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Disturbed spermatogenic signaling in pituitary adenylate cyclase activating polypeptide deficient mice

Running title: **Spermatogenesis in PACAP deficient mice**

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Abstract

PACAP is a neuropeptide with diverse functions in various organs, including reproductive system. It is present in the testis in high concentrations, and in addition to the stage-specific expression within the seminiferous tubules, PACAP affects spermatogenesis and the functions of Leydig and Sertoli cells. Mice lacking endogenous PACAP show reduced fertility, but the possibility of abnormalities in spermatogenic signaling has not yet been investigated. Therefore, we performed a detailed morphological analysis of spermatozoa, sperm motility, and investigated signaling pathways that play a role during spermatogenesis in knockout mice. No significant alterations were found in testicular morphology or motility of sperm in homo- and heterozygous PACAP deficient mice in spite of the moderately increased number of severely damaged sperms. However, we found robust changes in mRNA and/or protein expression of several factors that play an important role in spermatogenesis. Protein kinaseA expression was markedly reduced, while downstream phospho-ERK and p38 were elevated in knockout animals. Expression of major transcription factors, such as Sox9 and phospho-Sox9 was decreased, while that of Sox10, as a redundant factor, was increased in PACAP deficient mice. The reduced phospho-Sox9 expression was partly due to increased expression and activity of phosphatase PP2A in knockout mice. Targets of Sox transcription factors, such as Collagen type IV was reduced in knockout mice. In summary, our results show that lack of PACAP leads to disturbed signaling in spermatogenesis, which could be a factor responsible for reduced fertility in PACAP knockout mice, and further support the role of PACAP in reproduction.

Keywords: spermatogenesis, fertility, neuropeptide, neurotrophic factor, Sox transcription factors

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated as a hypothalamic neuropeptide that stimulates adenylate cyclase activity, and thus, the release of several hormones in the pituitary (Miyata et al. 1989). PACAP plays diverse roles in the endocrine system, including the gonadal axis (Bardosi et al. 2016, Counis et al. 2007, Egri et al. 2016, Vaudry et al. 2009, Koves et al. 2014, 2016). The modulatory effects on

gonadotropin secretion suggest a role for PACAP in reproduction (Apostolakis et al. 2005, Kanasaki et al. 2016, Sherwood et al. 2000). An important discovery regarding male reproductive system came soon after the isolation of PACAP, showing that the peptide can be found in the testis in high concentrations, similar in range to those in the CNS (Arimura et al. 1991). The high levels of PACAP in the testis suggest that it plays an important role in spermatogenesis and/or sperm functions (Daniel et al. 2000, Li and Arimura 2003, Li et al. 2000). Indeed, PACAP has been shown to influence the development and functioning of spermatozoa (Gozes et al. 1998, Li et al. 2004, Koh et al. 2003, 2006). PACAP expression was found mainly in immature and, to a smaller extent, in mature sperms (Hannibal and Fahrenkrug 1995). PACAP regulates the activity of the supporting Sertoli cells, and acts at the hormonal level influencing the synthesis of testosterone by Leydig cells. Epididymis-derived PACAP may also influence the final stages of spermiogenesis (Csaba et al. 1997, El-Gehani et al. 2000, Heindel et al. 1992, Lacombe et al. 2006, Li et al. 2004, Matsumoto et al. 2008, Rossato et al. 1997, Shioda et al. 1994, Tanii et al. 2011, Yanaihara et al. 1998, West et al. 1995).

In, mature sperms of golden hamster, it has been found that the addition of $PACAP_{7-27}$ hybrid antagonist results in a reduction in motility , implying the stimulatory effect of PACAP on sperm motility (Gozes et al. 1998). Indeed, PACAP has been reported to increase motility and penetration of ovum to promote fertilization in mice (Tanii et al. 2011). In an earlier study, we had confirmed this hypothesis in human sperms: we found that PACAP stimulated the slowly moving population, while it did not influence the cells with normal motility (Brubel et al. 2012). Furthermore, PACAP has been shown to regulate the synthesis of both secreted and intracellular proteins of spermatids and spermatocytes in vitro (West et al. 1995).

Mice deficient in PACAP are known to have several abnormalities during development (Nemeth et al. 2014, Sandor et al. 2016, Reglodi et al. 2012) and they also display reduced fertility (Reglodi et al. 2012, Sherwood et al. 2007). This is not limited to just one factor being abnormally altered but it seems that the reproduction of these animals is affected at several levels. PACAP deficient mice have been described to have impaired implantation (Isaac and Sherwood 2008), which, together with the increased rate of early postnatal death also leads to a reduced number of offspring (Gray et al. 2001, Wilson and Cumming, 2008). As PACAP is involved in several other processes, such as gametogenesis (Apa et al. 2002, Barberi et al. 2007, Canipari et al. 2016, Koppan et al. 2012, Li et al. 2004), placental development (Horvath et al. 2014, 2016) and gonadotropin regulation (Kanasaki et

al. 2016), it can be hypothesized that lack of PACAP may also influence reproduction in germ cells (Lacombe et al. 2006). In a previous study we found that the diameter of sperm heads in PACAP deficient mice was smaller, (Brubel et al. 2012), but no data was obtained as to whether this resulted in abnormal motility. Therefore, the aim of the present study was to perform detailed morphological analysis of spermatozoa, sperm motility, and to investigate signaling pathways playing a role during spermatogenesis in order to elucidate the role of endogenous PACAP at the molecular level.

Materials and Methods

Animals and genotyping protocol

Generation of male PACAP-deficient mice on CD-1 background was described earlier and it was demonstrated that heterozygous mice show a ~70% reduction of PACAP level (Hashimoto et al. 2001). Three-months-old wild-type (WT), heterozygous (HZ) and homozygous knockout (KO) mice were kept under light/dark cycles of 12/12 hours with free access to food and water. The study was carried out in accordance with ethical guidelines (ethical permission number for this study: BA02/2000-15024/2011, University of Pecs, Hungary). Genotyping was performed using Phire Animal Tissue Direct PCR Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primer sequences used for detection of wild-type and KO DNA signatures of PACAP were identical with those used earlier (Farkas et al. 2017, Hashimoto et al. 2001).

Light and electron microscopical morphology

Testes of 3-month-old wild-type (WT) (n=11), heterozygous (HZ) (n=10) and KO mice (n=8) were fixed in Bouin fixative containing (per 100 ml) 75 ml picric acid in saturated aqueous solution, 25 ml formalin (40% aqueous solution) and 5 ml glacial acetic acid, then embedded in paraffin. Serial sections were made and HE staining was performed (HE, Sigma-Aldrich, MO, USA). Photomicrographs were taken using an Olympus DP72 camera on a Nikon Eclipse E800 microscope. Sperms from a separate group of WT and KO animals were also investigated with electron microscopy (n=5-5 WT and KO). For electron microscopic evaluation, epididymis-derived sperms were fixed in 2% of formaldehyde and 2.5% of glutaraldehyde fixative overnight in 4^{o}C after dissection. Samples were washed and dehydrated. One drop of the supernatant was dropped on a glass surface and it was sputter

coated with gold. Samples were examined and photographed in a JEOL 1200EX electron microscope.

