Cell-type-specific regulation of genes involved in testicular lipid metabolism: fatty acid-binding proteins, diacylglycerol acyltransferases and perilipin 2

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Short title: FABPs, DGATs and PLIN2 during spermatogenesis

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Abstract

Male germ cell differentiation entails the synthesis and remodeling of membrane polar lipids and the formation of triacylglycerols (TAG). This requires fatty acid-binding proteins (FABP) for intracellular fatty acid traffic, a diacylglycerol acyltransferase (DGAT) to catalyze the final step of TAG biosynthesis, and a TAG storage mode. We examined the expression of genes encoding five members of the FABP family and two DGAT proteins, as well as the lipid droplet protein Perilipin 2 (PLIN2), during mouse testis development and in specific cells from seminiferous ephitelium. *Fabp5* was distinctive of Sertoli cells and consequently was higher in prepubertal than in adult testis. *Fabp3* expression increased in testis during postnatal development, associated to the functional differentiation of interstitial cells, but was low in germ cells. *Fabp9*, together with *Fabp12*, were prominently expressed in the latter. Their transcripts increased from spermatocytes to spermatids and, interestingly, were highest in spermatid-derived residual bodies. Sertoli and germ cells, which produce neutral lipids and store them in lipid droplets, expressed *Plin2*. Yet, while *Dgat1* was detected in Sertoli cells, *Dgat2* accumulated in germ cells with a similar pattern of expression as *Fabp9*. These results correlated with TAG levels also increasing with mouse germ cell differentiation to be highest in residual bodies, pointing to DGAT2 as the protein involved in the biosynthesis of such TAG. The age- and germ cell type-associated increases in *Fabp9*, *Dgat2* and *Plin2* levels are thus functionally related in the last stages of germ cell differentiation.

Keywords: DGAT; FABP; germ cells; lipid droplets; PLIN2; residual bodies; Sertoli cells; spermatids; spermatocytes; triacylglycerols

Introduction

During spermatogenesis, the extensive formation and remodeling of membranes associated with germ cell differentiation and maturation entails considerable quantitative and qualitative changes in their lipids. In rodents, classical studies have shown that testicular glycerophospholipids (GPL) and triacylglycerols (TAG) become increasingly rich in long-chain polyunsaturated fatty acid (PUFA) like docosapentaenoic acid during postnatal development, as spermatids contain more of this fatty acid in their GPL and TAG than spermatocytes (Beckman *et al.* 1978; Grogan & Huth 1983). Concomitantly, there are also significant changes in unique fatty acids of testicular sphingomyelins and ceramides destined to be components of spermatozoa (Zanetti *et al.* 2010): the proportion of 2-hydroxy very-long-chain PUFA with respect to their nonhydroxy counterparts increases considerably In both lipids from spermatocytes to spermatids (Oresti *et al.* 2010). The regulation of fatty acid biosynthesis and exchange among intracellular organelles of germ cells during spermatogenesis is thus essential for male fertility.

In the final stages of spermatogenesis, spermatids elongate and undergo a considerable cytoplasmic volume reduction via the formation and release of the membrane-bound vesicles known as "residual bodies". These densely packed structures, readily phagocytized by Sertoli cells (Kerr & De Kretser 1974), contain remains of cytoplasm, RNA, former organelles, and neutral lipid inclusions (Russell *et al.* 1990). The spermatid-derived residual bodies are clearly discernible by markers of neutral lipid droplets, in accordance to the finding that they are even richer in TAG than spermatids (Oresti *et al.* 2010).

The interstitium-located Leydig cells are involved in the production testosterone. The regulatory functions performed by Sertoli cells require a continuous cross-talk between them and the germ cells they nurse in the seminiferous epithelium. Both these functions are under autocrine, paracrine and endocrine regulation. The inter-dependent cellular structure and

functions of the testis thus provide a paradigmatic model to assess the regulation and relevance of the different genes encoding for proteins involved in lipid biosynthesis and catabolism, which require fatty acid traffic among membranes throughout development and cell differentiation.

Fatty acid binding proteins (FABPs) comprise a conserved family of small cytosolic proteins that reversibly bind long-chain fatty acids and their corresponding acyl-CoA esters. They promote the uptake and intracellular transport of fatty acids, guide them to specific metabolic pathways, and participate in cell growth by regulating the expression of specific genes (Haunerland & Spener 2004; Furuhashi & Hotamisligil 2008). In mammals, ten FABP members with different tissue-dependent patterns of expression have been reported (Yamamoto *et al.* 2009). All FABPs have the same primary role with potential differences in ligand selectivity for long-chain fatty acids, binding affinity and binding mechanism. The expression of FABPs in each particular cell type has been considered to reflect its lipid-metabolizing capacity (Chmurzynska 2006).

The predominant FABP in testis is widely accepted to be FABP9 (Kido & Namiki 2000; Selvaraj *et al.* 2010). Although other members of this family have also been reported, such as FABP3 (Watanabe *et al.* 1991), FABP5 (Kingma *et al.* 1998), and more recently FABP12 (Liu *et al.* 2008; Yamamoto *et al.* 2009), no studies comparing on the same basis the expression of different FABPs at definite periods of testis development and correlating this with their relative abundances in particular testicular cell types were available.

The biosynthesis of TAG involves several enzymatic steps. The last of these, in which a long-chain fatty acyl-CoAs is joined to a diacylglycerol molecule, is catalyzed by the enzyme acyl-CoA:diacylglycerol acyltransferase (DGAT) with ubiquitous activity in cells of most tissues (Lehner & Kuksis 1996). Two DGAT proteins have been described, DGAT1 and DGAT2, which probably differ in physiological function (Cases *et al.* 1998; Cases *et al.* 2001). To

date, the expression of genes encoding for each of these proteins in testis as a function of developmental stages and in differentiating cells of the seminiferous epithelium has not yet been reported.

Perilipin 2 (PLIN2), formerly termed adipose differentiation-related protein (ADRP) (Kimmel *et al.* 2010), is one of the proteins involved in maintaining lipid droplet structure and function (McIntosh *et al.* 2012). In the testis the protein was found to be present in Sertoli and Leydig cells, associated with TAG- and cholesterol ester-containing lipid droplets (Brasaemle *et al.* 1997; Heid *et al.* 1998). The abundance of lipid droplets containing TAG -but no cholesterol esters- in rat germ cells and residual bodies (Oresti *et al.* 2010) suggested the possibility that in our mice the expression of one of the *Dgats* would coincide with that of *Plin2* in increasing with germ cell maturation, the activity of these proteins being indirectly reflected in increased amounts of TAG and lipid droplets as germ cell differentiation proceeds.

In order to assess the co-regulation of genes involved in lipid metabolism in testis, in this study we analysed the expression of genes encoding for five FABPs, two DGATs, and PLIN2 in mouse testis during postnatal development and in specific testicular cell types. The inclusion of residual bodies in the germ cell analysis led to the biologically relevant finding that some of these mRNAs are highly concentrated in these particles, whose contents are destined to be recycled by Sertoli cells.

Materials and Methods

Animals

CD-1 mice were bred at the CIB-CSIC animal facility under specific pathogen-free, temperature (22±1ºC) and humidity-controlled (50-55%) conditions, on 12 h light/dark cycles and with *at libitum* access to food and water. Animals were treated according to the guidelines of the CSIC Bioethics Committee.

