Phosphoprotein enriched in astrocytes-15 is expressed in mouse testis and protects spermatocytes from apoptosis

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Abstract

Phosphoprotein enriched in astrocytes (PEA-15) is a 15 kDa acidic serine-phosphorylated protein expressed in different cell types, especially in the CN. We initially detected the expression of PEA-15 in primary cultures of Sertoli cells. To assess the presence and localization of PEA-15 in the mouse testis, we studied the expression pattern of the PEA-15 protein by immunohistochemistry and mRNA by *in situ* hybridization. Both the protein and the mRNA of PEA-15 were localized in the cytoplasm of Sertoli cells, all types of spermatogonia, and spermatocytes up till zygotene phase of the meiotic prophase. Subsequently, with ongoing development of the spermatocytes, the expression decreased and was very low in the cytoplasm of diplotene spermatocytes. To analyze the possible role of PEA-15 in the developing testis, null mutants for PEA-15 were examined. As the PEA-15 C terminus contains residues for ERK binding, we studied possible differences between the localization of the ERK2 protein in wild type (WT) and *PEA-15*^{-/-} mice. In the WT testis, ERK2 was localized in the cytoplasm of Sertoli cells, B spermatogonia, preleptotene, leptotene, and zygotene spermatocytes, whereas in the KO testis, ERK2 was primarily localized in the nuclei of these cells and only little staining remained in the cytoplasm. Moreover, in PEA-15-deficient mice, significantly increased numbers of apoptotic spermatocytes were found, indicating an anti-apoptotic role of PEA-15 during the meiotic prophase. The increased numbers of apoptotic spermatocytes were not found at a specific step in the meiotic prophase.

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Introduction

PEA-15 (phosphoprotein enriched in astrocytes, 15 kDa) has been originally identified in the CN (Araujo *et al.* 1993, Danziger *et al.* 1995, Estelles *et al.* 1996) as a multifunctional protein implicated in various physiological and pathological processes (Renault *et al.* 2003), playing an anti-apoptotic role in cellular pathways in several cell types (Condorelli *et al.* 1998, Kitsberg *et al.* 1999), including cancer cells (Hao *et al.* 2001). It contains a death effector domain (DED) at its N terminus, thus sharing homology with other proteins involved in apoptosis (Kitsberg *et al.* 1999). One of the apoptotic pathways, known as the extrinsic pathway, involves the tumor necrosis factor (TNF) receptor superfamily of proteins, including Fas and TNFR1, conveying the death signals through DEDs. Phosphorylation of Ser¹¹⁶

regulates the anti-apoptotic function of PEA-15 and modulates its targeting to the death-inducing signaling complex (Xiao *et al.* 2002).

PEA-15 inhibits cell proliferation (Formstecher *et al.* 2001) and is more abundantly expressed in terminally differentiated cells promoting survival (Renault-Mihara *et al.* 2006). The C terminus of PEA-15 contains residues required for ERK 1/2 binding (Ramos *et al.* 1998, Formstecher *et al.* 2001, Hill *et al.* 2002). Upon various stimuli, ERK1/2 translocates to the nucleus allowing it to phosphorylate several transcription factors, required for entry into the cell cycle (Brunet *et al.* 1999). The nuclear import and export of ERK1/2 are regulated by a variety of protein–protein interactions that therefore serve as control points in cell proliferation. Interestingly, PEA-15 is one of the proteins identified to bind to

ERK1/2 and to regulate its subcellular localization. By various mechanisms, PEA-15 expression leads to a retention of ERK in the cytoplasm (Formstecher *et al.* 2001, Whitehurst *et al.* 2004).

The amino acid sequence of PEA-15 is conserved in human, mouse, rat, and hamster, and this protein is expressed in many tissues including brain, breast, lung, and prostate (Araujo *et al.* 1993, Danziger *et al.* 1995, Estelles *et al.* 1996). Thus far, the expression of PEA-15 in the testis has not been studied. In preliminary studies (unpublished results), we found an increased expression of PEA-15 in Sertoli cells exposed to doxorubicin. In this study, we demonstrate the presence of PEA-15 in the testis and we studied some functional aspects of PEA-15 in spermatogenesis, also using PEA-15-deficient mice.