Evaluation of sperm quality

Sperms were collected from the caudal part of the epididymis after cervical dislocation of 3-month-old adult male mice (n=18 WT, n=9 HZ and n=16 KO). The dissected caudal epididymis was placed in phosphate buffered saline (PBS) supplemented with 10% BSA and torn apart into small pieces followed by 5-minutes of incubation on a warming plate at 37.5 °C. During incubation the spermatozoa had time to swim out of the epididymal duct. Obtained sperms were placed in PBS. Following 5 minutes of incubation on a warming plate at 37.5°C, sperm motility was measured with CASA System (Medealab™, Erlangen, Germany). For every sample, a total of 6 fields were examined giving an evaluation of approximately 900 cells per sample. Each field was recorded for 8 sec (total of 48 sec per sample). Sperms were divided into 4 groups based on motility: group A (rapid progressive), B (medium progressive), C (non-progressive), D (immotile). Sperm morphology (categories: normal, proximal or distal plasma droplets, detached head, bent tail, microcephaly, macrocephaly) and acrosome integrity (presence or absence of acrosome) were evaluated with Spermac™ staining (Beernem, Belgium). Minimum 200 cells were examined for the morphological analysis. After the measurement of untreated sperms, 5 µl of 100 nmol PACAP1-38 synthesized as previously described (Jozsa et al. 2005), dissolved in 495 µl PBS, was added to the samples which were incubated at 37^{o}C for 5 minutes in order to investigate the effects of the exogenous peptide on motility (Brubel et al. 2012).

Immunohistochemistry

Immunohistochemistry was performed on WT (n=5), HZ (n=5) and KO (n=5) testis samples to visualize the localization of P-Sox9, Sox10 and collagen type IV (Col. IV). Testes were fixed in Saint-Marie's fixative and washed in 70% ethanol. After embedding serial sections were made, deparaffination was then followed by rinsing in PBS (pH 7.4). Non-specific binding sites were blocked with PBS supplemented with 1% bovine serum albumin (Amresco LLC, Solon, OH, USA), following which the samples were incubated with polyclonal P-Sox9 (Sigma-Aldrich, MO, USA), Sox10 (Abcam, Cambridge, UK), or Col. IV (Abcam, Cambridge, UK) antibodies at a dilution of 1:600 at 4°C overnight. For visualization of the primary antibodies, anti-rabbit Alexa fluor 555 secondary antibody (Life Technologies Corporation, Carlsbad, CA, USA) was used at a dilution of 1:1000. Samples were mounted in

Vectashield mounting medium (Vector Laboratories, Peterborough, England) containing DAPI for nuclear DNA staining. For negative controls anti-rabbit Alexa fluor 555 was used without the primary antibodies, which did not result in any positive signal (not shown). Photomicrographs of the tissues were taken using an Olympus DP72 camera on a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan). Images were acquired using cellSens Entry 1.5 software (Olympus, Shinjuku, Tokyo, Japan) with constant camera settings to allow comparison of fluorescent signal intensities. Images of Alexa555 and DAPI were overlaid using Adobe Photoshop version 10.0 software. The contrast of images was increased equally without changing constant settings.

RT-PCR analysis

Testes of WT, HZ and KO mice (n=5) were mechanically ground and were dissolved in Trizol (Applied Biosystems, Foster City, CA, USA), after 30 min incubation at 4°C total RNA was isolated. RNA was harvested in RNase-free water and stored at −70°C. Reverse transcription was performed by using High Capacity RT kit (Applied Biosystems, Foster City, CA, USA). For the sequences of primer pairs and details of polymerase chain reactions, see Table 1. Amplifications were performed in a thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) as follows: at 95°C for 2 min, followed by 35 cycles (denaturation at 94°C for 30 sec; annealing for 45 sec at optimized temperatures as given in Table 1; extension, 72°C, 90 sec) and then 72°C, 7 min. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as internal control. PCR products were analyzed using a 1.2% agarose gel containing ethidium bromide. Optical densities of PCR product signals were determined by using ImageJ 1.40g freeware.

Western blot analysis

Testes of WT (n=5), HZ (n=5) and KO mice (n=5) were washed in physiological saline and stored at −70°C. Samples were mechanically disintegrated with a tissue grinder in liquid nitrogen. Then they were collected in 100 µL of homogenization RIPA (Radio Immuno Precipitation Assay) buffer (150 mM sodium chloride; 1.0% NP40, 0.5% sodium deoxycholate; 50 mM Tris, pH 8.0) containing protease inhibitors (Aprotinin (10 µg/mL), 5 mM Benzamidine, Leupeptin (10 µg/mL), Trypsine inhibitor (10 µg/mL), 1 mM PMSF, 5 mM EDTA, 1 mM EGTA, 8 mM Na-Fluoride, 1 mM Na-orthovanadate). The suspensions were sonicated by pulsing burst for 30 sec at 40 A (Cole-Parmer, Illinois, USA). Total cell

lysates for Western blot analyses were prepared. Twenty μg protein was separated in 7.5% SDS–polyacrylamide gels for the detection of PKA, ERK 1/2, P-ERK 1/2, p38, P-p38, Sox9, P-Sox9, Sox10, PP2A, Collagen type IV (Col. IV), Collagen type IX (Col. IX), Testatin and Actin. Proteins were transferred by electrophoresis to nitrocellulose membranes and exposed to the primary antibodies overnight at 4°C in the dilution as given in Table 2. After washing for 30 minutes with PBST, membranes were incubated with the peroxidase-conjugated secondary antibody anti-rabbit IgG in a 1:1500 (Bio-Rad Laboratories, CA, USA) or anti-mouse IgG in 1:1500 (Bio-Rad Laboratories, CA, USA) dilution. Signals were detected with enhanced chemiluminescence (Advansta Inc., Menlo Park, CA, USA) according to the instructions provided by the manufacturer. Actin was used as an internal control. Signals were developed with gel documentary system (Fluorchem E, ProteinSimple, CA, USA). Optical densities of signals were measured by using ImageJ 1.40g freeware.

In vitro protein phosphatase 2A (PP2A) activity assay

PP2A activity was assayed with ^{32}P-labelled myosin light-chain (5 μM) in testes of WT (n=5), HZ (n=5) and KO mice (n=5) as described previously (Erdodi et al. 1995). Briefly, pellets were suspended in 10 mM Tris/HCl (pH 7.4), 0.1 mM EGTA, 0.25 mM dithiothreitol, 0.1 mM PMSF, 0.1 mM DFP, 0.1 mg $\cdot$mL^{-1} leupeptin and 1 mM benzamidine (buffer A) and further diluted in buffer A supplemented with 1 mg $\cdot$mL^{-1} BSA. One unit of the protein phosphatase activity releases 1 μmol of P_i from the phosphosubstrate per min at 30°C.

