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Cell markers of spermatogenesis in mammalian (p63 and tACE)

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ABSTRACT

Spermatogenesis is a complex process by which spermatogonial stem cells self-renew and differentiate into spermatozoa under the elaborate coordination of testicular microenvironment. Sertoli cells (SC) are the most critical component of regular development of spermatogenesis. SC expressed various cellular markers specific for their development stages. In contrast GC do not expressed cellular makers which is an obstacle for investigation of GC development and differentiation.

Data about expression of p63 protein in the testicular cell during pre and postnatal periods is quite limited. Our study revealed stage specific pattern of expression of p63 proteins in spermatocytes later than middle pachytene stage of meiosis during the cycle of the seminiferous epithelium. P63 is suggested to have clinical importance playing a role in preventing testicular lesions.

Another cellular marker tACE is germ cell specific isoform that is important for male fertility. This isoform is expressed in germ cells during spermiogenesis and tACE is localized only in elongating spermatids and spermatozoa. Immunohistochemical analysis in our studies revealed stage-specific pattern of tACE expression in postmeiotic germ cells in rat testis. Changes in the expression of tACE were reported in some pathological conditions.

Key words: p63, tACE, spematogensis, testis

Introduction

p63 is a member of the p53 gene family and have structural similarities with p53. p63 encodes for multiple isotypes either with N-terminal transactivation domain (TAp63) or without it (DeltaNp63). In the mammalian testis, it has been shown that p53 plays important roles in the regulation of germ cell apoptosis and meiosis. However, little is known for the physiological function of p63 in the mammalian spermatogenesis (Nakamuta & Kobayashi, 2004). All three genes (p53, p63 and p73) regulate cell cycle and apoptosis after DNA damage. However, despite a remarkable structural and partly functional similarity among p53, p63, and p73, mouse knockout studies revealed an unexpected functional diversity among them. P63 and p73 knockouts exhibit severe developmental abnormalities but no increased cancer susceptibility, whereas this picture is revealed for p53 knockouts. The p53/p63/p73 family members are capable of interacting in many ways that involve direct or indirect protein interactions, regulation of same target gene promoter and regulation of each other's promoters (Murray-Zmijewsk et al., 2006).

Expression of p63 is absolutely essential for limb formation and epidermal morphogenesis including the formation of adnexa (teeth, hair, mammary and prostate glands, and sweat and lacrimal glands). The p63-null animals have defects of the apical ectodermal ridge and they show severe limb truncations or absence of limbs and absence of skin, teeth, mammary, lachrymal or salivary glands and craniofacial (De Laurenzi & Melino, 2000; Levrero et al., 2000).The animals do not survive beyond a few days postnatally.

The human and mouse p63 genes expressed as two major types: full-length proteins containing the TA domain and ΔN

proteins missing the TA domain. Each of them was express at least three alternatively spliced C-terminal isoforms (α, β, γ). P63 containing the transactivation domain (TAp63) and amino-deleted p63 isoforms (ΔNp63) exert distinct (often opposite) functions on stemness, cycle arrest, mobility and invasion (epithelial–mesenchymal transition) and senescence. This suggests that p63 is a marker of epithelial tumors such as ductal carcinoma in situ of the breast or prostatic intraepithelial neoplasia (Graziano & De Laurenzi, 2011).

Data about expression of p63 protein in the main testicular cell during pre and postnatal periods is quite limited. In addition, androgens are known to be essential for initiation of meiosis during puberty and testosterone suppression induced neonatally by DES or GnRHa inhibit meiotic differentiation of spermatocytes.

Angiotensin I-converting enzyme (ACE) plays an important role in male reproduction. ACE exists in two isoforms – somatic (sACE) and testis-specific (tACE) being differently distributed in the male reproductive system. Testicular ACE is germ cell specific isoform that is essential for male fertility. This isoform is expressed in germ cells during spermiogenesis and tACE is localized only in elongating spermatids and spermatozoa. Acting as dipeptidase tACE is responsible for release of GPI proteins from sperm membrane that is important for sperm-zona pellucida binding, necessary for fertilization. Therefore, tACE may serve as marker for fertilizing ability of spermatozoa. Studies on the human germ cells showed that tACE-mRNA was present in spermatocytes and the mRNA levels increased in spermatids (Yudong et al., 1996).