Materials and Methods

Experimental animals

Eight-week-old *PEA-15*^{-/-} mice on a C57Bl6 background and wild type (WT) littermates were generated as described previously (Kitsberg *et al.* 1999) and maintained at Collège de France, Paris, France.

Adult CD-1 mice were purchased from Charles River (Wilmington, MA, USA) and used and maintained according to the regulations provided by the Animal Ethics Committee of the University of Utrecht, which also approved the experiments.

The mice were killed by CO₂ asphyxiation and the testes were fixed in RNase-free Bouin's solution or 4% paraformaldehyde (PFA) for histology, immunohistochemistry, and *in situ* hybridization purposes.

Immunohistochemistry

Anti-PEA-15 antibody staining

For immunolocalization of PEA-15, 5 µm paraffin sections were mounted on 3-aminopropyl triethoxysilane (TESPA, Sigma)-coated glass slides and dried overnight at 37 °C. A 10-min antigen retrieval (sodium citrate buffer, 0.1 mM, pH 6) step was performed at 700 W in a microwave oven thrice (Polaron H2500 Microwave Processor, BioRad). Endogenous peroxidase was blocked with 0.35% H₂O₂ in PBS for 15 min. After blocking in 5% normal horse serum (Vector Laboratories, Burlingame, USA), slides were consecutively incubated with avidin and biotin blocking solutions (Vector Laboratories) and then with 1:1000 Ab-7 rabbit polyclonal anti-PEA-15 antibody (Sharif *et al.* 2004) in 1% BSA in PBS for 1 h at 4 °C (Sigma). Subsequently, the sections were incubated with PowerVision poly HRP-anti-mouse/rabbit/rat IgG (one component) ready-to-use secondary antibody (#DPVO-110HRP, Immunologic, ImmunoVision Tech. Co., Brisbane, CA, USA) for 30 min at room temperature. Antibody was finally detected using diaminobenzidine (DAB; Sigma) in

50 mM Tris-HCl (pH 7.6). Sections were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd, Hemel Hempstead, UK).

Rabbit IgG (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to replace the primary antibody in immunohistochemistry to check the specificity of the staining. Experiments were repeated at least thrice.

Anti-ERK2 staining

Paraffin sections were mounted on coated glass slides and dried overnight at 37 °C. Endogenous peroxidase was blocked with 3% H₂O₂ in PBS for 10 min. After blocking in 5% normal goat serum (Vector Laboratories) in 1% BSA in PBS (Sigma) for a minimum of 15 min, the sections were incubated with mouse monoclonal anti-ERK2 primary antibody (sc-1647, Santa Cruz Biotechnology) with 1:100 dilution in 1% BSA in PBS for 1 h at room temperature. Subsequently, the sections were incubated with PowerVision poly HRP-anti-mouse IgG, ready-to-use secondary antibody (#DPVM-110HRP, ImmunoVision Tech. Co.) for 30 min at room temperature, according to manufacturer's instructions. DAB was used as chromogen for detection. Sections were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd).

A blocking peptide specific for ERK2 (sc-1647-p, Santa Cruz Biotechnology) was used to monitor the specificity of the staining. Experiments were repeated at least thrice.

Preparation of RNA probes

An approximately 116 bp mouse PEA-15 cDNA insert, cloned in the vector pGEM-T-Easy (Promega), was linearized by *Pst*I and *Sph*I and *in vitro* transcribed to generate antisense and sense digoxigenin (DIG)-labeled cRNAs using DIG-RNA labeling mix (Roche), and T7 RNA polymerase and SP6 RNA polymerase respectively, according to manufacturer's instructions.

Cell culture

SK49 and TM4 Sertoli cell lines were grown at 37 °C in a humidified incubator under 5% CO₂, 95% air in MEM supplemented with 2.5% FBS, in culture flasks. Culture medium was replaced twice a week. At confluency, cells were passaged following trypsinization with 0.25% trypsin-EDTA solution (Life Technologies).