Statistical Analysis

All data presented are representative of at least three independent experiments. Statistical analysis was performed by ANOVA and unpaired Student's *t*-test. The threshold for statistically significant differences as compared to controls was set at $*P < 0.05$.

Results

Morphology of seminiferous tubules, sperm motility and morphology

The general characteristics of seminiferous tubules were visualized with HE staining and morphological comparisons were made in WT and PACAP KO mice. No alterations were seen in tubular structures, and the general location of the differentiating cells was similar in

all experimental groups (Fig.1A). Regarding sperm morphology, there was no difference in the electron microscopical structure (Fig.1B). No difference was seen in the structure of HZ mice either (not shown).

Results of groups A and B sperms (rapid and medium progressive) were combined as well as those from groups C and D (non-progressive and immotile). The ratio of the sperms with good motility (A+B) and weak or no motility (C+D) was 65.6/34.4% in the wild-type animals, 60.6/39.8 in HZ mice and 68.7/31.3 in KO sperms (Fig.1D). There was no significant difference between any of the parameters. Addition of PACAP did not significantly change motility in any of the groups (data not shown). These results show that neither exogenous nor endogenous PACAP changed sperm motility in mice. Examining the morphology, we could not confirm statistical differences, in spite of the 10% less normal sperms in KO mice (percentage of normal sperms was 69.6±3.1%, 68.8±8.1 and 58.8±6.7 in WT, HZ and KO mice, respectively). The standard deviation was high, but there were a few KO mice with no normal sperms at all, with the detached head being the most apparent abnormal sign (Fig.1C).

PKA and MAPK pathways had modified activation in gene-deficient animals

Expression of canonical targets of PACAP receptor activation such as PKA and MAPKs were examined by RT-PCR and Western blot. Although the mRNA expression of PKA did not show any alterations in HZ or KO mice, its protein expression was significantly decreased in KO animals, and it was expressed at higher levels in testis samples of HZ mice than in WT controls (Fig.2A and B). mRNA and protein expression of ERK1/2 were similar in the three experimental groups. The phosphorylated form of ERK1/2 represents a more active form of this MAPK, which may have an influence on cell proliferation. HZ and PACAP KO mice showed a significantly increased P-ERK1/2 level (Fig.2A and B). p38 is another possible MAPK which can be involved in PACAP-induced signaling pathways. Its mRNA expression did not show alterations, but significantly higher protein expression and P-p38 were detected in the HZ and KO mice than in WT controls (Fig.2A and B).

Possible downstream targets of PKA in PACAP KO mice

The activation of Sox9, a major transcription factor of testis development and spermatogenesis, can be regulated by PKA as a canonical downstream target of PAC1 receptor activation. The mRNA expression of Sox9 was not altered in HZ or KO animals (Fig.3A). While Sox9 protein expression was unchanged in HZ mice, it was significantly

decreased in PACAP KO mice. The level of the more active phosphorylated form of Sox9 also showed a reduction similar to the non-phosphorylated version in homogenous gene-deficient mice (Fig.3B), but again the protein expression was not significantly altered in HZ mice (Fig.3B). Next, we investigated the localization of P-Sox9 in seminiferous tubules and its immunopositivity was detected in the outer region of WT animals. In HZ animals expression of P-Sox9 was strongly localized close to the lumen of seminiferous tubules with a very weak peripheral presence (Fig.3D). Immunohistochemical results indicated lower immunopositivity of P-Sox9 detected in all parts of the seminiferous tubules of PACAP KO mice (Fig.3D). Sox10 is another important regulatory factor of testis development/spermatogenesis and it can substitute the function of Sox9. mRNA expression of this transcription factor was constant in the investigated samples (Fig.3A). Unexpectedly, Sox10 expression was significantly elevated in PACAP KO mice while it was not altered in HZ mice (Fig.3B). Immunopositivity of Sox10 in WT and HZ mice was very weak and randomly appeared in the cells of seminiferous tubules. On the contrary, strong Sox10 signals were detected in the middle portion of tubules in KO animals (Fig.3E).

Phosphatase function

The phosphorylation and, consequently, activation of Sox9 are regulated by the Ser/Thr phosphatase called PP2A, which can reversibly dephosphorylate the targets of PKA. As the decreased phosphorylated Sox9 expression can be the result of an increased PP2A activity we monitored the phosphatase function in the testis. Interestingly the mRNA and protein levels of PP2A showed a significant elevation in HZ and PACAP KO mice (Fig.3A and B). The increased expression of the phosphatase is not always followed by more intense activity; therefore, we measured the PP2A activity in the testis samples. In PACAP KO mice the phosphatase activity was dramatically increased. No differences were detected between the WT and HZ groups (Fig.3C).

Target genes of Sox transcription factors in testis development

Possible targets of Sox transcription factors can be collagen type IV (Col. IV), collagen type IX (Col. IX) or testatin, major components of the basal lamina of the tubules. The mRNA expression of testatin was not altered, while that of Col4a showed a significant decrease in HZ and KO mice (Fig.4A). However, the protein expression of this basement membrane component showed a significant decrease in HZ and PACAP KO mice (Fig.4B). The mRNA expression of Col. IX protein was moderately reduced in gene-deficient mice, while its

315 protein expression was elevated in both HZ and PACAP KO rodents (Fig.4A and B). The
316 other testis-specific basement membrane component is testatin, the protein expression of
317 which was dramatically increased in PACAP KO mice (Fig.4A and B). As these observations
318 may reflect a possible alteration in the basement membrane integrity, immunohistochemistry
319 was performed to detect structural differences of basal membrane Col. IV. In WT mice the
320 integrity of the basement membrane was strongly detected around the seminiferous tubules.
321 HZ animals showed a weaker Col. IV immunoreaction, which further decreased in PACAP
322 KO animals (Fig.4C).
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325 **Discussion**
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327 In the present study we described that several factors playing an important role in
328 spermatogenesis are significantly influenced by partial or complete lack of PACAP. In spite
329 of the unaltered or only slightly altered morphology and motility of spermatozoa of PACAP
330 deficient animals, the disturbed molecular mechanisms in spermatogenesis may account for
331 some of the known perturbations in the fertility of PACAP KO mice. The possible effects of
332 PACAP on signaling mechanisms are summarized in Figure 5 and discussed below.
333 Several studies have shown the presence of PACAP, its mRNA and receptors in the
334 testis and epididymis of different vertebrate species and in the human testis (Agnese et al.
335 2010, 2016, Konone et al. 1994, Rosati et al. 2014) after the pioneer study of Arimura and
336 coworkers (1991), which showed that testis contained the highest levels of PACAP among
337 the peripheral organs. Lv et al. (2011) described the gradual increase of PACAP mRNA in
338 the rat testis from day 20 after birth, reaching the maximum level on day 60, mainly in
339 spermatocytes and round spermatids. Similarly, the epididymis showed a gradual increase in
340 PACAP during puberty (Lv et al. 2011). Other studies found stage-specific expression of
341 PACAP: while high levels of expression could be found in most developing germ cells,
342 PACAP expression is lacking in mature spermatids according to some reports (Yanaihara et
343 al. 1998). Tanii and coworkers have described weak immunostaining in mature sperm heads
344 (Tanii et al 2011). The expression of PACAP has been shown to be influenced by several
345 factors, but its regulation in the testis is not yet entirely understood. Raising the temperature
346 to body temperature by inducing experimental cryptorchidism can lead to a dramatic decrease
347 of PACAP mRNA both in the testis and epididymis (Lv et al. 2011). It is possible that this
348 and other, yet unknown, influencing factors are responsible for the stage-specific expression