In this respect the aim of the present paper is to follow cellular localization and distribution of p63 and tACE in germ cells during development of the testis and in the course of the first spermatogenic wave in normal and experimental conditions.

Materials and Methods

Animals

Wistar rats, bred and maintained under standard conditions. We used experimental model for manipulation of neonatal hormonal environment by treatment with DES-10 μg. The testes and epididymides with the vas deferens attached were fixed for ~5 h in Bouins then transferred into 70% ethanol before being processed for 17.5 h in an automated Leica TP1050 processor and embedded in paraffin wax. Sections of 5 μm thickness were cut and floated onto silane coated slides dried at 50°C overnight before being used for morphological and immunohisochemical studies.

Immunohistochemistry

Unless otherwise stated, all incubations were performed at room temperature for 30 min. Sections were deparaffinised and rehydrated. Antigen retrieval procedure was applied by pressure-cooking for 5 min in 0.01M Citrate buffer, pH 6.0 at full pressure. At this stage and after all subsequent steps, sections were washed twice (5 min each) in Tris-buffered saline (TBS; 0.05M Tris-HCl, pH 7.4, 0.85% NaCl). Endogenous peroxidase activity was blocked by immersing sections in 3% (v/v) H_2O_2 in methanol. To block non-specific binding sites, sections were incubated for 30 min. with normal rabbit serum. Primary mouse monoclonal anti p63 antibody (sc0586 Santa Cruz Biotech, USA) was used in dilution 1:500 and sections were incubated overnight at 4°C in a humidified chamber. Biotinylated secondary anti-mouse IgG antibody (Dako) was used at 1:500 dilution in blocking mixture followed by incubation for 30 min. with avidin-biotin conjugated to horseradish peroxidase (ABC-HRP; Dako) diluted in 0.05M Tris-HCl, pH 7.4. Immunostaining was developed using 3,3′-diaminobenzidine (Liquid DABplus; Dako). All sections were then lightly counterstained with hematoxylin. Primary goat polyclonal anti tACE (sc12187, Santa Cruz Biotech, USA) was used at dilution 1:500 and sections were incubated overnight at 4°C in a humidified chamber. Incubating with secondary rabbit anti-goat IgG antibody (BA 5000, Vector) dilution 1:500 for 30 min. and the procedure proceeds in the same way as p63 immunohistochemistry.

Results

Our immunohictochemical studies on embryonal day 21.5 did not found any expression of p63 proteins in the fetal rat testes. The negative large gonocytes (prespermatogonia) are seen in the center of seminiferous cords. The similar negative reaction was observed in the testes on postnatal day 8th and differentiating spermatogonia that actively proliferate are located on the basal membrane of the cords.

First faint expression of p63 proteins appeared on day 15th in the nuclei of single pachytene spermatocytes adluminally located. On day 18th more immuno positive spermatocytes at stage middle pachytene were seen in the seminiferous tubules. Germ cells in earlier stages of meiosis (leptotene and zygotene) are negative.

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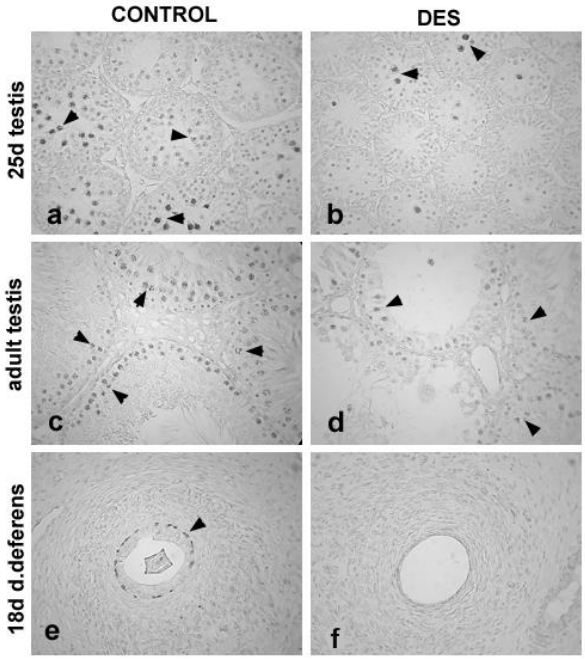