In situ hybridization

An *in situ* hybridization protocol was applied as described earlier (Mizrak *et al.* 2006). Initially, a post-fixation step was performed with 4% paraformaldehyde for 15 min at room temperature followed by a digestion step with proteinase K at a concentration of 10 µg/µl and

again a second post-fixation step. Then, a DNase (Roche; RNase free, 100 IU in PBS) control was introduced for 1 h at 37 °C. Hybridization was carried out overnight at 55 °C with 200 ng/ml PEA-15 digoxigenin-labeled RNA probes in the hybridization solution (10% dextran sulfate, 4×SSC (1×SSC consists of 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 50% formamide, 10 mM dithiothreitol (DTT), 0.25 mg/ml poly A, 0.25 mg/ml denatured and sheared herring sperm DNA, and 1×Denhardt's buffer), following 2 h of pre-hybridization step at 55 °C. After hybridization, sections were quickly washed with 1×SSC (containing 10 mM DTT) at room temperature, two successive washes of 15 min were done with 1×SSC (containing 10 mM DTT) at 55 °C, with further two washes of 15 min with 0.5×SSC (containing 10 mM dithiothreitol) also at 55 °C. RNase treatment was introduced immediately after the stringency washes. The slides were then washed thrice with Tris-buffered saline (TBS) and incubated for 30 min in a blocking solution (TBS+0.3% Triton X-100+2% normal sheep serum). The RNA probes were detected using a polyclonal anti-digoxigenin antibody (#1333089, Roche) in a 1:100 dilution in the blocking solution. After overnight incubation at 4 °C, the slides were washed four times with TBS (10 min each) and incubated with the biotinylated anti-sheep secondary antibody (Vector Laboratories) 1:100 dilution in PBS for 1 h at room temperature. Horseradish peroxidase–avidin–biotin complex reaction was performed according to manufacturer's protocol (Vector Laboratories). Finally, detection was done using DAB (Sigma) in 50 mM Tris–HCl (pH 7.6). Sections were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd). The *in situ* hybridization experiments were repeated at least thrice.

RT-PCR

RNA was extracted from mouse testis and mouse-derived SK49 (Walther *et al.* 1996) and TM4 (Mather 1980) Sertoli cell lines using the FastRNA Pro Green kit (Qbiogene, Illkirch Cedex, France), according to manufacturer's instructions. Two micrograms of each total RNA fraction were reverse transcribed in a 20 µl volume, using random hexamers and the Superscript II pre-amplification system (Invitrogen), according to manufacturer's instructions. RT reactions were performed with (+RT) and without (–RT) Superscript II reverse transcriptase.

Each type of cDNA was used as a template for PCR amplification using the PEA-15-specific forward primer 1663 (5'-CAGCTCAAGTCAGCCTGCAA-3') and reverse primer 1664 (5'-CTTGTGTGGCTCTCCAGGAA-3'). PCRs were carried out in 50 µl volumes, each containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, 200 µM of each dNTP, 50 pmol primer 1663, 50 pmol primer 1664, 1 µl cDNA, and 1 U

SuperTaq (HT Biotechnologies Ltd, Cambridge, UK). PEA-15-specific PCR products of ≈100 bp were visualized on 2% agarose gel.

TUNEL on PEA-15^{−/−} and WT testis

Bouin's fixed, paraffin-embedded testis sections were boiled for 5 min in 10 mM citric buffer (pH 6.0) at 98 °C and slowly cooled to room temperature. Endogenous peroxidase was blocked with 3% H₂O₂ in MilliQ for 5 min. Sections were washed thrice with PBS before 60-min incubation in TUNEL mix at 37 °C. TUNEL mix consists of 0.3 U/ml calf thymus terminal deoxynucleotidyl transferase (Amersham Biosciences), 6.66 µM/µl biotin dUTP (Roche) in terminal transferase buffer (Amersham Biosciences). The TUNEL reaction was stopped by incubation in 300 mM NaCl, 30 mM sodium citrate in Milli Q water for 15 min at room temperature. After washing in PBS, sections were blocked with 2% BSA (Sigma) in PBS at room temperature for 10 min. Sections were treated for 30 min at 37 °C in a moist chamber with a 1:20 dilution of ExtrAvidin peroxidase antibody (E2886, Sigma). After three washes in PBS, detection was performed with DAB+ (Dako, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd).

