermatogenesis in vivo. However systemically administered D-Asp fails to accumulate in rat testis [10, 11], and there have been no reports to date of a specific, direct effect of D-Asp on mammalian testis. The recent report of in vitro production of functional sperm in cultured neonatal mouse testis [12] represents a promising novel model for spermatogenesis. Here, we characterized the spatiotemporal determinants of D-Asp levels in mouse testis and evaluated the effect of D-Asp on spermatogenesis using the in vitro sperm production method.

## MATERIALS AND METHODS

### Animals

ICR mice were purchased from CLEA Japan, Inc. *Acr-green* fluorescent protein (GFP) mice [13] were provided by RIKEN BRC. For the culture experiments, 5.5 days postpartum pups were used, while for other experiments, the mice were used at an age appropriate to each experiment. Animal investigations had the prior approval of the Research Center for Animal Life Science of Shiga University of Medical Science.

### Western Blot Analysis

Tissues were homogenized in eight volumes RIPA buffer using a microtube pestle. Aliquots of homogenates (100 µg protein) were loaded and run on a SDS-PAGE using 4%–15% linear gradient gels (Bio-Rad), and the separated proteins were transferred onto a polyvinylidene fluoride membrane (Bio-Rad). The blot was probed with 1:250 diluted anti-DDO antibody (Human Protein Atlas) and 1:5000 diluted anti-β-actin monoclonal antibody (Sigma) at 4°C overnight. Horseradish peroxidase-conjugated secondary antibody, goat anti-rabbit immunoglobulin G (IgG) (Amersham), was diluted 1:5000 in 5% fat-free milk in PBS with 0.05% Tween-20 and incubated with the membrane at room temperature for 1 h. Immunoreactive bands were detected with an enhanced chemiluminescence detection system (Perkin Elmer Life Science).

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## Quantitative Reverse-Transcription PCR

Total RNA was extracted from mouse testis using an RNeasy Midi kit (Qiagen). First-strand cDNA was prepared from total RNA using Super Script III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems). Each reaction mixture (25  $\mu$ l) contained 12.5  $\mu$ l of Power SYBR Green PCR Master mix (Applied Biosystems), 0.4  $\mu$ M gene-specific primers, and 0.5  $\mu$ l of the cDNA template. The PCR protocol was as follows: 50°C for 2 min and 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 60°C for 1 min. The measurements were normalized to the expression levels of the glyceraldehyde 3-phosphate dehydrogenase homolog (Gapdh) or  $\beta$ -actin. The gene-specific primers used were as follows [14]: Mouse *Ddo* forward 5'-GGTTGGCAGATATTCCACAGC-3' and reverse 5'-TCTGCATCTGGGGATCGATTTC-3'; mouse GAPDH forward 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse 5'-GATTGCTGTTGAAGTCGAGGAG-3'; and mouse- $\beta$ -actin forward 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and reverse 5'-ATGGAGCCACCGATCCACA-3'.

## Assay of *Ddo* Activity

Mouse tissues were homogenized on ice with four volumes of 50 mM sodium pyrophosphate (pH 8.3) using a small glass homogenizer. A standard assay mixture (100  $\mu$ l) contained 50 mM sodium pyrophosphate (pH 8.3), 50 mM D-Asp, and 10  $\mu$ g of catalase (Roche Diagnostics). The reaction was initiated by the addition of a 5  $\mu$ l aliquot of each homogenate, run at 37°C for 120 min, and then stopped with the addition of 40  $\mu$ l of 12.5% trichloroacetic acid. After the addition of 10 nmol 2-oxoglutaric acid as an internal standard, the mixture was centrifuged at 5700  $\times g$  for 10 min at 4°C. The enzymatically formed oxaloacetate was then quantified as described previously [15]. Briefly, an aliquot of the supernatant (100  $\mu$ l) was treated with 3-methyl-2-benzothiazoline hydrazone hydrochloride (Sigma-Aldrich) and the resultant azine derivatives were analyzed by high-performance liquid chromatography (HPLC) on a Cosmosil 3C18 column (Nacalai Tesque, Inc.).