Tissue and cell isolation

Whole testes were obtained from mice at postnatal days 6, 10, and 18 (P6, P10 and P18, respectively) and from adult mice. Pachytene spermatocytes, round spermatids, elongating spermatids and residual bodies (abbreviated PS, RS, ES and RB, respectively, in figures) were obtained from adult mouse testes and enriched using BSA density gradients on STA-PUT (Bellve *et al.* 1977; Oresti *et al.* 2010; Paz *et al.* 2006; Romrell *et al.* 1976). Sertoli cells were obtained from testes of mice aged 14–18 postnatal days and enriched by primary culture. The cells were maintained for 2 weeks at 37 °C in a 5% CO_2/95% air atmosphere and cultured in Dulbecco's modified Eagle's medium: Ham-F12 medium (Gibco, BRL) (1:1), following standard procedures (Karl & Griswold 1990). The purity of isolated and cultured cells was 90–95%, as determined by morphological criteria (supplementary Fig. S1) and RT-PCR using cell-type specific primers as previously described (Gonzalez-Gonzalez *et al.* 2008).

Total RNA extraction and analysis of mRNAs by RT-qPCR

RNA from testis and germ cells was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer´s instructions. RNA was resuspended in RNase-free water and its concentration was assessed from the A260/280 absorbance ratio in a NanoDrop Spectrophotometer ND-1000 (NanoDrop). Samples were stored at −80 °C until use. Aliquots containing 0.2 μg total RNA were used to synthesize cDNA in reactions containing 2.5 μM Oligo dT17, 1X First-Strand Buffer (Invitrogen), 0.01 M dithiothreitol (DTT), 2 UI of RNase inhibitor (RNAsin Promega), 0.5 mM of each dNTP and 200 U of superscript II (Invitrogen). The reactants were taken to a final volume of 20 μl with RNase-free water.

The cDNAs resulting from retrotranscription were amplified by real-time quantitative PCR (qPCR). Gene expression levels were determined using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). RT-qPCR was performed in a final volume of 10 μl using iQ™ SYBR Green SuperMix (Bio-Rad) and 0.25 μM concentration of each primer. Primer combinations for the specific amplification of the analysed genes were designed in "Universal ProbeLibrary" (http://www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) and

purchased from Roche. The primers used in PCR are listed in Table 1. The RT-PCR products were assessed by 3% agarose gel electrophoresis. The efficiency of each primer pair was calculated for comparative and normalizing purposes.

The PCR reaction conditions were as follows: 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and a final extension step at 72 °C for 1 min. The PCR products were also examined by melting curve analysis and agarose gel electrophoresis.

Data analysis

A total of 12 measurements were done for each gene studied at each stage or cell type: three biological replicates, from which four technical replicates, were analysed. Data were normalized according to the previously described $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001), using peptidylprolyl isomerase A (*Ppia*) and H2A histone family member Z (*H2afz*) as reference genes. The relative level of each gene is expressed as the relative change in gene expression (Livak & Schmittgen 2001).

Antibodies and Western blot

For FABP9 protein expression analysis, a validated anti-FABP9 antibody (Kido *et al.* 2005) kindly donated by Dr. T. Kido (UCSF, USA) was used. Anti-FABP12 (M-12): sc-241412 and anti-Actin (I-19): sc-1616 (both from Santa Cruz Biotechnology) were used as primary antibodies. Tissue and cells were lysed and thoroughly homogenized in Tris-HCl (pH 6.8), 5 mM EDTA, 3% SDS and 1% of protease inhibitor cocktail (Roche Applied Science). Proteins were mixed with denaturing Laemmli buffer, heated to 95 ºC for 5 min, resolved by SDS-PAGE, and subjected to Western blotting using the primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody and detection with SuperSignal chemiluminescent substrate (Pierce). The intensity of bands was compared using the Image-J software (National Institutes of Health, USA), and data were expressed as arbitrary units relative to actin expression.

Nile Red staining and confocal microscopy

To detect neutral lipid-containing lipid droplets, testes were fixed at 4% in paraformaldehyde at 4ºC, rinsed overnight in PBS containing 30% sucrose at 4°C for cryoprotection, placed in a small amount of OCT compound Tissue Tek (Sakura Finetek U.S.A) and stored at -80ºC. Ten μm-thick frozen sections were prepared with a cryostat and picked up on poly-L-lysine-coated glass coverslips (Sigma). Then, tissue sections were incubated in PBS to remove OCT and post-fixed in 4% paraformaldehyde in PBS for 5 min, followed by incubation for 15 min at room temperature with 1.5 μg/ml Nile Red (Molecular Probes) in PBS. Samples were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories). All images were captured using a Leica TCS-SP5-AOBS confocal microscope with 40X oil immersion objectives.

GPL and TAG separation and fatty acids analysis

After collecting pachytene spertmatocyte, round spermatids and residual bodies by centrifugation, lipid extracts were prepared and partitioned (BLIGH & DYER 1959). After phase separation, the organic solvents were evaporated under N_2 and the samples dissolved in chloroform-methanol (2:1 v/v). Aliquots were taken for total lipid phosphorus determination.

Lipid extracts were spotted on TLC plates under N_2 along with commercial standards (Sigma). The neutral lipids were resolved with n-hexane:diethyl ether (80:20 v/v) to obtain the TAG, and the total polar lipid fraction (mostly GPL) was recovered from the origin of these plates. The amount of GPL and TAG in germ cells and residual bodies was estimated from their fatty acids. These were measured by gas chromatography (GC) after conversion of the eluted, dried lipids into fatty acid methyl esters. Before GC, the latter were routinely purified by TLC on (pre-washed) silica gel G plates, using hexane:ether (95:5 v/v) as solvent. Fatty acid analysis was performed using the conditions and instrumentation described in previous work (Oresti *et al.* 2010).

Statistics

The bars shown in figure histograms represent mean values ± standard deviation. One-way ANOVA was used to determine the significance of differences among mean values, which were compared using Bonferroni's test for multiple comparison. In both analyses, the minimum acceptable level of significance was $p < 0.05$. Statistics marks (letters) shown in figures compare the different expression rates of a particular gene at different postnatal days or cells. Statistical data comparing the expression rates of the genes studied in a particular postnatal day or among different cells are provided in Supplemental Table 1.

Results

Differential expression of fatty acid-binding proteins

Because germ cells at progressively advanced stages of differentiation appear at defined times after birth (Bellve *et al.* 1977) it was possible to follow the appearance or accumulation of the transcripts under study at specific stages of spermatogenesis. In mice at postnatal day 6 (P6), nearly 85% of the cells in the seminiferous epithelium correspond to Sertoli cells and 15% to primitive spermatogonia. Pachytene spermatocytes abound at P18, and elongated spermatids prevail in the adult testis, where they represent about 60% of the intra-tubular cells, accompanied by an abundant presence of residual bodies (Bellve *et al.* 1977). The pattern of expression of the five *Fabp* genes we studied differed markedly during spermatogenic development and cell differentiation (Fig. 1). Analysis by RT-qPCR revealed a relative high transcript accumulation of *Fabp3* and *Fabp5* during early stages of development (P6-P10), decreasing significantly at P18. However, from P18 to adult testes, *Fabp5* continued to fall whereas *Fabp3* showed a significant increase. The expression of *Fabp7* was almost residual in testis and cells (Fig. 1). The *Fabp* isoform that presented the highest rate of transcript accumulation in adult testis was *Fabp9* (1300-fold from P6 to adult). *Fabp12* mRNAs

remained virtually undetectable from P6 to P18 but was clearly more abundant in adult testis (Fig. 1).