The numbers of TUNEL-positive cells were counted in both testis types and calculated as numbers per 100 tubule cross sections. At least 100 tubules per animal were studied. The numbers were expressed as mean ± S.E.M. and statistical analysis was performed using the split-plot ANOVA (mixed model analysis). The model assumptions for ANOVA are equal variances in the groups which are compared and a normal distribution of the data. Both assumptions were checked.

For the analysis of possible differences per epithelial stage in PEA-15 KO and WT mice with respect to mean numbers of apoptotic cells, we used split-plot ANOVA. This analysis showed a significant interaction between the mouse type and the stage ($P=0.001$), and therefore, performed *post hoc* testing at each stage to determine whether there was a difference between the two mouse types. This was done by performing an ANOVA at each stage (in essence this is a two-sample independent *t*-test). Consequently, a Bonferroni correction was performed.

SDS-PAGE and western blotting

Protein lysates from PEA-15 KO and WT testes were prepared in radio-immunoprecipitation assay (RIPA) buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) including 1 mM phenylmethylsulfonyl fluoride. Of each sample, 50 µg were separated on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA). Western blots were blocked using

Blotto-A, containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in TBS (10 mM Tris; 150 mM NaCl, pH 7.6), including 0.05% Tween 20. Rabbit monoclonal anti-phospho-p44/42 MAPK (4376, Cell Signaling) antibody was diluted 1:500 in Blotto-A and incubated overnight at 4 °C. The blot was washed with TBS including 0.05% Tween 20. After incubation with a 1:1000 dilution of anti-rabbit-HRP secondary antibody (P-0260, DAKO Cytomation) for 1 h, blots were incubated with ECL and exposed to X-ray film (RX-omat, Kodak). For internal control, anti- α -tubuline antibody (MS-581-PO, Neomarkers, Freemont, CA, USA) was applied to the blot.

Results

Localization of PEA-15

Immunohistochemistry, applying the A-7 polyclonal antibody against PEA-15 (Sharif *et al.* 2004), resulted in the localization of the PEA-15 protein in the cytoplasm of Sertoli cells and all A spermatogonia, intermediate (In) spermatogonia, and B spermatogonia (Fig. 1A). Furthermore, there was a remarkable staining in the cytoplasm of preleptotene and leptotene spermatocytes (Fig. 1C and D). The staining that became weaker with the ongoing development was observed in the cytoplasm of pachytene spermatocytes, and staining was faint in the cytoplasm of diplotene spermatocytes (Fig. 1C). No staining was detected in spermatids (Fig. 1D). A PEA-15^{-/-} mouse testis was used as a negative control for the immunohistochemistry together with rabbit IgG in a concentration equal to the IgG concentration of the applied antibody. We did not detect any staining in testis

sections of PEA-15 null mice (Fig. 1B) and in the IgG controls (not shown).

The *in situ* hybridization analysis showed the PEA-15 mRNA to be present in germ cells and Sertoli cells in all stages of the cycle of the seminiferous epithelium (Fig. 2A). After hybridization, RNase treatment was carried out to see whether the signal was specific (Fig. 2C). As another control, the DIG-labeled sense riboprobe was applied in the same concentration as the antisense probe, and no signal was detected (Fig. 2B). PEA-15 mRNA was localized in the cytoplasm of all spermatogonial cell types, and preleptotene and leptotene spermatocytes (Fig. 2C and D). The PEA-15 mRNA was also detected in the cytoplasm of Sertoli cells (Fig. 2C and D). The signal started to diminish in pachytene spermatocytes and completely disappeared in diplotene spermatocytes. Round and elongated spermatids did not express PEA-15 mRNA (Fig. 2C and D).

The presence of PEA-15 in the mouse testis and mouse-derived SK49 and TM4 Sertoli cell lines was studied using the RT-PCR technique. Expression of PEA-15 was found in SK49 and TM4 Sertoli cell lines and in the mouse testis (data not shown).

