Expression of p63 in the Testis of Mouse Embryos

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ABSTRACT. Apoptosis of testicular germ cells during fetal development is regulated by both p53-dependent and independent mechanisms. However, its precise mechanisms are largely unknown. A member of p53 gene family, p63, is required for p53-dependent apoptosis and can induce apoptosis in the absence of p53 through the activation of p53-target genes. In this study, we examined the expression pattern of p63 in the mouse testis from embryonic day (E) 13.5 to E18.5 to clarify their possible role in the regulation of germ cell apoptosis. Immunostaining for p63 was found in the nucleus of germ cells at E13.5, and continuously observed until E18.5. The RT-PCR using specific primers for two p63 isotypes, those containing the transactivation domain and others not, showed that both transcripts were expressed into at least six protein isotypes divided into two groups, those containing the transcription activation domain (TA isotypes) and others not (ΔN isotypes) [15]. The TA isotypes can activate transcription of p53-reporter genes and others unable to activate transcription, and act as dominant negatives, inhibiting transcription activation by both p53 and TA isotypes [15]. Therefore, it is suggested that p63 is implicated in the regulation of both p53-dependent and independent apoptosis of the germ cells in the embryonic testes.

In this study, we examined the expression pattern of p63 in the testis of mouse embryos to clarify their possible involvement in the regulation of germ cell apoptosis. Nuclear staining was found in the testicular germ cells by immunohistochemistry from E13.5 to E18.5. Expression of both TA and AN isotypes in the embryonic testes were revealed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The results presented here suggest that p63 may be implicated in the control of growth and differentiation of germ cells in addition to their apoptosis in the testes of mouse embryos.

MATERIALS AND METHODS

Animals and tissue preparation: Timed-pregnant mice (Jcl:ICR) were purchased from CLEA Japan, and sacrificed by cervical dislocation under diethyl ether anesthesia. All procedures were approved by the committee for the use of laboratory animals at Kyushu Dental College, Japan. The embryos were collected in phosphate-buffered saline (PBS), fixed in Bouin’s fixative at room temperature (RT) for 6–24 hr, depending on the size of the embryo, and then dehydrated through a graded series of ethanol solutions and embedded in paraffin. Eight-µm-thick sections were made and mounted on 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO, U.S.A.)-precoated slides and processed for immunohistochemical experiments.

Antibodies: Monoclonal antibody anti-human p63 (clone 4A4) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A.). This monoclonal antibody, raised against amino acids 1–205 mapping at the amino terminus of ΔNp63, reacts with all known p63 variants [15]. Horse-radish peroxidase (HRP)-conjugated goat affinity purified antibody to mouse IgG was from ICN Pharmaceuticals (Ohio, U.S.A.).

Immunohistochemistry: Sections were de-paraffinized and re-hydrated with standard procedures. Subsequently, high temperature antigen unmasking was performed using 0.01 M citrate buffer (pH 6.0) containing 0.1% Tween-20 (Sigma). After cooling to RT, the quenching of endogenous peroxidase was carried out in 0.3% H2O2 in methanol for 30 min. Sections were subsequently treated with 1% normal goat serum in 0.01 M PBS for 10 min, incubated overnight with anti-p63 monoclonal antibody (diluted to 1:50) at 4°C, and then incubated with HRP-conjugated anti-mouse IgG (diluted to 1:100) for 30 min at RT. After washing in 0.01 M Tris-Cl buffer (pH 7.4) for 10 min, the sections were incubated with HRP-conjugated antibody to mouse IgG for 10 min, and then in 0.01 M Tris-Cl buffer (pH 7.4) containing 0.01% diaminobenzidine (DAB) and 0.01% H2O2 for 10 min. After washing, the sections were dehydrated through a graded series of ethanol solutions and embedded in paraffin. Eight-µm-thick sections were made and mounted on 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO, U.S.A.)-precoated slides and processed for immunohistochemical experiments.
**M PBS, the HRP-reaction was developed with DAB/H2O2 solution diluted in 0.05 M Tris-HCl buffer (pH 7.6). Sections were observed with a light microscope Nikon BIOPHOT and photographed using a digital CCD camera (CoolSNAP, Media Cybernautics, Silver Spring, MD, U.S.A.). In each experiment, substitution of the primary antibody with PBS served as negative-staining control.**