## Determination of Aspartate

Aspartate was measured as described previously [16] with slight modification. An aliquot of the tissue homogenate (10–50  $\mu$ l) was mixed with four or eight volumes of methanol, and 10  $\mu$ l of 1 mM L-homocysteic acid was added to the mixture. After 10 min of centrifugation at 12000  $\times g$  and 4°C, 100  $\mu$ l aliquot of the supernatant was transferred to a 1.5 ml Eppendorf tube and dried under reduced pressure at room temperature. The resultant residue was dissolved with 60  $\mu$ l of 10 mM sodium borate (pH 8.0), and an aliquot of the supernatant (10  $\mu$ l) was mixed with 0.5  $\mu$ l of 100% methanol containing 20 mM o-phthalaldehyde and 15 mM N-acetyl-L-cysteine, then incubated at room temperature for 2 min and the insoluble material removed by centrifugation. Then 10  $\mu$ l of the resultant amino acid derivatives were immediately separated on a Cosmosil 5C18-MS-II column (4.6  $\times$  150 mm) and quantified using the L-homocysteic acid peak as an internal standard. The solvent system was composed of 0.1 M sodium acetate containing 1% acetonitrile (pH 6.0) and 0.1 M sodium acetate containing 55% acetonitrile (pH 6.0). The amino acid derivatives were eluted by developing a 1% to 15% linear gradient of the 55% acetonitrile (pH 6.0) over 20 min. The flow rate was maintained at 1.0 ml/min. Aspartate of the feed and water that our mice take was also measured using the same methods.

## Flow Cytometric Analysis

The testes of *Acr-GFP* mice were treated with 2 mg/ml collagenase for 15 min, followed by 0.25% (wt/vol) trypsin plus 1 mM ethylenediaminetetraacetic acid digestion for 10 min at 37°C to dissociate cells. After passing through a cell strainer with a 40  $\mu$ m pore size (Becton Dickinson), the cells were suspended in PBS and subjected to flow cytometry to analyze GFP-expressing cells using a FACS Aria II (Becton Dickinson). The flow cytometric analysis was performed with CellQuest and FlowJo software.

## Culture Method

Testis tissues of *Acr-GFP* mice were cultured as described previously [12]. Briefly, pup testes were decapsulated and gently separated by forceps into two pieces. Then 1.5% (w/v) agarose gels (10  $\times$  10  $\times$  5 mm) were placed in each well of a 12-well plate (Sumitomo Bakelite) and presoaked overnight in the medium, after which each gel was loaded with one testis tissue fragment. The control culture medium (CCM) was composed of  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) containing 10% KSR (Life Technologies). Five media were

compared: CCM only, CCM supplemented with 1, 5, or 10 mM D-Asp, and CCM supplemented with 10 mM L-Asp. Medium change was performed once a week. The culture incubator was supplied with 5% carbon dioxide in air and maintained at 34°C. Cultured tissues were processed for histological and immunohistological examination.

## Observations of GFP Expressions

The cultured tissues were observed 4 wk after under a stereoscope equipped with an excitation light for GFP (Leica MZ FL3) to score the level of GFP expression of the tissues. To score, we used the previously reported scale for the grading of GFP expression [12].

## Immunohistochemistry

For histological examination, the specimens were fixed with Bouin fixative and embedded in paraffin. Sections were made for each specimen and stained with hematoxylin and eosin stain, with the exception of D-Asp staining, for which we performed perfusion fixation as described previously [17]. For immunohistochemistry, paraffin-embedded tissues were cut into 3- $\mu$ m thick sections and treated as described in our previous report [15]. Primary antibodies were rabbit anti-DDO antibody (1:150; Human Protein Atlas), rabbit anti-phospho Histone H3 antibody (1:500; Millipore), and rabbit anti-SYCP1 antibody (1:300; Abcam). The sections were incubated with a horseradish peroxidase-labeled goat antibody against rabbit IgG (Histofine Simple Stain MAX PO; Nichirei Bioscience). Counterstain was performed with hematoxylin as needed. For immunofluorescence staining, paraffin-embedded tissues were also cut into 3- $\mu$ m thick sections. For D-Asp staining, deparaffinized sections were incubated with 0.5% NaBH<sub>4</sub> in PBS for 20 min at room temperature. Primary antibodies were rabbit anti-conjugated D-Asp antibody (1:500; MoBiTec), rabbit anti-DDO antibody (1:150; Human Protein Atlas), rabbit anti-phospho Histone H3 antibody (1:500; Millipore), and rat anti-Tra98 antibody (1:200; Cosmo Bio). Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400), Alexa Fluor 555-conjugated goat anti-rabbit (1:400), and Alexa Fluor 594-conjugated goat anti-rat (1:400; Life technologies) were used as secondary antibodies for anti-DDO, anti-D-Asp, and anti-Tra98 antibodies, respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Dojindo). Specimens were observed with a light microscope (Nikon Eclipse 80i) or laser microscope (Leica TCS SP8).

## Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare differences between groups. Values with  $P < 0.01$  were considered significant.

## RESULTS

### Determination of D-Asp in the Mouse Testis

In contrast to the consistently elevated (0.27–0.48 nmol/gram wet weight) testicular levels of L-Asp, HPLC analyses demonstrated that testicular level of D-Asp were initially low and rose to a plateau around 0.20 nmol/gram wet weight at 10 wk of age ( $n = 4$ ) (Fig. 1). We observed an obvious increase of D-Asp level in mouse testis during similar period as spermatogenesis progressed. D-Aspartate was undetectable in the liver and kidney, although L-Asp was detected in those organs at a level similar to those in testis (data not shown). Accordingly, in contrast to its consistently low level in the liver and kidney, the testicular D/L-Asp ratio increased with age, reaching approximately 60% at 8 wk of age. HPLC analyses demonstrated that D-Asp was not included in the feed or water of mice in our animal center (data not shown).

### Localization of D-Asp in the Mouse Testis

We next used an immunohistochemical approach to study the distribution of D-Asp in mouse testis. Based upon a previous study [17] demonstrating the localization of D-Asp in the cytoplasm of elongate spermatids, we used 10-wk-old *Acr-GFP* Tg mice, which express GFP predominantly in the acrosome. D-Aspartate was found to be localized in close proximity to the GFP-expressing acrosomes (Fig. 2A).

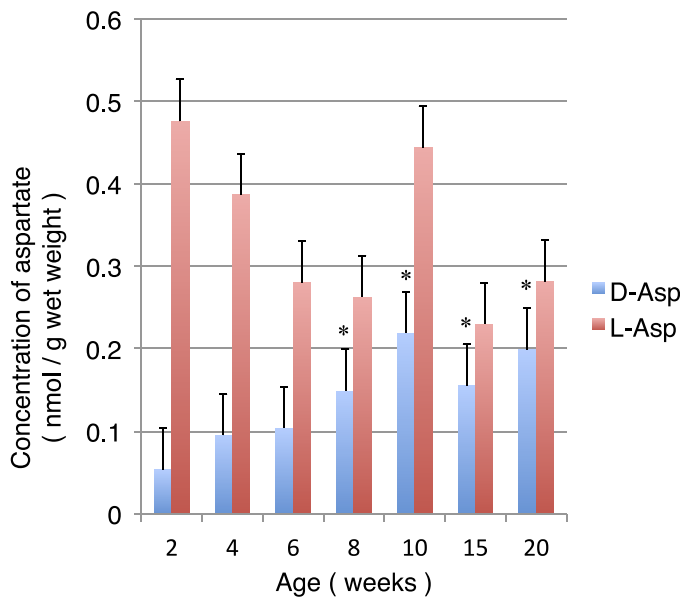


FIG. 1. Changes in aspartate levels in the testis of mice during development (means  $\pm$  SD,  $n = 4$ ). \* $P < 0.01$  compared with respective values of D-Asp at 2 wk of age.

Moreover, when *Acr*-GFP Tg mice testes were enzymatically dissociated into GFP-positive -negative cells by cell sorting, levels of D-Asp were found to be higher in the GFP-positive fraction (Fig. 2B). DAPI analysis showed that spermatid D-Asp was localized adjacent to, but not entirely overlapping with, the nucleus (Fig. 2C). In contrast, immunohistochemical (Fig. 2D) and HPLC (Supplemental Fig. S1; Supplemental Data are available online at [www.biolreprod.org](http://www.biolreprod.org)) approaches failed to locate D-Asp in the epididymis, suggesting that although D-Asp is present in spermatids initially, its levels fall during their maturation and migration to the epididymis.