Apoptosis of germ cells in WT and PEA-15 knockout mice

Complete spermatogenesis was observed in the PEA-15 KO mouse testis. However, the number of apoptotic germ cells seemed higher than that in controls. Therefore, we performed TUNEL labeling to detect apoptotic germ cells (Fig. 3A and B). Especially in the PEA-15-deficient testis, we observed apoptotic spermatogonia, spermatocytes, and round spermatids, whereas spermatocytes were the

Figure 1 Detection of PEA-15 protein in mouse seminiferous tubules. A: Cells in all tubules stain positive for PEA-15, B: No staining is observed in the PEA-15 null mouse testis, C and D: Sertoli cells show a cytoplasmic staining (thin arrow). Staining for PEA-15 is also observed in pachytene spermatocytes (arrow head), preleptotene spermatocytes (asterisk), leptotene spermatocytes (hollow arrow head), and B spermatogonia (thick arrow). No staining is present in round spermatids (star). Bars represent: A and B, 30 μ m; C and D, 10 μ m.

Figure 2 *In situ* hybridization for PEA-15 mRNA. A: Antisense probe hybridized slide, staining is present in all tubules. B: Sense riboprobe hybridized slide as a negative control, no staining is observed. C: RNase-treated control slide. mRNA localization can be seen in the cytoplasm of Sertoli cell (thin arrow) and in that of pachytene spermatocytes (arrow head). No mRNA localization in round spermatids is observed (star). D: Antisense probe hybridized slide, staining is seen in all tubules. mRNA localization is present in the cytoplasm of Sertoli cell (thin arrow), pachytene spermatocytes (arrow head), leptotene spermatocytes (hollow arrow head), and preleptotene spermatocytes (asterisk). No mRNA localization in round spermatids (star). Bars represent: A and B, 100 μ m; C, 50 μ m; D, 10 μ m.

most affected cell type. Therefore, we determined the numbers of TUNEL-positive spermatocytes per 100 seminiferous tubule cross sections. At least 100 tubules per animal were studied. We found a clear, statistically significant increase in the numbers of apoptotic spermatocytes in *PEA-15*^{-/-} versus WT testes (Fig. 4).

We also studied the distribution of the apoptotic spermatocytes over the epithelial cycle to see whether the increased apoptosis could be traced to a specific step in the development of the spermatocytes. For that purpose, we counted on an average 234 tubules per testis. The increase in the numbers of apoptotic spermatocytes was found to be not stage specific (Fig. 5). Differences in the numbers of apoptotic spermatocytes in stages IX and XI reached significance in *PEA-15*^{-/-} testes compared with WT testes (Fig. 5).

Localization of ERK2

We performed immunohistochemistry to localize the ERK2 protein in both WT and PEA-15 knockout mouse

testes. In the WT testis, ERK2 was localized in the cytoplasm of Sertoli cells, B spermatogonia, preleptotene, leptotene, and zygotene spermatocytes. Especially in spermatocytes, also some nuclear staining for ERK2 was found (Fig. 6A and C). In contrast, in the KO testis, ERK2 was primarily localized in the nuclei of B spermatogonia, preleptotene, leptotene, and zygotene spermatocytes, while only a weak staining was observed in the cytoplasm of these germ cells and Sertoli cells (Fig. 6B and D).

To study the active form of ERK2, western blotting of proteins of PEA-15 KO and WT testes was carried out. No difference was found in phosphorylated ERK2 expression in *PEA-15*^{-/-} versus WT mice testes (Fig. 7A and B). However, phosphorylated ERK1 expression, which was also detected by the antibody, decreased in *PEA-15*^{-/-} mice testes compared with WT testes (Fig. 7C).

Discussion

The present results show that PEA-15 is expressed in the testis, both in Sertoli cells and in germ cells up till shortly

Figure 3 TUNEL analysis of apoptotic cells (brown) in A seminiferous tubules of wild type mice, B the PEA-15 KO mouse testis showing increased numbers of apoptotic germ cells. Bars represent 50 μ m.

Figure 4 Numbers of apoptotic spermatocytes in wild type and PEA-15 KO testes.

Figure 5 Distribution of apoptotic cells in various epithelial stages of the seminiferous epithelium of wild type and PEA-15 KO mice.

before the end of the meiotic prophase and the meiotic divisions. In the testis too, it appeared to have an anti-apoptotic role as enhanced germ cell death occurred in the PEA-15-deficient testis.