To assess cell-type-specific gene expression, additional comparative analyses were performed in purified Sertoli cells, pachytene spermatocytes, round spermatids, and residual bodies (Fig. 2). Normalized data showed that mRNA levels of *Fabp3* were higher in whole adult testis than in any of the seminiferous tubule cell types studied (Fig. 2 left panel). These results indicated that *Fabp3* was mainly expressed in extra-tubular cells. This expression was mainly located in the interstitium and correlated with the increase of functional activity of Leydig cells in adults (see supplementary Fig. 2). In contrast, *Fabp5* expression was apparently restricted almost exclusively to Sertoli cells. Because the proportion of Sertoli cells relatively decreases with respect to the massive increase the number of germ cells undergoes from prepubertal to adult testis, this *Fabp5* localization explains the apparent progressive reduction observed for its transcript in testis with gonadal development (Fig. 1).

In clear contrast with *Fabp5*, both *Fabp9* and *Fabp12* transcript levels were low, virtually absent from Sertoli cells. Their expression was restricted to germ cells and residual bodies. Transcript levels of *Fabp9* and *Fabp12* increased from spermatocytes to spermatids and reached their maximum values in residual bodies (Fig. 2). In agreement with the lack of expression detected in developing testes, *Fabp7* transcripts were virtually absent in the different cell types analysed.

In view of the predominance of *Fabp9* followed by *Fabp12* in the germ cell line, we assessed their expression at the protein level during developmental stages and from isolated cell types (Fig. 3). In Western blots, FABP9 was detected as a single band of 15kD, in agreement with previous work in adult mice (Selvaraj *et al.* 2010). The protein was undetectable at P6, first appearing at around P18, i.e., associated with the emergence of pachytene spermatocytes in the first wave of germ cell differentiation. As did the mRNA transcript, FABP9 reached its highest amount in adult testes (Fig. 3A). Also in coincidence with its mRNA distribution,

FABP9 protein level was very low in Sertoli cells, considerably higher in germ cells, and as copious in the latest (elongating) spermatids as in residual bodies (Fig. 3A).

FABP12 was detected as two bands of 15 and 17 kd. This may be ascribed in part to post-transcriptional modifications or, as previouly suggested for other FABP (Ockner *et al.* 1982; Spener *et al.* 1990), to alternate forms due to this protein binding different amounts of fatty acids or other substrates. FABP12 increased in the testis with postnatal maturation, its level being higher at earlier than at later stages of development (Fig. 3B). This tendency correlated with the distribution of FABP12 among cells: its level was lowest in Sertoli cells and highest in pachytene spermatocytes and round spermatids, being lower in elongated spermatids and residual bodies (Fig. 3B). This finding points to an interesting difference between FABP9 and FABP12 in relative rates of mRNA and protein expression during germ cell differentiation.

Dgat and Plin2 Expression, Neutral Lipid Droplets, and TAG levels

The expression profiles shown by *Dgat1* and *Dgat2* indicated that both genes were subjected to different regulatory mechanisms (Fig. 4A). Although both isoforms were abundantly expressed in the testis at all developmental stages, *Dgat2* was the predominant one in the adult. *Dgat2* mRNA maintained a low level between P6 and P18, but increased 30-fold from P18 to adult (Fig. 4A). This suggested a preferential association of *Dgat2* with spermatids which was confirmed by the profiles of *Dgat1* and *Dgat2* expression among cell types (Fig. 4B). Sertoli cells had a low but detectable *Dgat1* while virtually lacked *Dgat2* expression. Conversely, in germ cells *Dgat1* mRNA levels were lower, and increased less, than those of *Dgat2* with their differentiation. In comparison with spermatocytes, the amount of *Dgat2* was 3- and 4-fold higher in round spermatids and residual bodies, respectively (Fig. 4B).

PLIN2 is one of the proteins associated with neutral lipid-storing intracellular lipid droplets (Brasaemle *et al.* 1997). Its testicular mRNA levels underwent a total increase of 3-fold from P6 to adult mouse testes (Fig. 4A), in agreement with the finding that *Plin2* was expressed in

both Sertoli and germ cells (Fig. 4 B). In the latter and residual bodies, its transcript levels were twice as large (Fig. 4 B) as in whole testes (Fig. 4A).

In rat testis, the intensity of lipid droplet staining with Nile Red increases from isolated pachytene spermatocytes to round spermatids and from these to residual bodies (Oresti *et al.* 2010). This allowed linking this attribute with the increase in TAG concentration per cell or particle, which followed the same order. In the mouse, we observed similar attributes, with some qualitative and quantitative differences. Three populations of lipid droplets, one extra-tubular and two within seminiferous tubules, were identified (Fig. 5). The interstitially located ones were considerably larger and more abundant in mice than in rats. Of the intra-tubular droplets, those larger, basally located ones were mostly those included in Sertoli cells, and the tiny ones, facing the tubule lumena, were considerably smaller and even more numerous in mice (Fig. 5) than in rats (Oresti *et al.* 2010).

The extra-tubular lipid droplets are expected to contain mostly the cholesteryl esters (Brasaemle *et al.* 1997) that Leydig cells store as a source of the cholesterol involved in steroidogenesis. Those of Sertoli cells are probably mixed in nature, since these cells produce both cholesterol esters and TAG, whereas those of germ cells mostly contain TAG as the main neutral lipid (Oresti *et al.* 2010). In the present study we also analysed the content and fatty acid composition of the TAG of our mouse germ cell preparations and residual bodies, as a manifestation of the TAG-biosynthesizing activity of germ cells.

Concomitantly with the cell location of lipid droplet staining, the expression of *Fabp9*, *Dgat2* and *Plin2* coincided with the levels of TAG concentration (Fig. 5) in that all of them increased from pachytene spermatocytes to round spermatids and from these to residual bodies. The differentiation-related increase in TAG amount was mostly accounted for by these neutral glycerides becoming enriched in specific PUFA (Fig. 6A), as was the case of docosapentaenoic and docosahexaenoic acids (22:5n-6 and 22:6n-3), both showing a significant accumulation in the TAG of spermatids, and especially of residual bodies. For

comparison purposes Fig. 6B shows that as mouse spermatogenic germ cells differentiation proceeds the major membrane GPL became (as TAG) more richer in 22:5n-6 and 22:6n-3 fatty acids and these molecular species were highly accumulated in residual bodies.

Discussion

FABPs are ubiquitously expressed proteins with the various members of this family differing in tissue expression patterns (Haunerland & Spener 2004; Yamamoto *et al.* 2009). The present quantitative study in the mouse testicular tissue showed, in our knowledge for the first time, cell-compartmentalized and time-dependent variations in the gene expression of five simultaneously assessed FABP members indicative that there is a differential regulation of their expression at specific stages of spermatogenesis.

Our finding that *Fabp3* increased 8-fold from the first spermatogenic wave to adult testis is in agreement with the increase in FABP3 previously reported in a proteomics study (Paz *et al.* 2006). The developmental profile we observed for *Fabp3* expression was consistent with this protein mostly localizing in Leydig cells (Heuckeroth *et al.* 1987; Watanabe *et al.* 1991; Zschiesche *et al.* 1995) and with the changes of intratesticular testosterone concentrations during mouse testis postnatal maturation. Thus, levels of this hormone (O'Shaughnessy *et al.* 2002), as did *Fabp3* (present results), showed a decline between P10 and P20, followed by an increase at P25. Using immunofluorescence, we confirmed that FABP3 protein was interstitially located and that it displayed low signal level at P6, was almost undetectable at P18, and was plentiful in adult testis (see Fig. 2 in Supplemental Material). Interestingly, the *Fabp3* gene is down regulated in Leydig cells as a result of exposure to a known endocrine disruptor (Chauvigne *et al.* 2011).