Figure 1. *Immunoexpression of p63 in the germ cells (spermatocytes) of control rat and DES treated testes on day 25 (a, b) and in adulthood (c, d). Positive controls from ductus deferens from control and DES treated rats on day 18 (e, f). Note basal epithelial cells are labelled.*

Strong immunoreactivity of p63 was evident on day 25th and some stage specificity can be seen as four type tubules can be distinguished based on the different association of germ cell types (Figure 1a). Spermatocytes at stage late pachytene are more intensively stained compared to the spermatocytes at stage middle pachytene.

Stage specific pattern of expression of p63 proteins is obvious and reaction is confined to the primary and secondary spermatocytes in the tubules from middle (VII-VIII) to late stages (IX-XIV) (Figure 1c). Early pachytene spermatocytes in stages I-VI are negative for p63. Primary spermatocytes at middle pachytene stage of meiosis are intensively stained. Strong immune-reactivity continues in late pachytene spermatocytes in stages IX-XII of spermatogenic cycle.

The testes from rats treated neonatally with DES showed suppressed spermatogenesis manifested by dramatic reduction in germ cell number, especially evident for primary spermatocytes on day 18th and day 25th. In seminiferous tubules form 25 day old DES treated testes single middle pachytene spermatocytes can be seen that exhibit strong immuno-expression for p63 comparable to that in controls (Figure 1b). In adult DES treated testes of spermatocytes were less intensively stained compared to the controls (Figure 1d).

Paraffin sections from ductus deferens of 18 day old control rats were used as positive control where strong expression is shown in basal epithelial cells (Figure 1e). For validation of the DES treatment sections form ductus deferens of 18 day old DES treated rats were used where lack of p63 and altered basal cells differentiation were reported in our previous study (Figure 1f, Figure 2).

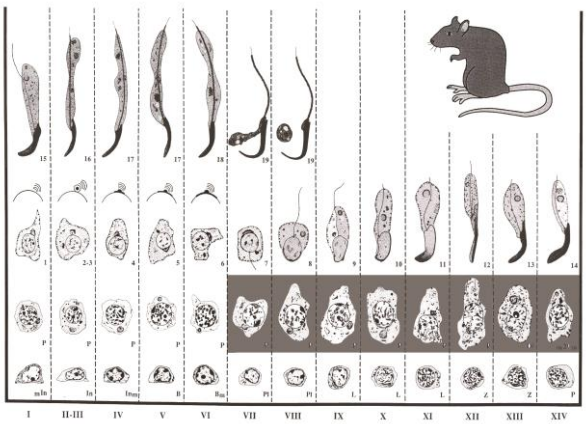

Figure 2. *Schematic presentation of immunoexpression of p63 during spermatogenic cycle.*

Detailed immunohistochemical analysis in our studies revealed stage-specific pattern of tACE expression in postmeiotic germ cells in rat testis. The cycle of the seminiferous epithelium in the rat comprises of fourteen stages and spermiogenesis involved 19 steps. Schematic and semi-quantitative expression of tACE was shown on Figure 3.