#### Developmental Changes of Activity and Localization of Ddo in Mouse Testis

Consistent with developmental fluctuations in D-Asp levels, we found that Ddo activity (HPLC, Fig. 3A), protein levels (Western blots, Fig. 3B, and immunohistochemistry, Fig. 3D), and gene expression (real-time PCR, Fig. 3C) were highest just after birth and decreased with age, and were barely detectable or undetectable at 10 wk of age. In contrast, Ddo activity of liver and kidney increased with age to a level similar to that in testis just after birth (Supplemental Fig. S2). Immunohistochemical analysis using antibodies against Ddo and a Tra98 antigen expressed exclusively in the germ cell indicated that Ddo was expressed in mouse Sertoli cells at 2 wk of age (Fig. 3E).

#### Effect of D-Asp on In Vitro Spermatogenesis

Next, we established the previously described in vitro sperm production model [12] and verified that GFP, which was an index of sperm differentiation, was expressed in testis tissues after 4 wk culture under established conditions. Hematoxylin-eosin staining demonstrated tubular growth in the peripheral area and the formation of sperm in cultured testis tissue at a level similar to that previously reported [12] (Supplemental Fig. S3). We proceeded to examine the dose-dependent effect of aspartate in this culture system using medium supplemented with D- or L-Asp. Flow cytometry analysis showed that

compared with controls, testicular GFP expression was significantly lower in testis treated with D-Asp, but not L-Asp (Fig. 4, A and B). Next, we used an anti-phospho Histone H3 antibody to evaluate the effect of aspartate on the mitotic activity of testis tissue cultured for 14 days and found that mitosis was inhibited in the presence of 10 mM D-Asp. Similarly, immunohistochemical analysis using an antibody against SYCP-1 [18], a meiotic marker protein, found that culturing with 10 mM D-Asp for 21 days reduced the number of meiotic cells in the testis. Moreover, hematoxylin-eosin staining analysis showed that compared with controls, culturing with 10 mM D-Asp reduced the number of germ cells in the testis tissue but had no significant effect on the structure of the seminiferous tubules (Fig. 4, C and D). In concert, our results indicate that, by a mechanism that is yet to be determined, exogenous D-Asp suppresses germ cell differentiation in mouse testis. This inhibitory effect was also observed when D-Asp was cultured together with 10 mM L-Asp (Supplemental Fig. S4).

## DISCUSSION

To date, only a small number of substances have well-defined effects on spermatogenesis. A study using short-term culture of Leydig cells showed that the presence of D-Asp in the culture medium stimulated synthesis of testosterone by promoting expression of the gene encoding *Star*, a factor related to the synthesis of testosterone [8, 19]. Based on this result, we speculated that D-Asp has a positive effect on spermatogenesis, although to date this has not been verified. To our knowledge, the present study, which is focused on germ cells using a method based on in vitro spermatogenesis [12], is the first report of a testis-specific effect of D-Asp on spermatogenesis. Although we initially hypothesized that D-Asp would upregulate spermatogenesis, our results in fact show the opposite. We speculate that levels of D-Asp are strictly controlled in the testis in vivo because addition of D-Asp to cultured medium has no positive impact on spermatogenesis. In addition to the results in a previous report ([5] and references cited therein) regarding D-Asp in mammals, the present study shows that adult mouse testis contains levels of D-Asp that are comparable to those seen in other organs such as kidney and liver. Substantial amounts of free D-Asp have been detected in various kinds of animals. For example, it has been reported that adult mouse testis has higher D-Asp levels than prepubertal testis [17]. Consistent with this, our longitudinal analysis of D-Asp content in mouse testis indicates a remarkable increase in testis D-Asp levels with age.