Together, the above commented results suggest a fine regulation of *Fabp3* expression associated with the functional activity of Leydig cells in testosterone production. A trait of the protein FABP3 is its higher affinity for arachidonic than for palmitic acid (Murphy *et al.*

2005). Arachidonic acid (AA) is the fatty acid specifically involved in the mechanism of cholesterol transport from the outer to the inner mitochondrial membrane in Leydig cells, the first step in steroidogenesis (Albert *et al.* 1980; Castillo *et al.* 2006). Taken together, these observations allow the speculation that FABP3 could play a role in testosterone production by transporting the required AA from other cellular sites to mitochondria. Although there are studies using *Fabp3*-knock-down mice to define FABP3 functions in different tissues (Schachtrup *et al.* 2008; Shioda *et al.* 2010), no one has yet informed on the effects of such gene change on testicular function.

Fabp5 expression was here detected as significant in Sertoli cells. The developmentally associated decrease of its expression in whole testis may be ascribed to the relatively small proportion of these cells with respect to germ cells in the adult, and with the almost negligible expression of this gene in germ cells (Figs. 1 and 2). As a protein, FABP5 was previously observed in bovine Sertoli cells and retinal Müller cells, both having in common that they are supportive of other cell types in their respective tissues (Kingma *et al.* 1998). A function of Sertoli cells is to remove, by means of phagocytosis, apoptotic bodies evolving from supernumerary spermatocytes and residual bodies released from condensing spermatids. Once these lipid-containing bodies have been incorporated, and their content hydrolyzed in lysosomes, a variety of fatty acids —from saturates to long-chain and very-long-chain PUFA— need to be transported to subcellular organelles such as the endoplasmic reticulum, mitochondria or peroxisomes to be used in biosynthetic and/or catabolic pathways. The high level of *Fabp5* expression that we found in mouse Sertoli cells points to a highly active intracellular fatty acid traffic among organelles. The fact that this protein has a low ligand specificity, since it binds fatty acids with widely differing levels of unsaturation with relatively high affinities (Kingma *et al.* 1998), agrees with this possibility.

The mRNA of *Fabp7*, mainly detected in brain, was included in our study because this isoform was occasionally detected in the zebrafish (*Danio rerio*) testis (Liu *et al.* 2003) and

because it is involved in the transport of 22 carbon PUFA like docosahexaenoic acid (Balendiran *et al.* 2000; Mita *et al.* 2010). This fatty acid, like docosapentaenoic acid, abounds in mouse testis lipids (Furland *et al.* 2003), highly concentrated in membrane phospholipids, and especially in TAG, of germ cells and residual bodies (present results). However, in addition to being expressed at much lower levels than other FABP isoforms, *Fabp7* remained virtually constant throughout testicular development and showed no remarkable variations among the studied cell types. Further work is required to clarify the apparent discrepancy between the faint expression of *Fabp7* and the abundance of 22 carbon PUFA in mouse testis.

FABP9 is the best characterized member of the FABP family in the testis. Our results quantifying *Fabp9* mRNA and protein levels in different developmental stages and specific cell types concur with, and extend, previous reports. The fact that both increase in ascendant order from pachytene spermatocytes to round spermatids, from these to elongating spermatids, and from these to residual bodies explains why *Fabp9* mRNA and protein accumulate during postnatal maturation in the testis. The presence of FABP9 protein at the latest stages of spermatid differentiation agrees with previous observations using immunohistochemistry in testis sections (Korley *et al.* 1997; Selvaraj *et al.* 2010). Our results detecting this protein in isolated elongating spermatids and residual bodies agrees with the presence of this protein in mouse spermatozoa (Korley *et al.* 1997). Interestingly, FABP9 is one of the proteins whose phosphorylation state increases during *in vitro* sperm capacitation (Platt *et al.* 2009).

The Sertoli cells used in the present study, in addition to being obtained at a relatively early developmental stage (P18), were cultured for two weeks in order to exclude remainders of germ cells as possible sources of this gene. Although these conditions may be considered with caution, since the transcripts could have been degraded during that time, the same conditions served to demonstrate high and specific *Fabp5* (and other mRNAs) expression by

these cells. Our observation that FABP9, both at the mRNA and protein levels, were completely undetectable in Sertoli cells supports the notion that it is not endogenous to these cells. The fact that it is detectable in Sertoli cells after a massive apoptosis of spermatocytes is induced, is probably a result of Sertoli cell phagocytic activity (Kido & Namiki 2000).

It was intriguing that during the last stages of germ cell differentiation, both *Fabp9* and *Fabp12* expression increased at the mRNA level but apparently not both at the protein level. Problems with the immunodetection are not likely, since both proteins were expressed at similar rates during meiosis (pachytene spermatocytes) and up to early steps of spermiogenesis (round spermatids), the main difference being established in elongating spermatids and residual bodies. This discrepancy could bear a relationship with post-transcriptional regulation via RNA, whereby the latter is silenced concomitantly with mouse germ cell differentiation (Gonzalez-Gonzalez *et al.* 2008), possibly affecting each of these isoforms to different extents.

In general, FABPs participate mainly in the uptake and intracellular traffic of fatty acids and acyl-CoAs prior to their utilization in cells (Liou & Storch 2001). Because *Fabp9* not only abounded but increased in cells in the late stages of spermatogenesis as well as residual bodies, we suggest that the protein could contribute to the intracellular transport of those fatty acids that are required for the synthesis of the membrane sphingolipids and glycerophospholipids that will exit from the testis together with the mature gametes. It could also contribute to the conservation of fatty acids within the testis by transporting the surplus of fatty acids discarded during spermatid volume reduction to the subcellular site of TAG synthesis. This possibility is suggested by the fact that these neutral glycerides concentrate a lot of the most metabolically costly PUFA (e.g. 22:5n-6 and 22:6n-3). Further studies are still needed to establish the nature of the hydrophobic ligands of FABP9 and clarify its role in spermatogenesis. Intriguingly, mice lacking *Fabp9* are viable and fertile, and the total lipid fatty acid profile of their spermatozoa is not apparently affected (Selvaraj *et al.* 2010). It is

then possible that the deficiency in FABP9 could be compensated by overexpression of *Fabp12* in the germ cells of *Fabp9*-null mice, thereby enabling spermatogenesis to continue.

Our finding that that the testis contains transcripts encoding for both *Dgat1* and *Dgat2* and that these genes differ in their degree of upregulation with testicular development and in their distribution among cells, suggests their involvement in TAG biosynthesis as part of the testicular lipid homeostasis. TAG are the main neutral lipids present in adult rodent seminiferous tubules (Furland *et al.* 2003). That *Dgat2* was increasingly expressed from spermatocytes to spermatids and from these to residual bodies is in agreement with the concurrently progressive increase of TAG levels we observed in rodent testis in this sequence ((Oresti *et al.* 2010) and present results).

The biosynthesis of TAG was proposed to function as one of the strategies used by developing spermatids while reducing their volume to become thinner and longer before being released as spermatozoa into the tubular lumen (Oresti *et al.* 2010). Intracellular organelles such as Golgi membranes, ribosomes or peroxisomes, which have important biosynthetic functions in germ cells during earlier stages, are no longer required in gametes. In the last stages of spermiogenesis, all surplus material in each spermatid including lipids is tightly condensed and enclosed in a subacrosomal cytoplasmic lobe, that is then released in compact form as a residual body (Russell *et al.* 1990). The volume occupied by membrane glycerophospholipids may be efficiently reduced by converting them partly into diacylglycerols and partly into free fatty acids, then combining these products into TAG, and confining such TAG into lipid droplets.