First faint immunoreactivity appeared in the cytoplasm of round spermatids step 8 (stage VIII of the cycle) in a round shape manner. Weak intensity was found in elongating spermatids step 9 at stage IX of the cycle of seminiferous epithelium. Immunoexpression became strong later than steps 12 of spermiogenesis (stage XII of the cycle) and reached maximum in steps 17-19 (stages IV-VIII of the cycle) (Figure 4). No immunoexpression was observed in other germ cell types (spermatogonia, spermatocytes) as well as in somatic cells (peritubular cells, Leydig and Sertoli cells). Stage specificity of tACE localization during spermatogenic cycle characterizes tACE as a good marker for stage of spermatid

differentiation. In the rat testis expression of tACE start and reaches maximum in androgen dependent stage VIII of spermatogenic cycle that implies androgen regulation of enzyme production in germ cells. Localization pattern of tACE revealed the importance of elongation phase of spermatids in male germ cell differentiation with respect to gene expression and not only to morphological modifications. Expression of tACE in postmeiotic germ cells is an example for specific gene activation and translation during spermiogenesis. In the course of the first spermatogenic wave tACE is a marker for developmental stage of germ cell differentiation. tACE could serve as a marker for germ cell depletion during experimental and pathological conditions.

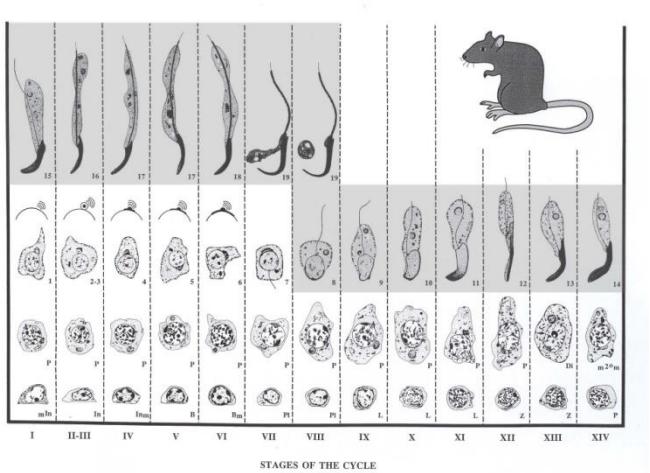

Figure 3. *Schematic presentation of expression of tACE during stages of seminiferrous epithelium. On the scheme of the rat spermatogenesis all germ cell types and fourteen stages of the spermatogenesis are illustrated. Expression of tACE spanned from step 8 to step 19 of spermiogensis (marked with grey).*

ACE activity in the testicular complex is possibly linked with androgens and is involved with spermatogenesis and sperm maturation. Testicular ACE as marker for postmeiotic stages of GC differentiation turn out suitable, because in DES-10 treatment rats we found out abundance of tubules with no expression of tACE.

Using immunohistochemistry analysis for tACE we refine the degree of loss of elongated spermatids in the cycle of seminiferous epithelium. Germ cell depletion was manifested by loss of elongating and round spermatids and in most severe cases by lack of spermatocytes. Sertoli cell only tubules (SCO) containing only Sertoli cells and few spermatogonia were also observed. No reaction was found in germ cell depleted tubules in which elongated spermatids step 8-19 were absent or the staining was weaker than in controls. In some early stages we found failure of round spermatids and spermatocytes. This data characterize tACE as suitable marker for evaluation of the depletion of seminiferous epithelium from postmeiotic elongated spermatids in neonatal induced hormonal imbalance.