of PACAP during the spermatogenic cycle and are responsible for the discrepancies found between descriptions (Rosati et al. 2014). A testis-specific splice variant of the PAC1 receptor has also been reported (Daniel et al. 2001). Interestingly, unlike in other tissues, PACAP38 and 27 were found to have the same levels in human testis, shown by radioimmunoassay (Tamas et al. 2015). No differences were found in the level of PACAP peptides between normal tissues and seminomas (Tamas et al. 2015), but a different distribution pattern was observed in different tumor biopsies by Nakamura et al. (2014). In addition to the local production of PACAP, circulating PACAP can additionally contribute to testicular functions of the peptide, as it has been shown that PACAP can cross the blood-testis barrier (Banks et al. 1993, Mizushima et al. 2001). Testis functions in PACAP gene-deficient animals had only been studied by Lacombe et al. (2006). This study revealed interesting results in the testis: unlike in any other organ/system investigated so far, testicular aging was delayed in PACAP deficient mice with a decreased level of apoptosis and decreased oxidative stress markers (Lacombe et al. 2006). The authors argued that the stimulatory effect of PACAP on steroidogenesis may result in a higher level of its byproduct reactive oxygen species production, leading to testicular aging, but also supporting the role of PACAP played in normal testicular function at younger ages. Our present results of normal testicular histology in PACAP KO mice at a young age are in accordance with the findings of Lacombe and coworkers (Lacombe et al. 2006).

The high level of PACAP and its stage-specific expression pattern indicate that the peptide plays important roles in spermatogenesis. It has been described that PACAP influences hormonal secretion via regulating Leydig- and Sertoli cell growth and functions, as well as acrosome reaction during fertilization (Tanii et al. 2011). Influencing motility is also among the possible functions of PACAP, as our previous report showed that PACAP could increase the motility of abnormally slow-moving human sperms (Brubel et al. 2012). The results of Gozes et al. showing that addition of a PACAP antagonist peptide led to a decrease in motility provided a proof for the possible endogenous stimulatory effect of PACAP (Gozes et al. 1998). However, our present finding that mice deficient in PACAP show normal motility implies that even if present, the endogenous effects of PACAP on motility can be compensated by other factors. Furthermore, exogenous PACAP did not alter the sperm motility in the present study. This observation is in accordance with our earlier description in human sperms (Brubel et al. 2012), where we found that the motility of only slow-moving sperms could be increased by PACAP with normal moving sperms not influenced. Our previous study also described that sperm heads from PACAP deficient mice

were significantly smaller than those from wild types (Brubel et al. 2012). This observation further implies that PACAP is necessary for normal spermatogenesis. Our present findings also support this observation: the percentage of normal sperms was 10% lower in PACAP KO mice than in the other two groups, even if due to the high deviation this was not statistically significant. Among the abnormal sperms, more sperms had detached heads in the PACAP KO group. In spite of some differences, our present results show that reduced fertility in PACAP KO mice is most probably not due to altered morphology or motility, since differences were slight. However, we revealed marked differences in the molecular factors and targets of PACAP-induced signaling pathways.

PACAP, binding to its receptors, induces intracellular cAMP accumulation, and subsequently activates PKA, also known as the canonical downstream signaling pathway. As expected, the expression of PKA was reduced in the samples of KO mice. PKA has a crucial role in germ cell development and differentiation, therefore its lower activity can result in lower fertility, decreased sperm number, or reduced capacitation capability (Burton and McKnight, 2007). The inactivation of PKA by the lack of PACAP may influence spermatogenesis or spermiogenesis. However, PKA pathways have several cross-talks, thus the lack of PKA has compensation possibilities.

It is also known that PACAP receptor activation can communicate with other kinases such as ERK1/2 and p38 (Racz et al. 2007), which may influence cell survival, proliferation and differentiation. Therefore, we monitored the expression of these MAPKs. Protein expression of p38 and of the more active phosphorylated form of ERK1/2 and p38 increased in PACAP deficient testes. ERK1/2 functions have been described in the testis: it can regulate cell renewal in goat spermatogonia (Niu et al. 2015) and affects Sertoli cell junction, blood-testis barrier and division of spermatogonia (Siu et al. 2005, Wong and Cheng, 2005). Our data suggest that the elevated expression of P-ERK1/2 can be a compensatory effect of the lower PKA activation, ensuring the spermatogenesis with the activation of other downstream signalization in a PKA independent manner. p38 also plays role in the Sertoli cell adhesion stability and has some regulatory effect on elongated spermatids (Wong and Cheng, 2005) triggering normal motility. Thus, the effect of p38 in PACAP KO animals can be dual in testis, partly maintaining a functional blood-testis barrier and supporting the normal cell motility of spermatids. On the one hand, the increased phosphorylation of p38 alters the apoptotic processes in spermatogonia resulting in an abnormal differentiation with altered sperm morphology. On the other hand, PKA can phosphorylate several transcription factors, which play unique roles in testis development and spermatogenesis.

The members of SoxE transcription factors such as Sox8, Sox9 and Sox10 have fundamental functions in several developmental processes, including testis development (Barrinuevo and Scherer, 2010, Georg et al. 2012). Sox9 is required for maintenance of the integrity of Sertoli cells in adult testis (Barrinuevo et al. 2016). Loss of function of Sox10 had no effect on testis development (Barrinuevo and Scherer 2010) but a cross-regulation has been proven between Sox9 and Sox10 in other cells, such as melanocytes (Shakhova et al. 2015). The reduced expression and phosphorylation of Sox9 in PACAP KO mice suggest a direct signalization with PKA as it has been shown in chondrogenic cells (Zakany et al. 2005). Moreover, it has been demonstrated that PAC1 receptor activation increases the level of P-Sox9 in chondrogenic cell cultures (Juhasz et al. 2014a), which further supports the possible PKA-Sox9 signalization axis. The elevated Sox10 expression proves a possible substitution of Sox9 function, therefore, the increased level of Sox10 may compensate for the lower activity of Sox9, resulting in a normal blood-testis barrier. On the other hand, expression profiles in HZ animals were disturbed, also suggesting an interrupted Sox9-Sox10 crosstalk. A recent study has demonstrated that some signs of neurobehavioural development are more disturbed in mice partially lacking PACAP than in mice with a complete lack of the peptide (Farkas et al. 2017), implying disturbed compensatory processes in some signaling pathways.