In addition to diglycerides, DGAT2 requires fatty acids or acylCoAs to be carried to the site of TAG synthesis, a function that could be played by FABP9 —or alternatively by FABP12— Subsequently, TAG need to be transported to, and stored in, lipid droplets. The increasing amounts of these subcellular inclusions with germ cell differentiation, detected in tissue sections with Nile Red, agrees with the finding that *Plin2* mRNA level was higher in

spermatids and residual bodies than in spermatocytes. In other words, the differentiation-related increase in the amounts of TAG and lipid droplets, concomitant with the increase in *Fabp9*, *Dgat2* and *Plin2* expression as spermatogenesis progresses suggests a functional connection between the products of these transcripts. The finding of a high concentration of these three mRNAs in residual bodies is intriguing, but reasonable if one considers that they have terminated their function related to protein synthesis in spermatids. In fact, these mRNAs are in fact a minor part of the total RNA that residual bodies were shown to contain (Shin *et al.* 2007). Although the ultimate biochemical fate of the RNA molecules confined in these particles remains to be established, the possibility that some of their hydrolysis products or related metabolites could serve regulatory functions relevant to spermatogenesis in Sertoli cells cannot be excluded.

Declaration of interest

The authors declare that there is no conflict of interest.

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Ref Type: Generic

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FIGURE LEGENDS

FIGURE 1. RT-qPCR analyses of *Fabp3*, *Fabp5*, *Fabp7*, *Fabp9* and *Fabp12* transcripts during postnatal testicular development in the mouse (P6, P10, and P18 refer to postnatal days). Data were normalized to *Ppia* and *H2afz* as internal reference using the $2^{-\Delta\Delta Ct}$ method (described in Materials and Methods). Values with different letters (a-d) are significantly different ($P < 0.05$). Statistical significance between different expression rates of all genes studied in a particular postnatal day is shown as supplemental data.

FIGURE 2. RT-qPCR analyses of *Fabp3*, *Fabp5*, *Fabp7*, *Fabp9* and *Fabp12* transcripts in different cell types purified from mouse seminiferous tubules. SC, Sertoli cells; PS, pachytene spermatocytes; RS, round spermatids; RB, residual bodies. Data were normalized to *Ppia* and *H2afz* as internal reference using the $2^{-\Delta\Delta Ct}$ method. Values with different letters (a-d) are significantly different ($P < 0.05$). Statistical significance between different expression rates of all genes studied in a particular cell type is showed as supplementary tables.

FIGURE 3. FABP9 and FABP12 protein expression in mouse testis. Western blots from samples obtained from testis during postnatal development (A) and in different cell types obtained from adult testes. (B): SC, Sertoli cells; PS, pachytene spermatocytes; RS, round spermatids; ES, elongating spermatids; RB, residual bodies.

FIGURE 4. RT-qPCR analyses of *Dgat1*, *Dgat2* and *Plin2* transcripts during postnatal development in the mouse testis (A) and in cells from the seminiferous epithelium of adult mice (B). SC, Sertoli cells; PS, pachytene spermatocytes; RS, round spermatids; RB, residual bodies. Data were normalized to *Ppia* and *H2afz* as internal reference using the $2^{-\Delta\Delta Ct}$ method. Values with different letters (a-d) are significantly different ($P < 0.05$). Statistical significance between different expression rates of all genes studied in a particular postnatal day or cell type is shown in supplementary tables.

FIGURE 5. Testicular lipid droplets during postnatal testis development as revealed by Nile red staining and fluorescence microscopy (40X, bar = 100 μm). P6, P10, and P18 refer to postnatal days; AD, adults. In the panel labeled as AD 63X, the localization of different lipid droplets is illustrated, showing their intense concentration in the interstitium (asterisks). Within seminiferous tubules, two populations of lipid droplets are evident: a smaller one typically facing the tubular lumen (arrow heads) and a less numerous, larger and more disperse type, located close to the basal membrane (arrows) of tubules. The lowest panel on the left corner shows the concentration of triacylglycerols (TAG) in the mouse pachytene spermatocytes (PS), round spermatids (RS) and residual bodies (RB) employed in this study. TAG were quantified by their fatty acids, and their concentration is expressed on the basis of a fixed amount of lipid phosphorus (P).

FIGURE 6. Amounts of main fatty acids in TAG and glycerophospholipids (GPL) in the mouse germ cells and residual bodies employed in this study. PS, pachytene spermatocytes; RS, round spermatids; and RB, residual bodies.

Table 1. Sequences of primers for RT-qPCR

Gen	*Primer*	*Sequence*	*Amplicon Length*
Fabp3	Forward Reverse	atccatgtgcagaagtggaa cactgccatgagtgagagtca	(91 nt)
Fabp5	Forward Reverse	acggctttgaggagtacatga ctcggttttgaccgtgatg	(122 nt)
Fabp7	Forward Reverse	caagaacacagagatcaatttcca catccaaccgaaccacaga	(92 nt)
Fabp9	Forward Reverse	cactgcagacaaccgaaaag tctgtttgccaagccatttt	(86 nt)
Fabp12	Forward Reverse	ttgaaaactacatgaaggaattgg agtgggctttgccagaca	(71 nt)
Dgat1	Forward Reverse	ggtcaaggccaaagctgtc gatctcggtaggtcaggttgtc	(95 nt)
Dgat2	Forward Reverse	tactccaagcccatcaccac ggcatggtacaggtcgatgt	(93 nt)
Plin2	Forward Reverse	cagccaacgtccgagatt actgtgctggctacagaatcc	(107 nt)