We find that D-Asp is localized in differentiated spermatids. These results indicate that when the number of spermatids reaches a steady state, the total amount of D-Asp in testis reaches a plateau. Consistent with this, the plateau in D-Asp levels coincides with the period when mice produce sufficient amounts of sperm. Regarding the localization of D-Asp in testis, immunohistochemical analysis using an anti-conjugated D-Asp antibody shows that D-Asp is present specifically in the cytoplasm of elongate spermatid [17]. Putting these findings together with our results, we speculate that D-Asp initially localizes in the cytoplasm of elongate spermatid and accumulates in the residual body of spermatid. In the liver and kidney, where D-Asp levels are low, we find that expression of Ddo is robust at birth and that the Ddo activities in these organs increase with their growth. Interestingly, in this study, fluctuations in Ddo activity and in the amount of D-Asp in the testis have an opposite result. Based upon these results, D-Asp does not undergo Ddo-

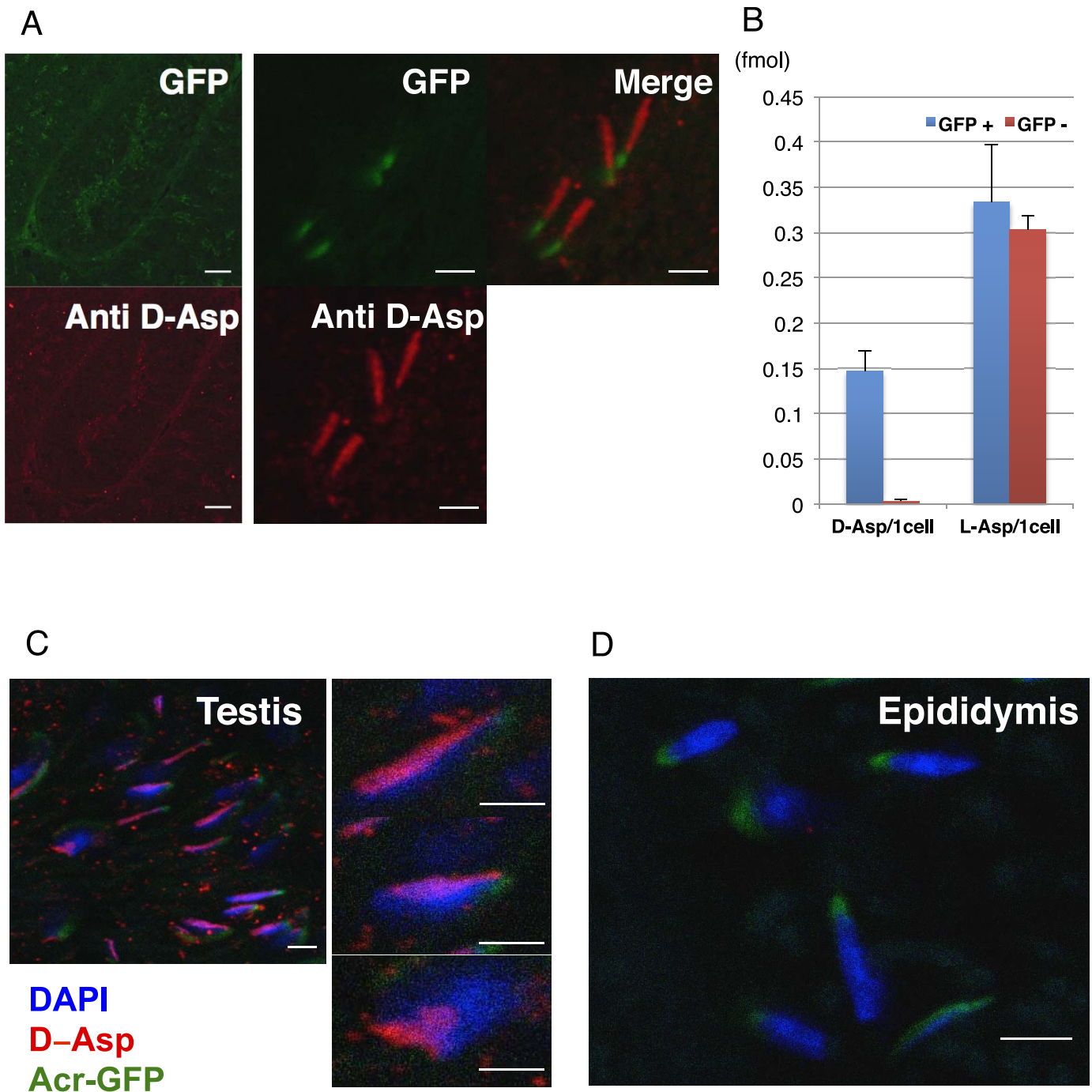


FIG. 2. D-Aspartate localization in mouse testis and epididymis. **A**) Immunohistochemistry of a testis from *Acr-GFP* mouse. GFP (green) and D-Asp (red) are shown. Bars = 10  $\mu$ m. **B**) D- and L-Aspartate levels in the GFP-positive and -negative cells (means  $\pm$  SD,  $n = 4$ ). **C**) Immunohistochemistry of a testis from *Acr-GFP* mouse. GFP (green), D-Asp (red), and DAPI (blue) are shown. Bar = 5  $\mu$ m. **D**) Immunohistochemistry of an epididymis from *Acr-GFP* mouse. Bar = 5  $\mu$ m.

catalyzed degradation in adult mouse testis due to low Ddo expression levels. We speculate that minute control of D-Asp level in the testis by the regulation of Ddo expression is critical to its physiological function. Although localization of Ddo and changes in its activity in the testis have not previously been reported, an inverse relationship between D-Asp levels and Ddo expression has been reported in many organs of adult mice [5, 20, 21]. Consistent with our observations, Hung et al. [22] have reported that, compared with wild-type mouse testis, D-Asp levels are elevated in *Ddo*<sup>-/-</sup> mouse. Future studies will clarify the significance of

D-Asp localization in the spermatids as well as the synthesis and transport mechanism of D-Asp.

Using an in vitro spermatogenesis assay system [12], we find that an exogenous D-Asp has a negative effect on cell division in premeiotic germ cells. Considering this effect on premeiotic germ cells, and the localization of D-Asp in the spermatids, we hypothesize that D-Asp is transported to the spermatids, or synthesized there de novo, without acting on the premeiotic germ cells. If, as previous reports suggest [6–8], D-Asp directly acts on Leydig cells, then D-Asp incorporated into Sertoli cells may be released to the