Transcription factors phosphorylated by PKA can be dephosphorylated by a Ser/Thr phosphatase, PP2A (Juhasz et al. 2014b; Zakany et al. 2002). PP2A was demonstrated influencing spermatid maturation (Hatano et al. 1993) and differentiation of Sertoli cells (Levallet et al. 2013). Furthermore, the inhibitory effect of PACAP on PP2A has been detected in chromaffin cells (Bobrovskaya et al. 2007) but no data is available for the testis. According to our results the absence of PACAP results in an increased PP2A activity, which can partly be responsible for the lower phosphorylation of Sox9 (Zakany et al. 2002). Therefore, the transcriptional inactivation can be a consequence of lower PKA activity, and a parallelly increased PP2A function. Sox9 can be translocated into the nuclei of Sertoli cells and can regulate certain gene expression. Col. IV is one of the major elements of the basement membrane in the seminiferous tubules, and regulates the formation of the blood-testis barrier (Harvey et al. 2006). Col. IX also has some functions in the maintenance of the basement membrane, consequently the blood-testis barrier (McClive and Sinclair, 2003). Furthermore, testatin has also been proven to have a role in the regulation of testis development (Georg et al. 2012). The genes of these proteins may be regulated by Sox9 as a termination of the PAC1-PKA-Sox9 axis. As we demonstrated, the protein level of Col. IV in

the basement membrane of the seminiferous tubules was decreased, therefore, the basement membrane was not well discernible in PACAP KO animals. On the other hand, the expression of testatin and Col. IX was elevated, suggesting a possible upregulation by other members of the SoxE family such as Sox10. On the contrary the mRNA expression of Col. IX and testatin was decreased indicating an altered transcriptional activation as it has been demonstrated in chondrogenic differentiation (Juhasz et al. 2014a). Our findings also support the idea that SoxE family members, especially Sox9, are important transcription factors in blood-testis barrier dynamics, which can be precisely regulated by PACAP. Indeed, we detected elevated expression of Sox 10 in testes of PACAP KO mice.

In summary, our findings suggest a role of PACAP in Sox9 expression and phosphorylation in mouse testis. In addition to the direct effects of Sox9 on testis determination and male germ cell production, PACAP seems to play a role in the maintenance of proper molecular composition of the basement membrane of seminiferous tubules. These results could be additional factors responsible for the reduced fertility in PACAP knockout mice as well as further supporting the role of endogenous PACAP in reproductive functions.

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Declaration of Interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure Legends

Fig. 1.

(A) Representative microphotographof WTand KO seminiferious tubules stained with haematoxylin and eosin. Overview of several tubules and higher magnification view of the wall of one seminiferous tubule.

(B) Electronmicroscopical analysis of WT andKO sperms.

(C) Representative images of spermatozoa showing normal and most common abnormal morphologies. White arrow: plasma droplet; white arrowhead: hairpin tail; black arrowhead: acrosome deficiency; asterisk: detached head; n: normal morphology.

(D) Motility of sperms: A (rapid progressive), B (medium progressive), C (non-progressive), D (immotile). Data are represented as A+B (progressive) and C+D (non-progressive and immotile) together. Data are given as mean±SEM.

Fig. 2.

Optical density of signals was measured and results were normalized to the optical density of controls. For panels (A) and (B) numbers below the signals represent integrated densities of signals determined by ImageJ software. Asterisks indicate significant (*P<0.05) alteration of expression as compared to the respective control. Representative data of 3 independent experiments.WT (+/+), HZ (+/-), PACAP KO (-/-). For RT-PCR reactions GAPDH and for Western blot actin were used as controls.

Fig. 3.

Optical density of signals was measured and results were normalised to the optical density of controls. For panels (A) and (B) numbers below the signals represent integrated densities of signals determined by ImageJ software. Asterisks indicate significant (*P<0.05) alteration of expression as compared to the respective control. Representative data of 3 independent experiments.(C) PP2A enzyme activity determined in a cell-free *in vitro* assay from lysates of WT, HZ and PACAP KO samples. Significant difference between PP2A enzyme activity in HZ and PACAP KO samples *vs*. control lysates is marked by asterisks (*$P < 0.05$). (D) (E) Immunohistochemistry of P-Sox9 and

Sox10 in seminiferous tubules. Magnification was made with 20× objective. Scale bar: 50 μm. WT (+/+), HZ (+/-), PACAP KO (-/-). For RT-PCR Gapdh and for Western blot reactions actin were used as controls.

