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Stage-specific expression of p63 in rat germ cells – marker of meiotic phase of spermatogemesis in normal and experimental conditions

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Abstract: P63 protein isoforms are found in adult male and female urogenital organs and mammary glands and they are essential for male and female reproduction. Data about expression of p63 protein in the main testicular cells during pre and postnatal periods is quite limited. The present paper aimed to follow cellular localization and distribution of p63 in germ cells during development of the testis and in adulthood in normal and experimental conditions. 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. Our data demonstrated that p63 is developmentally regulated in the testis 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.

Key words: P63 protein, spermatocytes, meiosis, germ cell, spermatogenesis

Introduction

The p53 family includes the three genes p53, p63, and p73. They have a modular structure consisting of the transactivation (TA), the DNA-binding (DBD), and the oligomerization domain. All three genes 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. However, the existence of p53-like and p53-inhibitory versions of TP73 and TP63 genes, plus intimate functional cross-talk among all family members, endows these genes with both tumor suppressor and oncogenic roles [9].

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. Although the proteins and their isoforms are

expressed at various levels depending on tissue type and developmental stage, the presence of an isoform at low levels does not necessarily mean it is insignificant [11]. The p53 family members and their isoforms can bind differentially to promoters and it may well prove that the ratio between isoforms is an important cell fate determinant. The changes upon stimuli of the balance and interactions between the isoforms are likely to be fundamental to our understanding in the transition between normal cell cycling and the onset of tumour formation.

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 [2, 8]. The animals do not survive beyond a few days postnatally. Similar defects are found in children affected by ectrodactyly, ectodermal dysplasia and facial clefts (EEC syndrome) and recently an autosomal dominant rare mutation in p63 gene has been shown to be responsible for this syndrome.

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. TAp63 induces cell death and cell cycle arrest with tumor-suppressor features, whereas ΔNp63 exerts oncogenic properties and is generally overexpressed in cancer. TAp63 and ΔNp63 (and their ratio) regulates chemosensitivity that is of clinical importance for cancer diagnosis and prognosis [9]. Generally, the more aggressive metastatic tumors lose p63 expression, suggesting that p63 loss accelerates tumorigenesis and metastatic spread. Correspondingly, disruption of p63 in squamous cell lines results in upregulation of genes associated with increased invasiveness and metastasis in tumors. This suggests that p63 is a marker of epithelial tumors such as ductal carcinoma in situ of the breast or prostatic intraepithelial neoplasia [4].

In postnatal epidermis, p63 expression is restricted to the nuclei of basal cells of normal epithelia (skin, esophagus, tonsil, prostate, urothelium, ectocervix, and vagina) and to certain populations of basal cells in glandular structures of prostate, breast, and bronchi [8]. In the female reproductive tract, all six splice variants of p63 were expressed in cervical/vaginal epithelium, oocytes in ovary and in a subset of epithelial cells in the ampulla of oviduct. Moreover, an antibody specific for ΔN forms detected proteins only in cervical/vaginal epithelium but not in the uterus, ovary and oviduct whereas TA splice variants were detected in oocyte.

In contrast, testicular germ cells were unreactive for ΔN or α isoforms, but reactive with anti-pan-p63 antibodies. This confirms that ΔNp63 isoforms are expressed in squamous/ basal epithelial and myoepithelial cells, while TAp63 forms are expressed in germ cells. Protein for α-isoforms was expressed in squamous epithelial tissues and oocytes. These expression patterns suggest functional differences in p63 isoforms in adult male and female urogenital organs and mammary gland [7, 12, 13, 14, 15].

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. In this respect the **aim** of the present paper is to follow cellular localization and distribution of p63 in germ cells during development of the testis and in the course of the first spermato-

genic wave in normal and experimental conditions. Our study is focused on the expression of p63 during the cycle of the seminiferous epithelium and on stage specific pattern of the p63 protein.

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 and paraffin embedded tissue samples were provided by the Centre for Reproductive Health in Edinburgh. Briefly, the testes and epididymides with the vas deferens attached were fixed for ~5h 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 5μm 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 at 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. The intensity of immunostaining was scored on an arbitrary scale ranging from negative (-) through weakly positive (+) to intensely positive (++++).

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 immunopositive spermatocytes at stage middle pachytene were seen in the seminiferous tubules. Germ cells in earlier stages of meiosis (leptotene and zygotene) are negative.

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 (Fig. 1a). Spermatocytes at stage late pachytene are more intensively stained compared to the spermatocytes at stage middle pachytene.