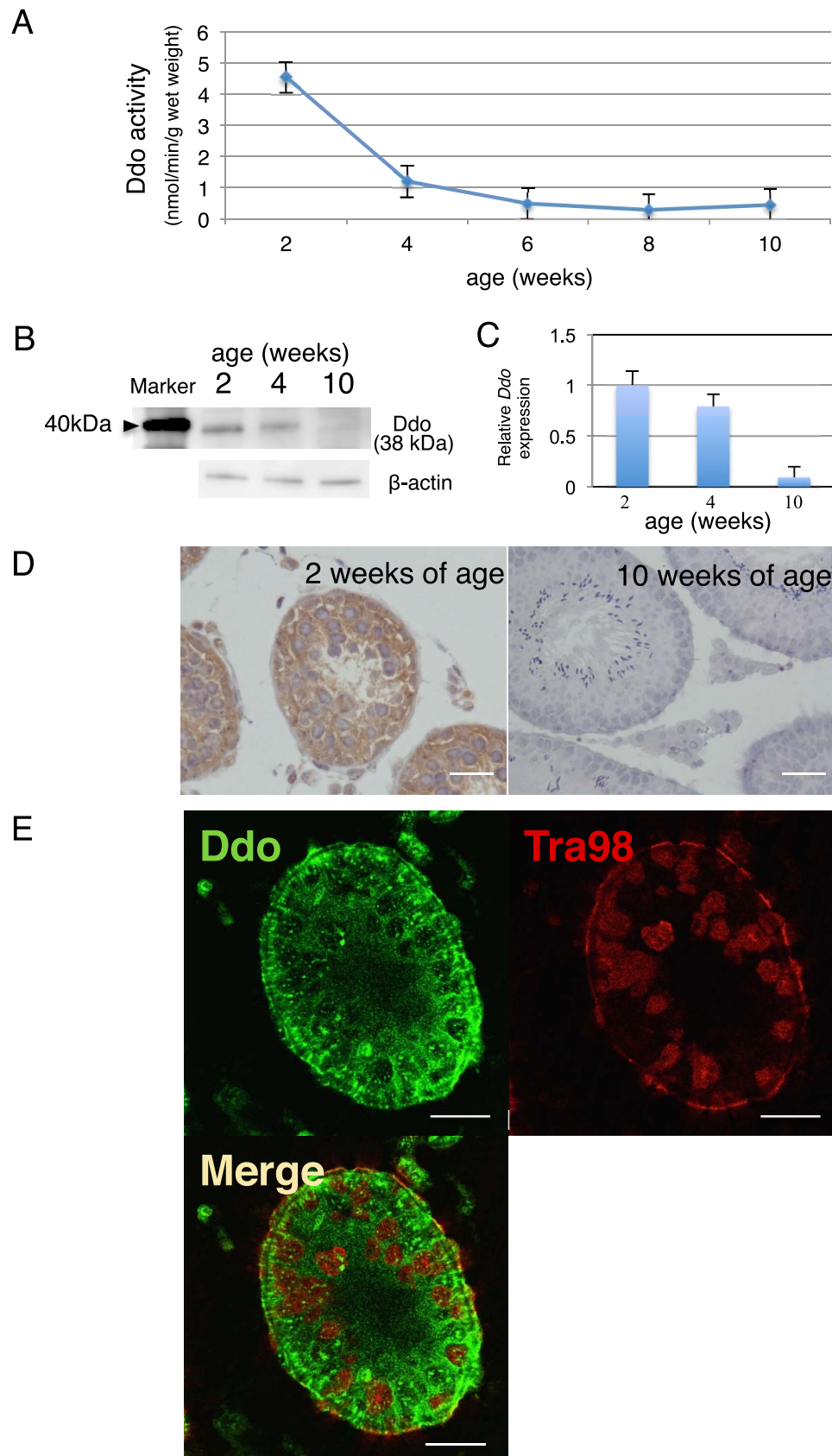


FIG. 3. Decrease of Ddo activity during development. **A)** Ddo activity in the testis of mice (means  $\pm$  SD,  $n = 4$ ). **B)** Western blot detection of Ddo in mouse testis homogenates with the anti-DDO antibody. **C)** Quantitative RT-PCR detection of *Ddo* in the mouse testis homogenates (means  $\pm$  SD,  $n = 4$ ). **D)** Ddo immunolocalization in the testis of mouse at 2 wk of age. Bars = 20  $\mu$ m. **E)** Comparison between testis at 2 and 10 wk with respect to Ddo expression using immunohistochemistry. Bars = 20  $\mu$ m.