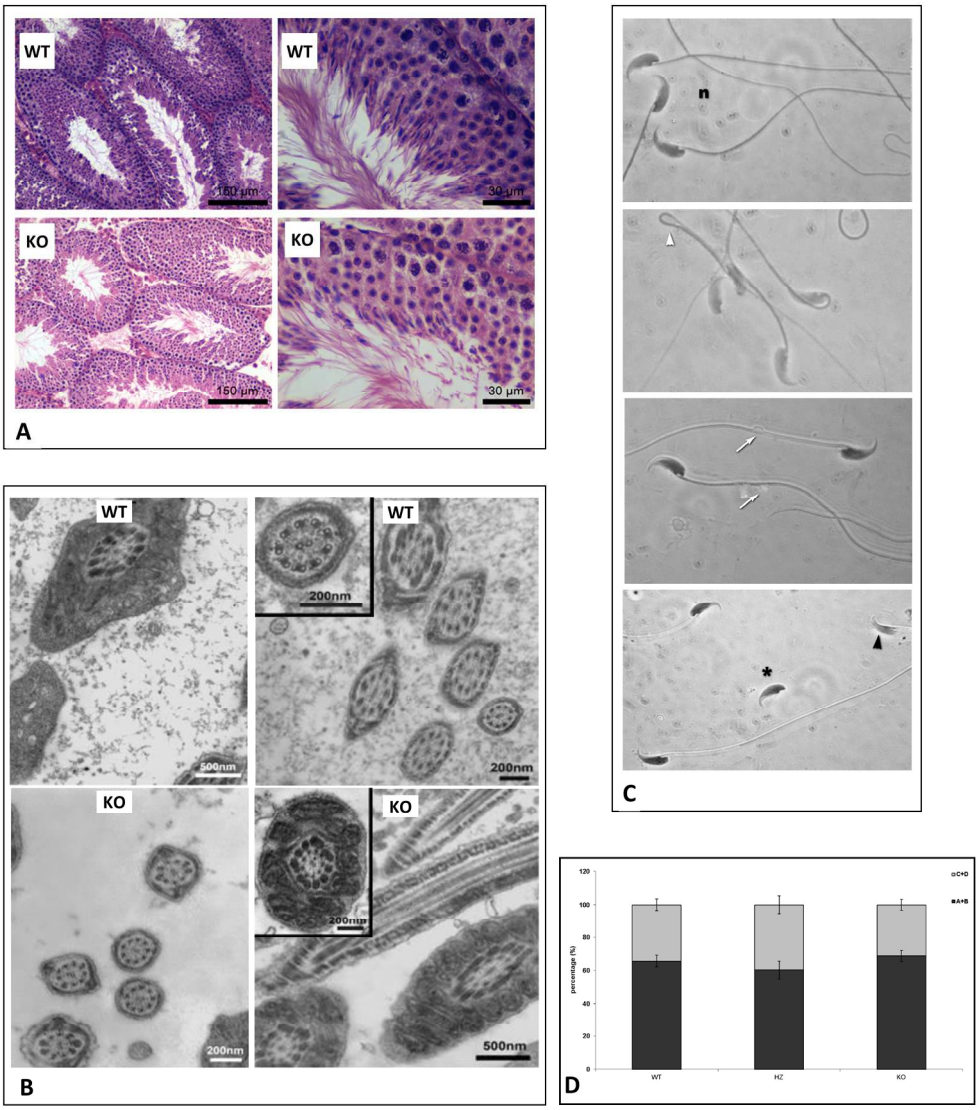
Fig. 4.

Downstream targets of Sox transcription factors altered in PACAP knockout mice. mRNA (A) and protein (B) expression of Col. IV, Col. IX and testatin. For RT-PCR Gapdh and for Western blot reactions actin were used as controls. Optical density of signals was measured and results were normalized to the optical density of controls. For panels (A) and (B) numbers below the signals represent integrated densities of signals determined by ImageJ software. Asterisks indicate significant (*$P<0.05$) alteration of expression as compared to the respective control. Representative data of 3 independent experiments. (C) Immunohistochemistry of Col. IV in seminiferous tubules. Magnification was made with 20× objective. Scale bar: 50 μm. WT (+/+), HZ (+/-), PACAP KO (-/-).

Fig. 5.

Schematic drawing of the possible signaling pathways regulated by PACAP in spermatogenesis

PAC1 receptor activation leads to the increase of intracellular cAMP, which activates PKA. Downstream targets of PKA are partly regulated by Ser/Thr reversible phosphorylation. PP2A can regulate the dephosphorylation of the transcription factor Sox9, which can modify the expression or activation of Sox10. Additionally, PKA can influence ERK1/2 phosphorylation. Parallel with these events, p38 activation can also take place. Main targets of Sox9 transcription factor can be Collagen type IV, Collagen type IX or Testatin. These reversible phosphorylation events may regulate proliferation, motility, or formation of blood-testis barrier.



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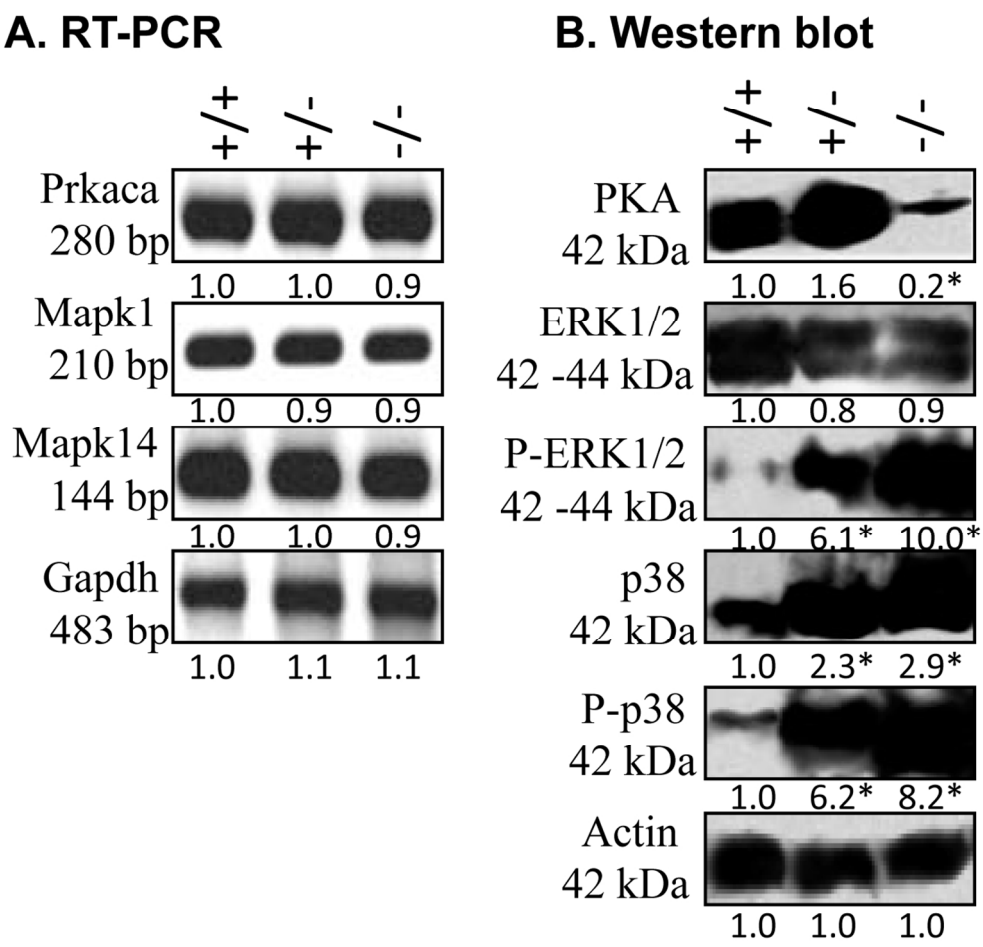