The PEA-15 protein and mRNA were found in the cytoplasm of Sertoli cells and all A, In, and B

spermatogonia, preleptotene, and zygotene spermatocytes. Subsequently, in the cytoplasm of pachytene spermatocytes, the expression decreased with ongoing development of these cells and expression was very weak in diplotene spermatocytes. In these cell types, the PEA-15 protein and mRNA were found to be present simultaneously.

To learn more about the function of PEA-15 in the testis, we studied testes of PEA-15-deficient mice. Spermatogenesis was found to be apparently normal, except for a virtual doubling in the number of apoptotic spermatocytes in PEA-15-deficient mice compared with WT mice. Counts of apoptotic spermatocytes revealed that the increase in apoptosis was not restricted to a specific step in spermatocyte development but occurred throughout the epithelial cycle. Apparently, PEA-15 has an anti-apoptotic function throughout the meiotic prophase. This is in contrast to the situation in mice deficient in various other proteins with a function during meiosis, such as *Msh5*, *Atm*, *Dmc1*, and *Spo11*, in which massive apoptosis of spermatocytes occurs specifically in epithelial stage IV (de Vries *et al.* 1999, de Rooij & de Boer 2003, Hamer *et al.* 2004, Barchi *et al.* 2005).

Spermatocytes have been found to express the FAS receptor and Sertoli cells are known to express and secrete FAS ligand (FasL; Lee *et al.* 1999). Soon after and dependent on the ligation of FasL to its receptor, FasL induces caspase-8 or -10 activation (Ohta *et al.* 1996, Blanco-Rodriguez & Martinez-Garcia 1998, Lee *et al.* 1999, Lue *et al.* 1999), and in this process, PEA-15 functions by blocking caspase-8 activation (Tschopp *et al.* 1998). In view of this, it is possible that the increased germ cell apoptosis in the PEA-15 KO mouse

Figure 6 Detection of ERK2 protein in WT and PEA-15 KO mouse seminiferous tubules. A WT mouse seminiferous tubule in stages V-VI and step 15 spermatids are depicted (hollow star). Cytoplasmic staining present in Sertoli cells (thin arrow) and pachytene spermatocytes (arrow head), B PEA-15 KO mouse seminiferous tubule in epithelial stage VII and step 16 spermatids are depicted (square). Cytoplasmic staining present in Sertoli cell (thin arrow), and also a nuclear staining is seen in preleptotene and pachytene spermatocytes (arrow head), C WT mouse seminiferous tubule in epithelial stages I-III and step 13 spermatids (10-point star) and stage XI are depicted. Cytoplasmic staining is shown in early pachytene spermatocytes (thick arrow) and zygotene spermatocytes (wavy arrow), D KO mouse seminiferous tubule in stages I-III and step 13 spermatids (10-point star) and stage X are depicted. Weak cytoplasmic staining is shown in Sertoli cell (thin arrow) and very dense nuclear staining in early pachytene spermatocytes (thick arrow). Bars represent 25 µm.

Figure 7 A: Western blot analysis of phosphorylated ERK1/phosphorylated ERK2 protein expression and internal control, α -tubuline expression in WT versus PEA-15 KO mouse testes, B: Quantification of western blot analysis of phosphorylated ERK1/phosphorylated ERK2 protein expression in WT versus PEA-15 KO mouse testes compared with α -tubuline expression, C: Quantification of western blot analysis of phosphorylated ERK1 protein expression in WT versus PEA-15 KO mouse testes compared with α -tubuline expression.

testis is due to the lack of inhibition of caspase-8 activation in spermatocytes, subsequently causing apoptosis of some of these cells.

Previously, we found an increased expression of PEA-15 in Sertoli cells exposed to doxorubicin, which was shown to be a reprotoxicant capable of altering gene expression (Bonilla & del Mazo 2003). Possibly, the upregulation of PEA-15 is a cellular response to toxicants to protect the cells from apoptosis.