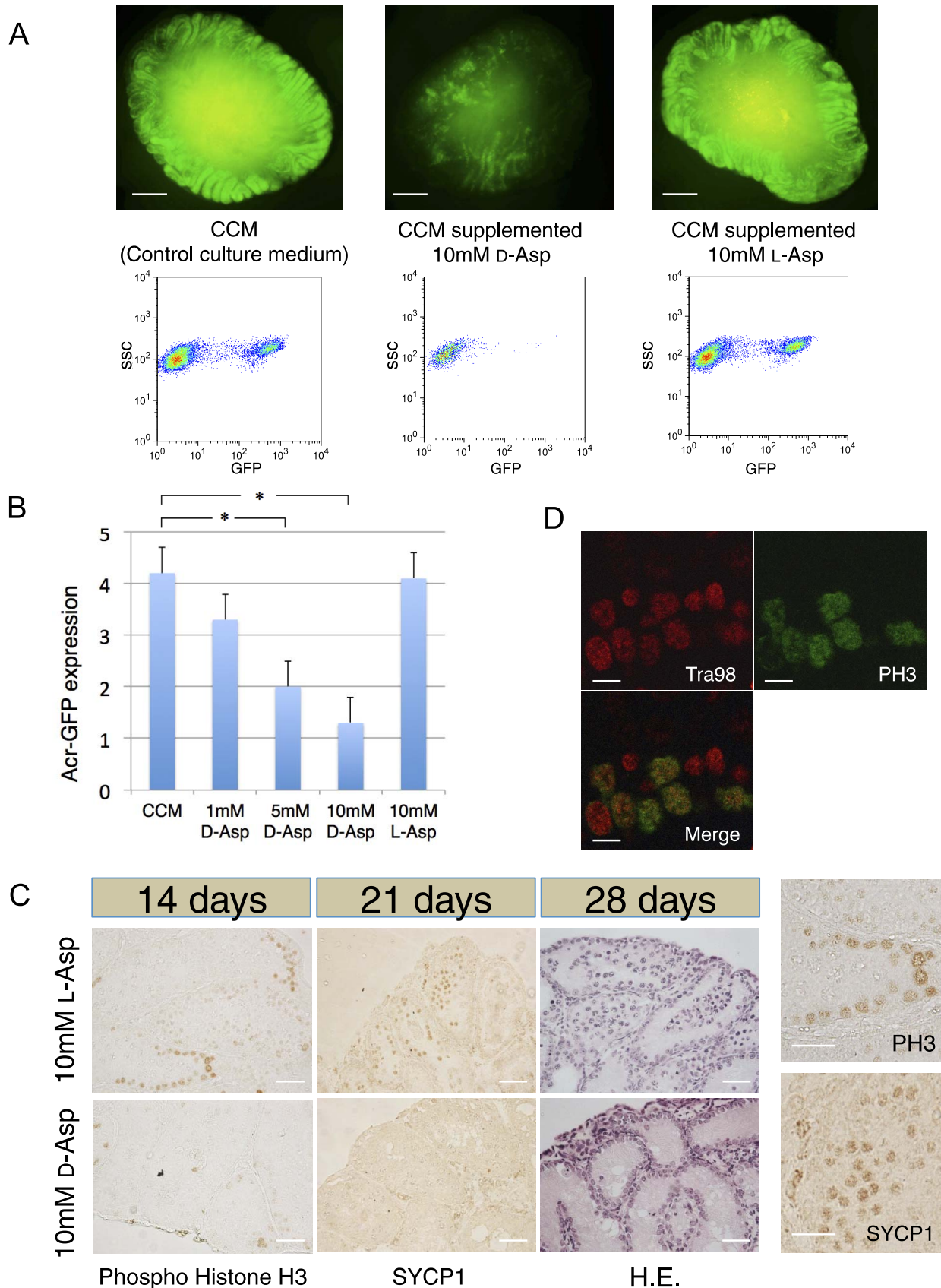


FIG. 4. Effect of D-Asp on cultured mouse testis tissues. Five media were compared: control culture medium (CCM), CCM supplemented with 1, 5, or 10 mM D-Asp, and CCM supplemented with 10 mM L-Asp. **A**) The expression of *Acr-GFP* in mouse testis on Culture Day 28 (upper panels). Flowcytometric analysis of cells of cultured enzymatically dissociated testis tissues (lower panels). **B**) *Acr-GFP* expressions were scored using the grading scale [12] on Culture Day 28 (means  $\pm$  SD,  $n = 10$ ,  $*P < 0.01$ ). **C**) Histological changes in testis tissue culture with L- or D-Asp. Bar = 100  $\mu$ m. The first panels on the right are the higher magnification image of cultured tissues for 21 days with 10 mM L-Asp. Bar = 30  $\mu$ m. **D**) Immunohistochemistry of cultured testis tissue for 14 days with 10 mM L-Asp. Tra98 (red) and phospho Histone H3 (PH3, green) are shown. Bar = 10  $\mu$ m.

interstitial region of testis. Based on our results and on previous findings, we therefore speculate that D-Asp is transported to or synthesized in the spermatid and ultimately acts on the Leydig cells. This has been proposed in the past [5], and the results obtained in this study extend these earlier ideas. Our results have clearly defined the region in which D-Asp is present and is presumed to act and provide important information on the origin of D-Asp in the testis. Recently, Santillo et al. [23] found that D-Asp stimulated proliferative pathways in GC-1 cell, a cell line of type-B mouse spermatogonia. In this study, we find a negative effect of D-Asp on differentiation of premeiotic germ cell. The reason of this discrepancy is not clear, but it might be due to different effects on the different stages of spermatogenesis or from the different methods for assays. Further investigation is needed to understand the physiological role of D-Asp.

To date, with the exception of several reports on the influence of D-Asp on the hormonal function of Leydig cells [6–8], the effect of D-Asp on spermatogenesis has not been elucidated. Our findings on the relative dynamics of testicular levels of D-Asp and the activities of its degradative enzyme, Ddo, may help our understanding of their physiological role in spermatogenesis. Ultimately, clarification of the synthesis and transport mechanism of D-Asp will lead to the development of approaches to regulating its local levels in the testis, which will have longer term clinically valuable significance.

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