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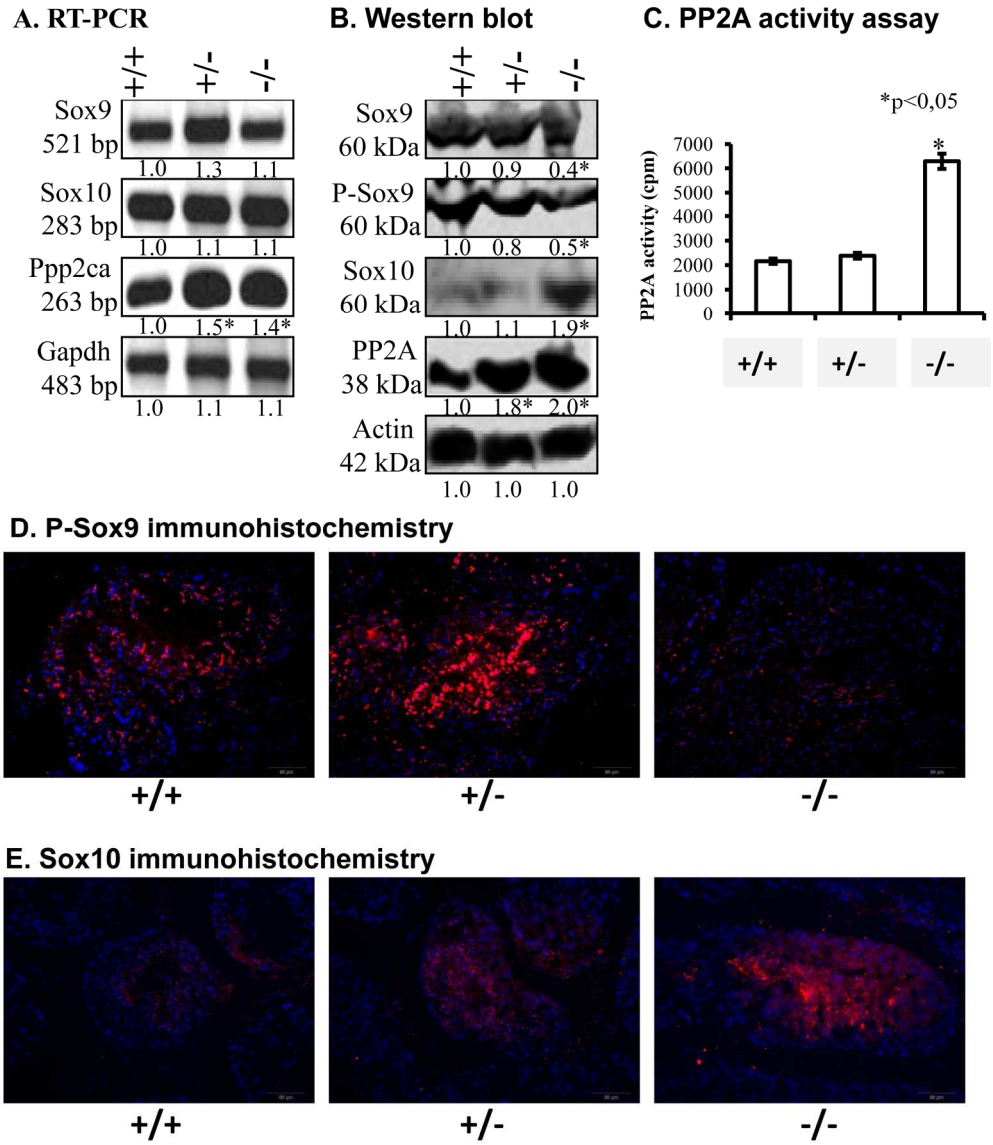

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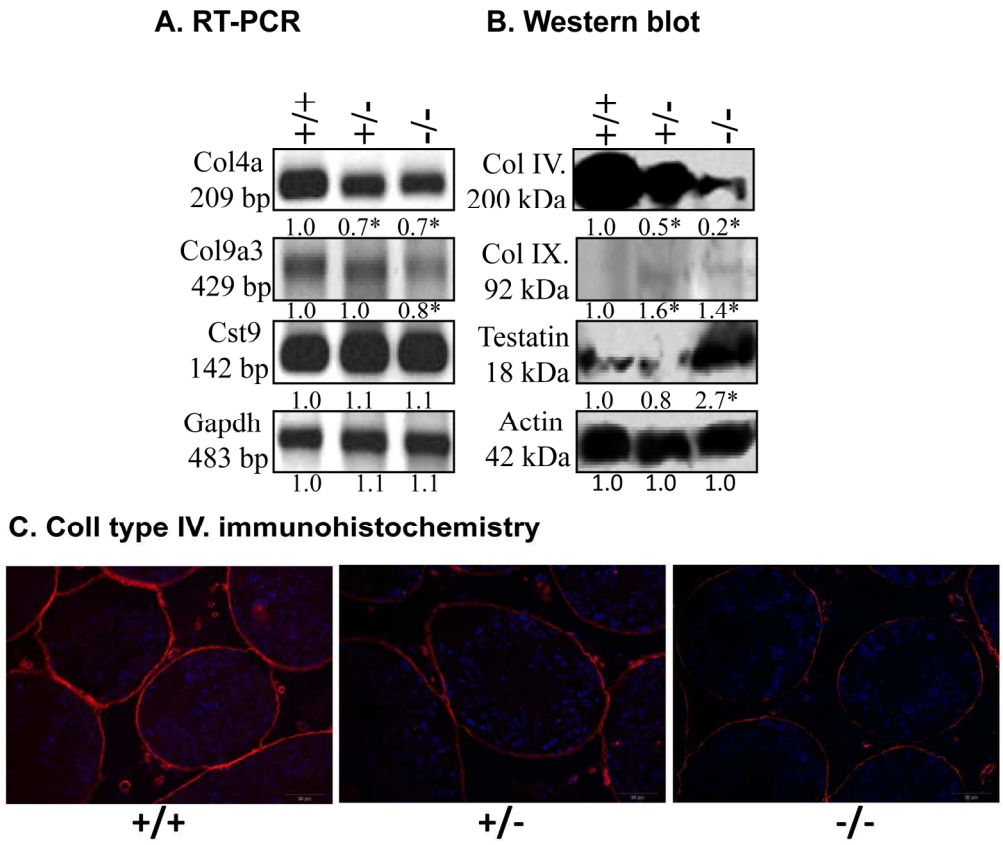