ERK1 and ERK2 are the two major isoforms of ERK and they are activated by phosphorylation (Blenis 1993). ERK2 seems to be the most important isoform as its knockout, but not that of ERK1, is embryonic lethal in mice (Wong & Cheng 2005). PEA-15 has a function in the MAPK pathway by controlling ERK2 subcellular localization, i.e., maintaining ERK2 in the cytoplasm of cells, and thereby inhibiting its proliferation-stimulating function (Goldberg 1999, Formstecher *et al.* 2001, Janssens & Goris 2001, Janssens *et al.* 2005). Therefore, we studied ERK2 localization in the WT testis, where it was found mainly in the cytoplasm of Sertoli cells, B spermatogonia, and early spermatocytes, although some immunoreactivity was found in the nuclei of these cells. In contrast, in the PEA-15 KO testis, ERK2 was mainly localized in the nuclei of Sertoli cells, B spermatogonia, and early spermatocytes, and only little staining was observed in the cytoplasm of these cells. The massive translocation to the nucleus of the activated ERK2 in the testis of PEA-15-deficient mice in spermatocytes may also have led to increased apoptosis of these cells. The meiotic prophase is a complex and strictly regulated process and the abnormal presence and proliferation-stimulating activity of ERK-2 in the spermatocyte nuclei may be fatal to some of these cells. Despite the difference in subcellular localization of ERK2, western blotting did

not detect any difference in the amount of phosphorylated ERK2 protein expression in PEA-15^{-/-} versus WT mice testes.

Okadaic acid (OA) causes meiotic progression and chromosome condensation in pachytene spermatocytes (Wiltshire *et al.* 1995, van den Ham *et al.* 2003). It has been shown that preincubation of cultured pachytene spermatocytes, with a selective inhibitor of ERK-activating kinases, MEK1/2, completely blocks the ability of OA to induce chromosome condensation and progression to meiotic metaphase (Sette *et al.* 1999). This suggests that ERK1 is specifically activated during G₂/M transition in mouse spermatocytes, that it contributes to the mechanisms of maturation promoting factor activation, and that it is essential for chromosome condensation associated with progression to meiotic metaphases (Sette *et al.* 1999). Interestingly, in the present study, phosphorylated ERK1 expression was found to be decreased in PEA-15^{-/-} mice testes compared with WT testes. This might be another cause for increased apoptosis of spermatocytes in PEA-15 knockout mice.

Inselman & Handel (2004) investigated the expression of ERK1 and ERK2 during spermatogenesis by western blot analysis of isolated cell types using an ERK antibody which recognizes both 44 kDa ERK1 and 42 kDa ERK2 proteins with similar specificity. ERK2 protein was demonstrated to be present in all types of germ cells in the testis. In the same study, ERK1 and ERK2 were found to be localized throughout the chromatin structure of spermatocytes. In the present study, we have performed immunohistochemistry to investigate the cellular localization pattern of ERK2 protein with an antibody that was used for immunocytochemistry technique by Inselman & Handel (2004), which only recognizes ERK2.

We detected the ERK2 protein in Sertoli cells, B spermatogonia, preleptotene, leptotene, and zygotene spermatocytes, but not in spermatids. We have also used p-MAPK antibody for western blotting that was used for immunocytochemistry purpose by [Inselman & Handel \(2004\)](#). The difference between our study and the previous one ([Inselman & Handel 2004](#)) in finding ERK2 in spermatids may lie in the use of same antibodies in different techniques.

In conclusion, for the first time, we show that PEA-15 is present in the testis in Sertoli cells, all types of spermatogonia, and spermatocytes up till just before meiotic divisions. Spermatogenesis in PEA-15 knockout mice is grossly normal except for a clear increase in spermatocyte apoptosis. The increase in spermatocyte apoptosis in PEA-15 knockout mice may be related to a lesser inhibition of the apoptosis executioner caspases or the observed increased activation of ERK2 in spermatocytes. In that case, the increased apoptosis of spermatocytes is caused by a direct effect of the PEA-15 deficiency on spermatocyte viability. While this possibility seems to be the most likely one, we cannot exclude that PEA-15 deficiency may also compromise Sertoli cell function in such a way that spermatocyte development is insufficiently supported in PEA-15-deficient mice, causing the increased spermatocyte apoptosis. This will have to be studied in further detail. Finally, in contrast to spermatocytes, spermatogonia were apparently not affected by the PEA-15 deficiency. Possibly, PEA-15 in spermatogonia is of less importance or its function can be taken over by other proteins.

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