In the adult rat testes spermatogenesis is complete and fourteen stages of cycle of the seminiferous epithelium are present. 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) (Fig. 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. Primary spermatocytes at diplotene stage in XIII stage of the cycle are less immune-reactive that pachytene germ cells. Weak expression can be seen in the nuclei of secondary spermatocytes in stage XIV. More advanced postmeiotic germ cells, spermatids do not express p63.

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 (Fig. 1b). In adult DES treated testes of spermatocytes were less intensively stained compared to the controls (Fig. 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 (Fig.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 (Fig. 1f) [1].

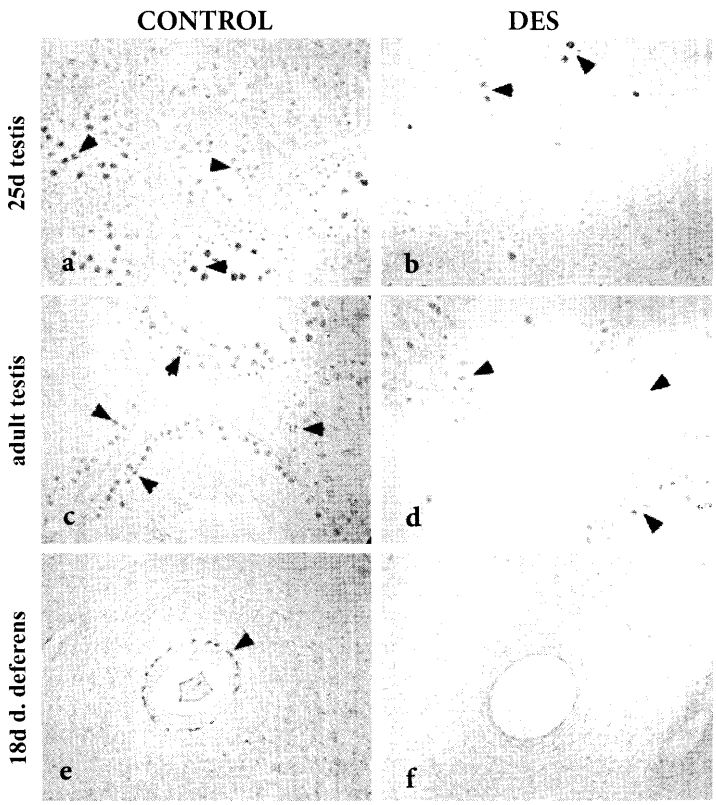

Fig. 1. Immunoexpression of p63 in the germ cells (spermatocytes) of control rat and DES treated testes on day 25 (a, b) and in adulthood (c, d). Positive controls from ductus deferens from control and DES treated rats on day 18 (e, f). Note basal epithelial cells are labelled

Semi-quantitative and schematic presentation of immunoexpression of p63 proteins during the stages of spermatogenic cycle is shown on is shown on table 1 and fig. 2.

Table 1. Semi-quantitative immunoexpression of p63 in the stages of spermatogenic cycle

Stages of the cycle	I-VI	VII-VIII	IX-XII	XIII	XIV
Type of spermatocyte	early pachytene	middle pachytene	late pachytene	diplotene	secondary spermatocytes
Intensity of immune reaction	-	+++	+++	++	+