**RT-PCR:** Total RNA was isolated from testicular tissues of mouse embryos using Isogen (Nippon Gene, Tokyo, Japan), an RNA isolation reagent. For RT-PCR, comple-

Fig. 1. Immunohistochemical localization of p63 in the embryonic mouse testis. Positive reaction for anti-p63 monoclonal antibody was observed in the nuclei of germ cells from E13.5 to E18.5. Bar = 10 µm.
RESULTS

Immunohistochemical localization of p63 in the embryonic mouse testis: The localization of the p63 protein was studied by immunohistochemistry on serially sectioned mouse embryos from E13.5 to E18.5. As shown in Fig.1, immunostaining for p63 was detected in the nuclei of testicular germ cells through the developmental period we examined. No distinct immunoreactivity was found in the somatic cells in the testes. Control sections, in which the primary antibody was omitted, showed no positive staining (data not shown).

RT-PCR analysis for p63 mRNA expression: We prepared total RNA from testicular tissues of embryonic mouse and performed RT-PCR reactions specific for the two different p63 amino termini, TA and ΔN. This analysis revealed the presence of transcripts encoding both p63 variants in the embryonic testes (Fig. 2). The RT-PCR amplification showed a 248 bp band corresponding to ΔNp63 mRNA from E13.5 to E18.5. The TAp63 transcript was detected in the samples from E16.5 to E18.5. Samples run without reverse transcriptase generated no PCR products (data not shown).

DISCUSSION

The present study shows that testicular germ cells of mouse embryos express p63 from E13.5 to E18.5. This is the first report of p63 expression by the germ cells of mouse embryos. As shown in Fig. 1, p63 protein was localized in the nuclei of germ cells soon after the testis became morphologically distinguishable from the ovary. In the adult mouse testes, the p63 protein has been immunohistochemically localized to the nuclei of pachytene spermatocytes from stage VII onwards and round spermatids [7]. Therefore, it is suggested that p63 plays some roles in the development of the spermatogenic cells both in the prenatal and postnatal development. Although the p63 knockout mouse demonstrated a crucial role for the p63 in the development of various organs [10, 16], the role of p63 in the testicular development has not been addressed.

As shown in Fig. 2, RT-PCR analysis revealed the expression of both TAp63 and ΔNp63 isoforms in the embryonic testes. Expression of TAp63 in the embryonic testis from E16.5 to E18.5 coincides with the time of p53 protein in the mouse testes [9]. In addition, p63 is required for p53 to bind to promoters of apoptosis-related genes and activate transcription [6]. Therefore, the data presented here support the notion that p53 promotes germ cell apoptosis in the embryonic testis after E16.5 [9].

As ΔNp63 expression was detected from E13.5 testes, it is suggested that p63 plays some roles in the development of testicular germ cells in addition to the control of p53-dependent apoptosis. Recently, it has been shown that ΔNp63 acts as a positive regulator in the β-catenin signaling pathway [12]. β-catenin is one of the targets of Wnt signaling cascade, which plays an important role in many cancers. Intra-nuclear accumulation of β-catenin leads to the activation of β-catenin-responsive transcription resulting in the cell proliferation and differentiation [1, 8]. ΔNp63 associates with the B56α regulatory subunit of protein phosphatase 2A (PP2A) and glycogen synthase kinase 3β (GSK3β), leading to a dramatic inhibition of PP2A mediated GSK3β reactivation [12]. The inhibitory effect of ΔNp63 on GSK3β mediates a decrease in phosphorylation levels of β-catenin, which induces intranuclear accumulation of β-catenin and activates β-catenin-dependent transcription [12]. In the nucleus, β-catenin binds to the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors and activates its downstream target genes [2]. Therefore, proliferation and differentiation of embryonic germ cells may be regulated through the activation of ΔNp63 expressed in the embryonic mouse testes. In support of this hypothesis, testicular germ cells of mouse embryos express β-catenin [5].

In conclusion, expression pattern of p63 was studied in the testis of mouse embryos to clarify their possible involve-
ment in the regulation of p53-dependent germ cell apoptosis. The results presented here suggest that p63 may be implicated in the control of growth and differentiation of germ cells in addition to their apoptosis in the testes of mouse embryos through the regulation of β-catenin signaling pathway.

REFERENCES