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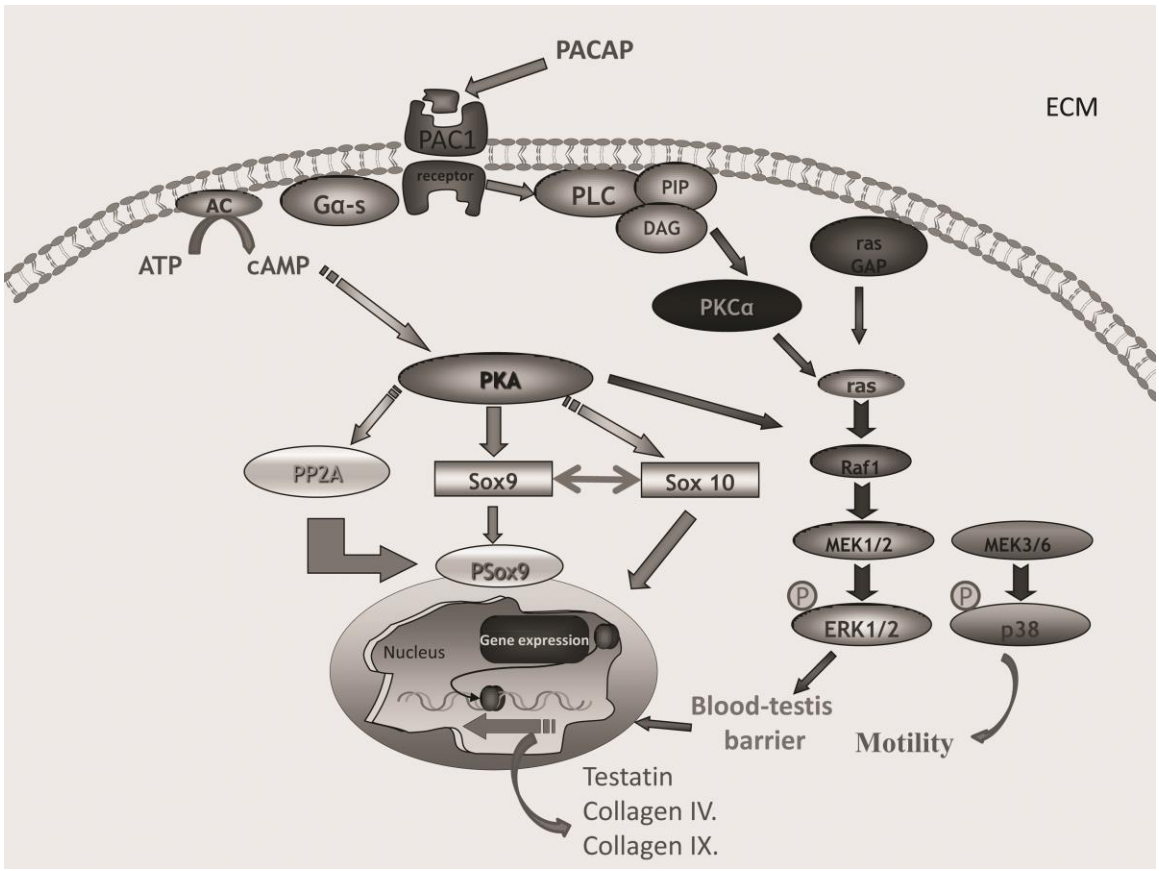
PACAP
ECM
PAC1
receptor
AC
Gα-s
PLC
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PP2A
Sox9
Sox 10
Raf1
MEK1/2
MEK3/6
PSox9
P
ERK1/2
P
p38
Nucleus
Gene expression
Blood-testis
barrier
Motility
Testatin
Collagen IV.
Collagen IX.

Table 1. Nucleotide sequences, amplification sites, GenBank accession numbers, amplimer sizes and PCR reaction conditions for each primer pair are shown.

Gene	*Primer*	*Nucleotide sequence (5'→3')*	*GenBank ID*	*Annealing temperature*	*Amplimer size (bp)*
PKA (Prkaca)	sense	GCA AAG GCT ACA ACA AGG C (847–865)	**NM_001277898.1**	54°C	280
	antisense	ATG GCA ATC CAG TCA GTC G (1108–1126)			
ERK (Mapk1)	sense	CCG TGA CCT CAA GCC TTC C (1732–1750)	**NM_011949.3**	58°C	163
	antisense	ATG CAG CCC ACA GAC CAA A (1877–1894)			
p38 (Mapk14)	sense	GAG GTG CCC GAA CGA TAC (453–470)	**NM_011951.3**	53°C	144
	antisense	TTT GGC GTG AAT GAT GGA (579–596)			
Sox9	sense	GTA CCC GCA TCT GCA CAA CG (378–397)	**NM_011448**	62°C	521
	antisense	GTG GCA AGT ATT GGT CAA ACT CAT T (874–898)			
Sox10	sense	ACG ACT GGA CGC TGG TGC (535–552)	**NM_011437.1**	58°C	283
	antisense	CGC CGA GGT TGG TAC TTG TAG (797–817)			
PP2A (Ppp2ca)	sense	CTC TGC GAG AAG GCT AAA (288–305)	**NM_017039.2**	54°C	436
	antisense	TGA TTC CCT CGG AGT ATG (706–723)			
Collagen IV (Col4a1)	sense	TCG GCT ATT CCT TCG TGA TG (4963 – 4982)	**NM_009931.2**	52°C	209
	antisense	GGA TGG CGT GGG CTT CTT (5154 –5171)			
Collagen IX (Col9a3)	sense	CAG GTT CCG ATG GTC TTC C (1357-1375)	**NM_009936.2**	55°C	492
	antisense	CTG TTG CTC CCT TGT CCC (1831-1848)			
Testatin (Cys9)	sense	CTG GAG GGA GAA GGT AAA (274-291)	**NM_009979.1**	51°C	142
	antisense	CAG GCA GGT GAA GGT ATT (398-415)			
GAPDH	sense	TGG CAA AGT GGA GAT TGT TG (238–258)	**NM_008084.2**	59°C	486
	antisense	GTC TTC TGG GTG GCA GTG AT (775–793)			

Table 2. Tables of antibodies used in the experiments.

Antibody	*Host animal*	*Dilution*	*Distributor*
Anti-Col. IX.	rabbit, polyclonal,	1:500	Abcam, Camridge, UK
Anti-Col. IV.	rabbit, polyclonal,	1:800	Abcam, Camridge, UK
Anti-Testatin.	rabbit, polyclonal,	1:300	Santa Cruz Biotechnology, Dallas, TX, USA
Anti-PKA C	rabbit, polyclonal,	1:800	Cell Signaling, Danvers, MA, USA
Anti-ERK1/2	rabbit, polyclonal,	1:1000	Sigma-Aldrich, St. Louis, MO, USA
Anti-P-ERK/12	mouse, monoclonal,	1:800	Sigma-Aldrich, St. Louis, MO, USA
Anti-p38	rabbit, polyclonal,	1:1000	Cell Signaling, Danvers, MA, USA
Anti-P-p38	rabbit, polyclonal,	1:800	Cell Signaling, Danvers, MA, USA
Anti-Sox9	rabbit, polyclonal,	1:800	Abcam, Camridge, UK
Anti-P-Sox9	rabbit, polyclonal,	1:800	Sigma-Aldrich, St. Louis, MO, USA
Anti-Sox10	rabbit, polyclonal,	1:500	Abcam, Camridge, UK
Anti-PP2A C	rabbit, polyclonal,	1:600	Cell Signaling, Danvers, MA, USA
Anti-Actin	mouse, monoclonal,	1:10000	Sigma-Aldrich, St. Louis, MO, USA