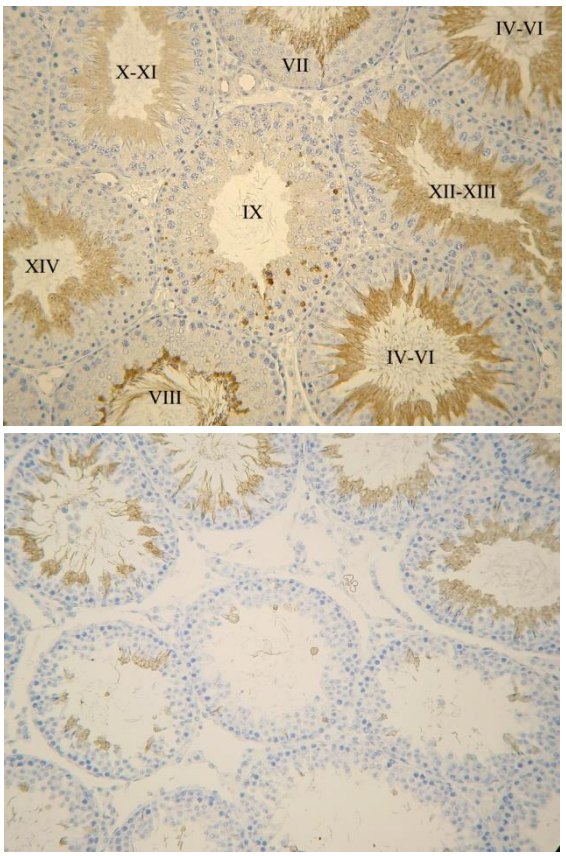

Figure 4. *Immunohistochemistry of tACE in testis of control and neonatal DES-10 treated (75-days) rats in adulthood. In experimental group observed full and part depletion of elongated spermatids.*

Discussion

All the three members of p53 family were expressed in the mouse testis (Nakamuta & Kobayashi, 2003). In the mammalian testis, it has been shown that p53 plays important roles in the regulation of germ cell apoptosis and meiosis. P53 is expressed in spermatocytes demonstrated by immunohistochemistry and *in situ* hybridization (Schwartz et al. 1993).

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Immunohistochemical studies by Hamer et al. (2001) revealed presence of p73 in the cytoplasm of spermatogonia, spermatocytes, residual bodies, as well as in the nuclei of spermatocytes and round spermatids. In contrast to the p73 -/- mice, in which no structural abnormalities were found in reproductive organs of either male or female by histology, the function of p63 in spermatogenesis is obscure, since p63 null mice born with severe developmental defects and die soon after birth (Nakamuta & Kobayashi, 2003).

Our developmental study demonstrated specific reaction for p63 protein in the nuclei of meiotic germ cells (spermatocytes) and is in concern with data by Hayashi et al. (2004) in rat and by Nakamuta and Kobayashi in mice (Nakamuta & Kobayashi, 2003). Moreover, our detailed observation on the expression of p63 during the cycle of seminiferous epithelium provide new data about stage specific localization of p63 protein in primary spermatocytes from middle pachytene till diplotene stage of prophase I of meiosis and in secondary spermatocytes, as well. On day 25 (mid puberty) four type/stages of seminiferous tubules can be distinguished where different intensity of immune reaction was found. In adult testes we observe expression of p63 in stages VII-XIV of the spermatogenic cycle. Nuclear localization of p63 proteins at specific stages of spermatogenesis suggests their involvement in the regulation of cellular function during spermatogenic cell differentiation.

Our study on developing and adult rat testes does not find any localization of p63 in postmeiotic stages of spermatogenesis - round spermatids as it was reported in mice by Nakamuta and Kobayashi (Nakamuta & Kobayashi, 2003). This discrepancy could reveal some species specificity in expression of p63 proteins.

A study by Petre-Lazar et al. (2007) followed ontogeny of each p63 mRNA isoforms during testis development to demonstrate correlation between their expression and gonocyte activity (proliferation/apoptosis versus quiescence). As p63γ mRNA and protein are strongly expressed in quiescent gonocytes, the γ isoforms appears to be the determining factor in these processes, rather than the balance between p63 N-terminal isoforms (TA and ΔN). P63 is suggested to be involved in spontaneous apoptosis in the germ cell lineage. There are many pro-apoptotic factors that are up-regulated by Tap63γ in different models and the Bcl2 and the Notch families may be also involved in apoptosis of postnatal germ cells.