FIGURE 1

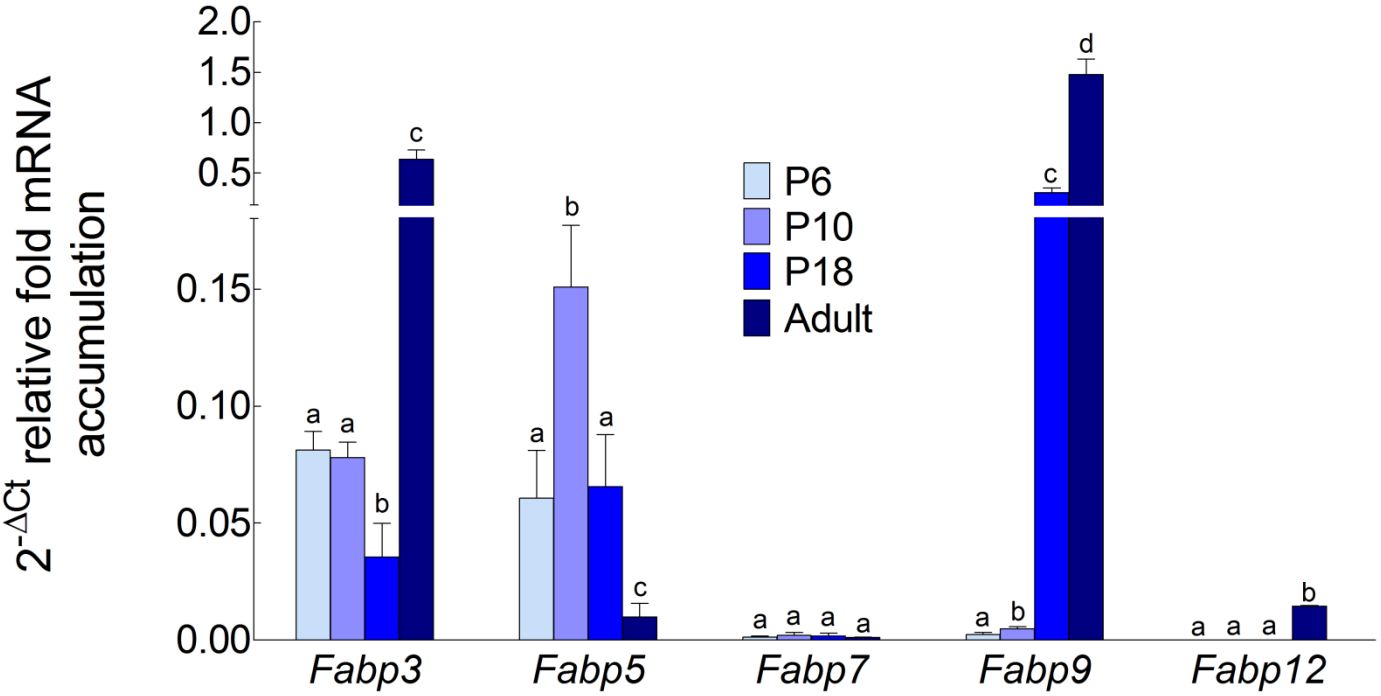

2-ΔCt relative fold mRNA accumulation
2.0
1.5
1.0
0.5
0.15
0.10
0.05
0.00
P6
P10
P18
Adult
a a b c
a b a c
a a a a
a b c d
a a a b
Fabp3
Fabp5
Fabp7
Fabp9
Fabp12

FIGURE 2

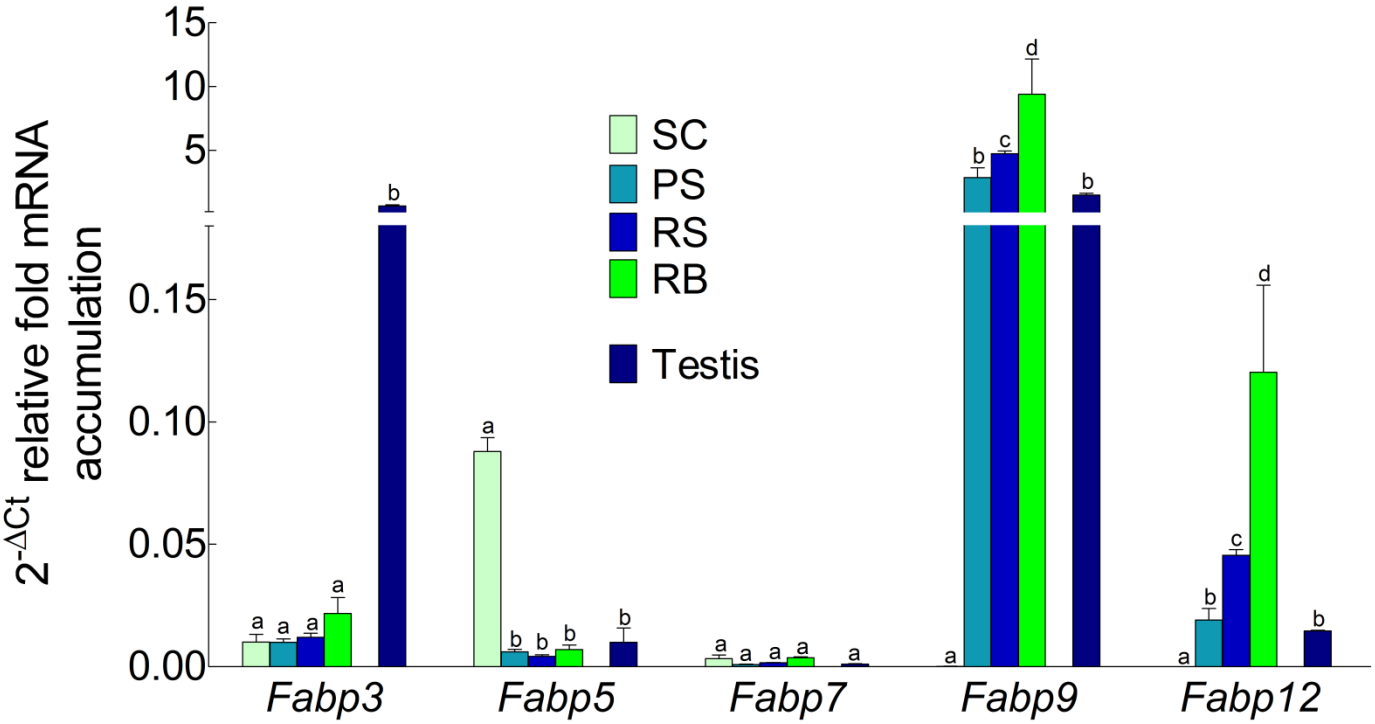

2-ΔCt relative fold mRNA accumulation
15
10
5
0.15
0.10
0.05
0.00
SC
PS
RS
RB
Testis
Fabp3
Fabp5
Fabp7
Fabp9
Fabp12