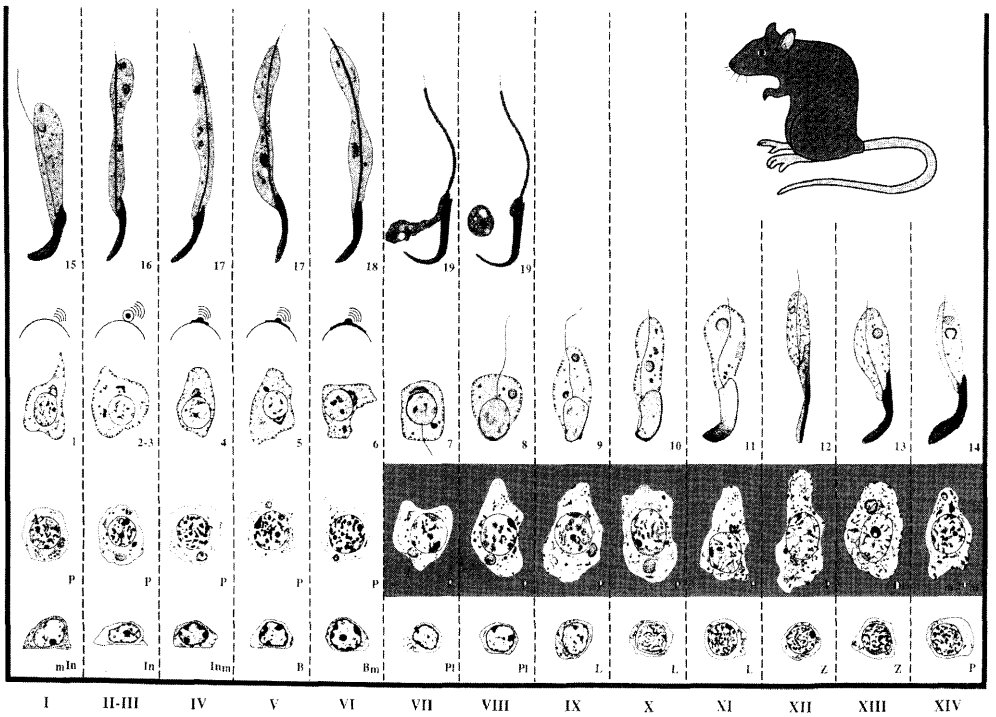

Fig. 2. Schematic presentation of immunoexpression of p63 during spermatogenic cycle

Discussion

All the three member of p53 family were expressed in the mouse testis [12]. 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 [18]. The knockout of p53 gene results in increased number of abnormal gametes due to suppression of spontaneous apoptosis [19]. In the testis, however, participation of p63 to DNA damage-induced apoptosis has not proved yet.

Immunohistochemical studies by Hamer et al. [5] 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 [12].

In our study we used an anti-p63 antibody (4A4 Santa Cruz Biotechnology, California, USA) raised against the amino terminus of ΔNp63 (amino acids 1-205). Since 15-205 amino acid region of ΔNp63 is a DNA binding domain which coincides with 111-301 amino acids of Tap63, this antibody reacts with all six p63 variants of mouse, rat and human origin in Western blotting and immunohistochemistry.

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 [6] in rat and by Nakamuta and Kobayashi [13] in mice. As expression of p63 in primary spermatocytes at early puberty coincides with appearance of Notch 1 and its ligand Jagged 2 [6], p63 was suggested to governs the balance between development, differentiation and apoptosis of germ cells through the Notch signaling system and p53 target genes. 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. On the other hand p53 is also expressed in spermatocytes [18]. Since TAp63 can transactivate p53-response genes and induce apoptosis, and the localization of p63 in developing testis was coincided with those of p53, TAp63 might induce the transcription of genes required for the cell cycle regulation or apoptosis of germ cells synergistically with p53 [13].

In our study we did not found any reaction of p63 in mitotic dividing germ cells -spermatogonia of developing and adult testes and they confirm observations in rat and mice by the authors mentioned above [6, 13]. However, Nakamuta and Kobayashi [12, 14] provide data for early expression of p63 since embryonal day 8.5 (e8.5) in primordial germ cells in hindgut to e11.5 in genital ridge that continues later in fetal male and female gonads. An important role of p63 in migration of germ cells and their colonization to the gonads is suggested. There are no differences between males and females as for the role of p63 in primordial germ cells before the germ cells are determined their different developmental fates to the testis or ovary. As opposite events occur in the fetal male and female gametogenesis specific pattern of p63 expression in mouse fetal gonads are found. In the fetal testes p63 was seen in the proliferating prespermatogonia from e12.5 to e18.5 and then protein expression declines and diminishes during quiescent period prior to resumption of germ cell development after birth. In contrast, germ cells in fetal ovary enter meiosis before birth, and arrest at the prophase of the first meiotic division, which do not complete until a few hours before ovulation. Moreover p63 protein is confined to the oocytes of primordial and primary follicles and expression is lost as follicles develop [16]. Despite the significant differences between male and female gametogenesis, there is temporal and spatial expression of p63 protein in germ cells involving early fetal events and resumption of cell cycle progression at puberty. Hence, an important role for p63 in cell cycle control and in regulation of germ cell development/meiosis is suggested.

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 [13]. This discrepancy could reveal some species specificity in expression of p63 proteins.

A study by Petre-Lazar et al. [16] 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 [16] performed *in vitro* studies using tissue fragments of fetal testes from p63 -/- and p63 +/+ mice. Invalidation of p63 resulted in an increase 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) [3] 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 estrogens or xenoestrogens [17]. The potent synthetic estrogen, diethylstilbestrol also has been reported to perturb p63 expression in the Mullerian duct [7] and in basal cells of developing rat epididymis and ductus deferens [1].

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.

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