As p63-/- mice died at birth Petre-Lazar et al. (2007) performed in vitro studies using tissue fragments of fetal testes from p63 -/- and p63 +/+ mice. Invalidation of p63 resulted in an increase of number of gonocytes during the culture period of 3 days due to a decrease in spontaneous apoptosis. Lack of p63 also caused abnormal morphology of germ cells (giant cells) that was found in p63 +/- adult male mice. These giant germ cells are reported in rat neonatal testes after treatment with phthalate (DBP) (Fisher et al., 2003) as well as in human testicular carcinoma in situ which is thought to originate from the abnormal differentiation of fetal gonocytes, possibly after exposure to estrogen or xenoestrogens (Rorth et al., 2000).

In conclusion, our results demonstrated that p63 is developmentally regulated in the testis as well as throughout the spermatogenic cycle and possibly changed with apoptotic and mitotic activity of germ cells. p63 is suggested to have clinical importance playing a role in preventing testicular lesions as apoptosis provides a mechanism for removing incorrectly differentiated gonocytes, which are thought to give rise to germ cell tumors.

Testicular ACE is germ cell specific isoform that is essential for male fertility. This isoform is expressed in germ cells during spermiogenesis and tACE is localized only in elongating spermatids and spermatozoa. The role of tACE in fertilization is proved by knockout models in mice lacking ACE gene. ACE null mice lacking both somatic and testicular ACE are infertile independently of normal testis weight, normal sperm count and morphology. Infertility is due to altered sperm migration in the oviduct and their ability to bind zona pellucida (Smith & Nothnick, 1997).

Studies on the human germ cells showed that tACE-mRNA was present in spermatocytes and the mRNA levels increased in spermatids. The gene for tACE could be activated by C-AMP response element modulators (CREMα and CREMτ). In vitro analyses of the testis ACE promoter have identified two important transcriptional motifs within the promoter region TATA box and the other motif is highly homologous to the consensus cAMP-response element (CRE). The consensus CRE or its variants have been found in the promoter regions of cAMP-responsive genes. Upon hormonal stimulation, a signal transduction cascade leads to the phosphorylation of a number of CRE-binding proteins, which then exert positive or negative effects on the transcription of cAMP-responsive genes. A unique member of this group of transcription factors is CREM. The CREM gene encodes both transcriptional repressors and activators. CREMτ, functioning as a transcription activator is abundant

in male germ cells (Yudong et al., 1996).

In our study we found that tACE activity in testicular complex is possible linked with androgens and using synthetic form of female hormone estrogens (DES) we found significant abundance of tubules with lack of expression of tACE in early and late stages of cycle of seminiferous epithelium. In control rats first immunoreactivity we observed in cytoplasm of round spermatids step 8 and it became stronger reaching maximum in steps 17-19. With one exception our results are consistent with the data by Sibony et al. (1994). Discrepancy is related to weaker expression of tACE in elongating spermatids at step 15-17 compared to earlier steps. In another study by Langford et al (Langford et al., 1993) tACE immunoreactivity in mouse testis was detected later than step 10 of spermatogenesis. The difference between these author groups could be explained by using different protocols antibodies against the portion common to the testicular and somatic ACE.

Changes in the expression of tACE were reported in some pathological conditions such as hypertension, cancer or presence of endocrine disruptor. In our study in rats treated with DES suggested relationship between disturbance of spermatogenesis and changes in androgen production. In adult DES rats (75-day) destructive changes in testicular histology were seen manifested by germ cell depletion, and reduced diameter of seminiferous tubules. Loss of tACE expression in germ cell depleted tubules is due to absence of corresponding stages of spermatid differentiation. In microscopic observation of the DES-treated animals, degeneration of germ cells and tubular atrophy in the testis were noted by Shin and Kim (Shin et al., 2009).

Therefore, tACE can be used as a marker for germ cell depletion due to hormonal disruption. Expression of tACE in postmeiotic germ cell, specifically altered by DES, suggested possible involvement of androgens and estrogens in the process of spermiogenesis. In conclusion, our results demonstrated that p63 is developmentally regulated in the testis as well as throughout the spermatogenic cycle and possibly changed with apoptotic and mitotic activity of germ cells. Testicular form of ACE also could serve as a marker for germ cell depletion during experimental and pathological conditions.

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