FIGURE 3

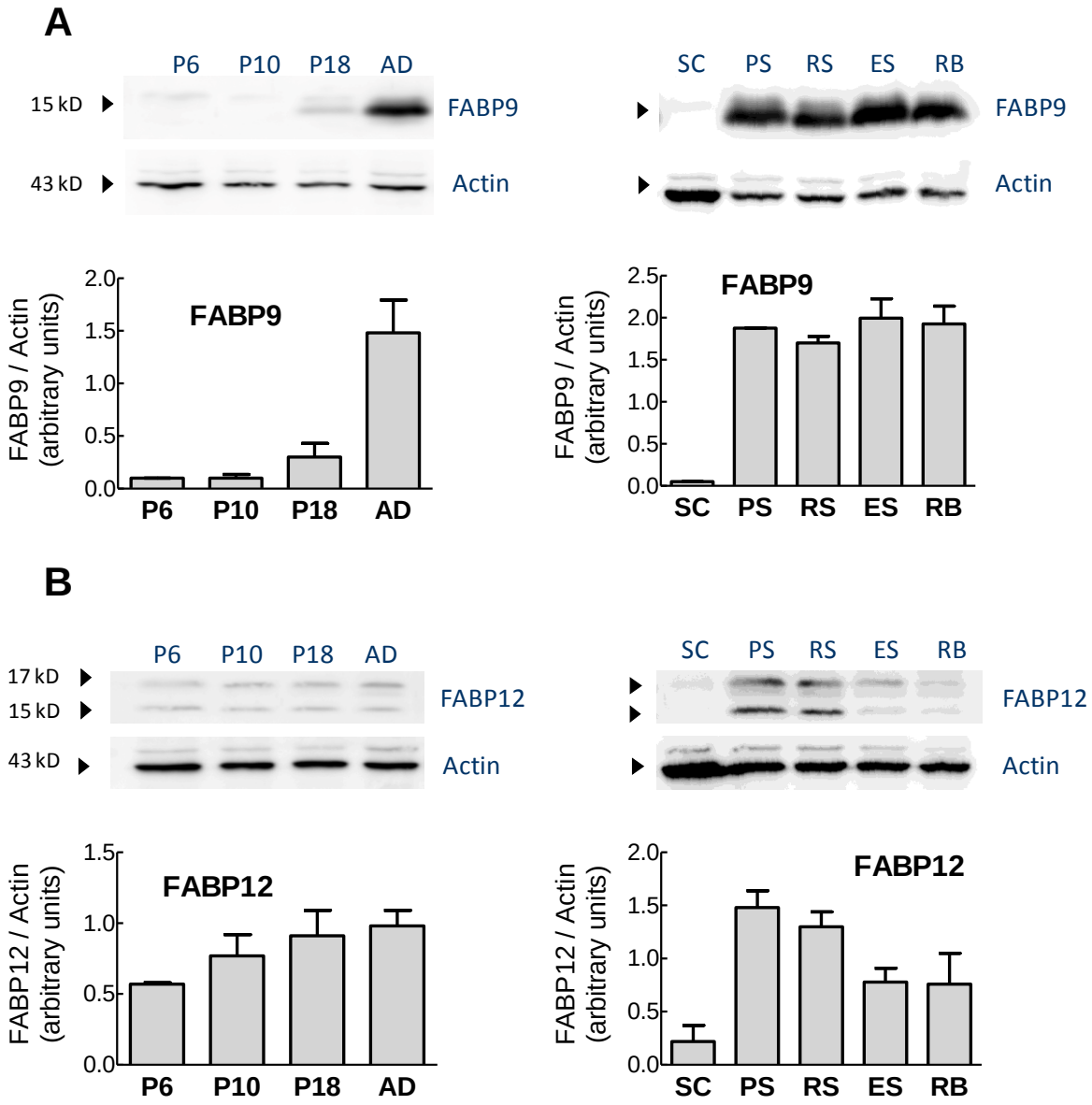

A
P6
P10
P18
AD
15 kD
FABP9
43 kD
Actin
SC
PS
RS
ES
RB
FABP9
Actin
FABP9 / Actin (arbitrary units)
FABP9
2.0
1.5
1.0
0.5
0.0
P6
P10
P18
AD
FABP9 / Actin (arbitrary units)
FABP9
2.5
2.0
1.5
1.0
0.5
0.0
SC
PS
RS
ES
RB
B
P6
P10
P18
AD
17 kD
15 kD
FABP12
43 kD
Actin
SC
PS
RS
ES
RB
FABP12
Actin
FABP12 / Actin (arbitrary units)
FABP12
1.5
1.0
0.5
0.0
P6
P10
P18
AD
FABP12 / Actin (arbitrary units)
FABP12
2.0
1.5
1.0
0.5
0.0
SC
PS
RS
ES
RB

FIGURE 4

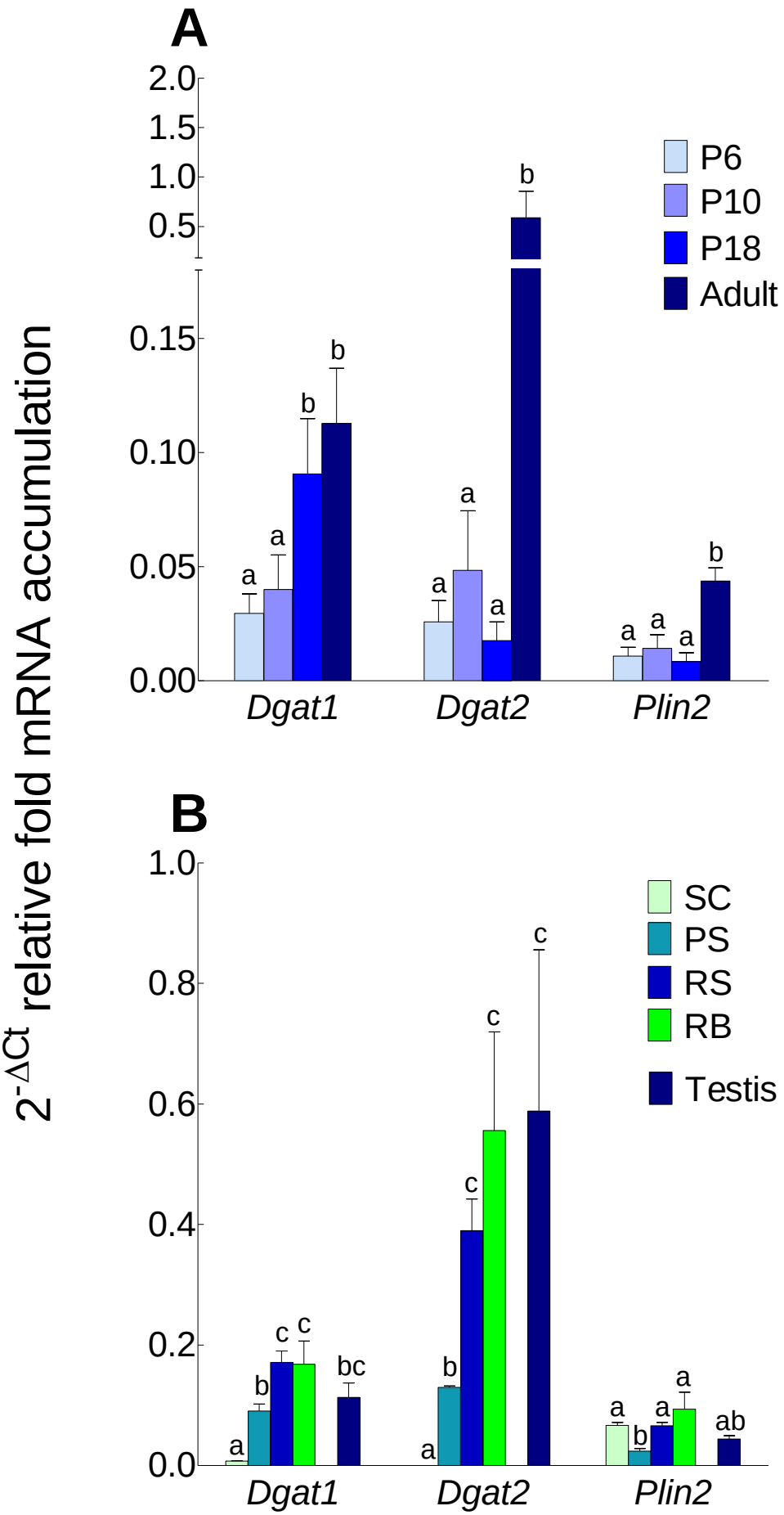

A
2.0
1.5
1.0
0.5
0.15
0.10
0.05
0.00
P6
P10
P18
Adult
Dgat1
Dgat2
Plin2
B
1.0
0.8
0.6
0.4
0.2
0.0
SC
PS
RS
RB
Testis
Dgat1
Dgat2
Plin2
2-ΔCt relative fold mRNA accumulation

FIGURE 5

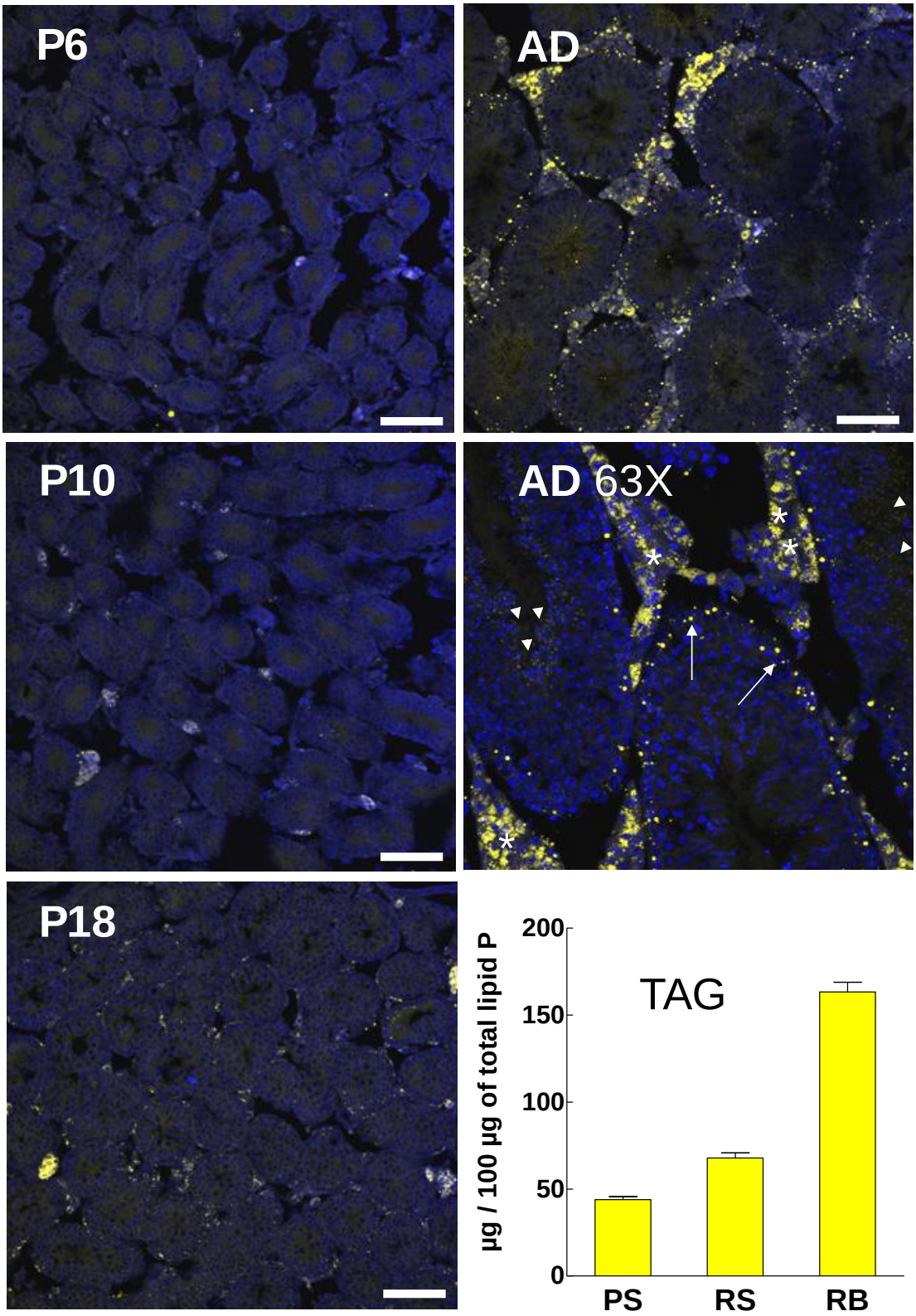
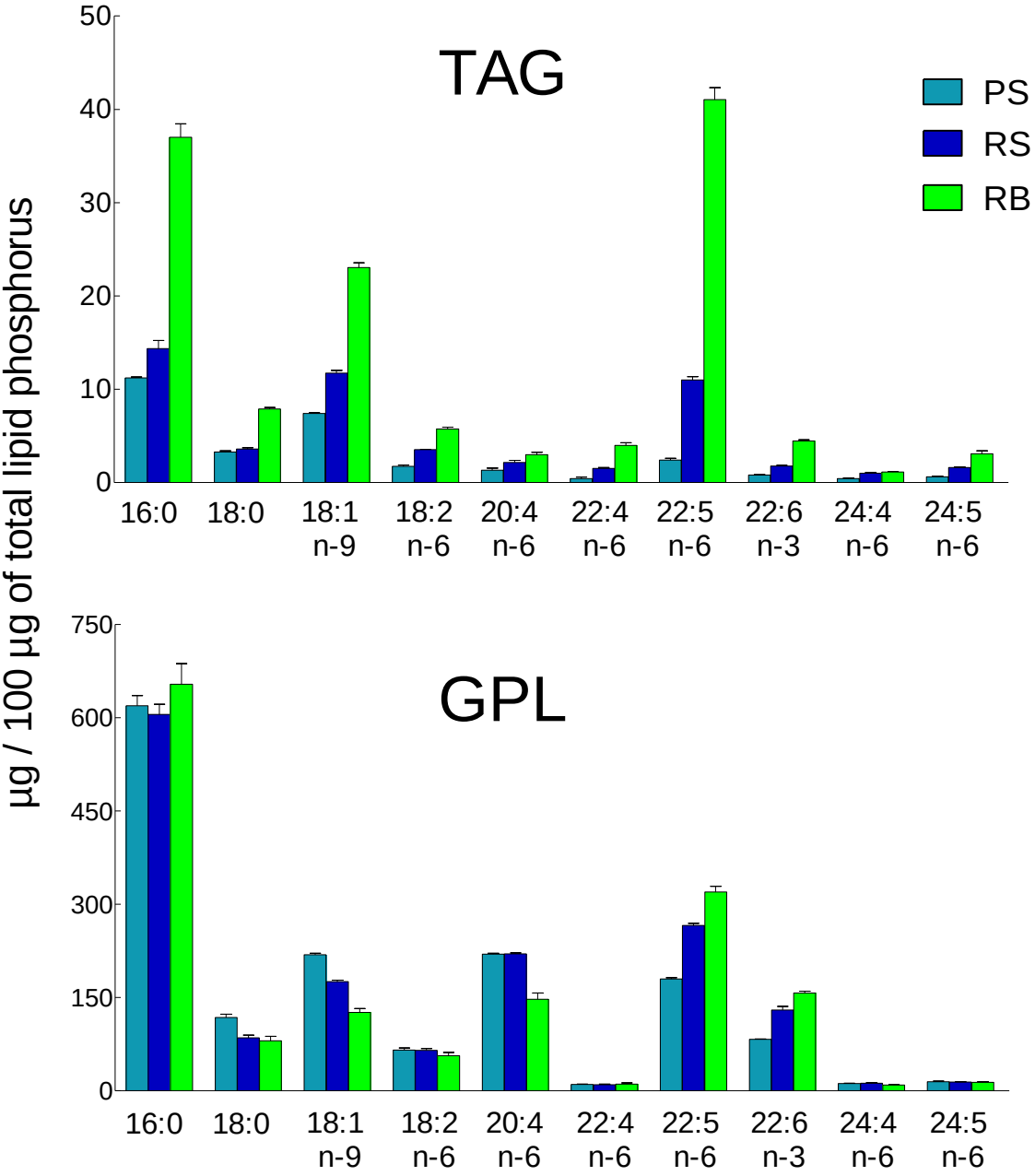

P6
AD
P10
AD 63X
P18
TAG
µg / 100 µg of total lipid P
200
150
100
50
0
PS
RS
RB

FIGURE 6


TAG
GPL
PS
RS
RB
µg / 100 µg of total lipid phosphorus
50
40
30
20
10
0
750
600
450
300
150
0
16:0
18:0
18:1 n-9
18:2 n-6
20:4 n-6
22:4 n-6
22:5 n-6
22:6 n-3
24:4 n-6
24:5 n-6