

## Expression of p63 in the Testis of Mouse Embryos

Nobuaki NAKAMUTA<sup>1)</sup> and Shigeru KOBAYASHI<sup>1)</sup>

<sup>1)</sup>First Department of Oral Anatomy, Kyushu Dental College, Manazuru 2-6-1 Kokurakita, Kitakyushu 803-8580, Japan

(Received 8 January 2003/Accepted 8 April 2003)

**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 in the fetal gonads. Possible roles of p63 in the embryonic testes were discussed.

**KEY WORDS:** immunohistochemistry, mouse, p63, RT-PCR, testis.

*J. Vet. Med. Sci.* 65(8): 853–856, 2003

A portion of testicular germ cells undergoes apoptosis during their prenatal development in the physiological context [3, 4, 14]. In the mouse testis, apoptotic germ cells are seen on day13 through day17 of embryonic development [4]. The number of cells with typical features of testicular germ cell apoptosis is highest at 13 days of gestation [14]. Apoptosis of testicular germ cells were also shown in the rat embryos by TUNEL-staining methods [3].

The p53 tumor suppressor gene product mediates cell cycle arrest and promotes apoptosis in various types of cells through the activation of p53-target genes including *p21* and *bax* [13]. Testicular expression of p53 mRNA and proteins in the mouse embryos suggests their involvement in the regulation of germ cell apoptosis in the embryonic testis [9]. Indeed, it has been shown that p53 promotes apoptosis of fetal testicular cells with the observation of fewer TUNEL-positive cells in the p53-deficient mice than in the wild-type mice [9].

The p63 gene, a member of the p53 gene family, is expressed into at least six protein isotypes divided into two groups, those containing the transcription activation domain (TA isotypes) and others not ( $\Delta$ N isotypes) [15]. The TA isotypes can activate transcription of p53-reporter genes and induce apoptosis [11, 15]. On the contrary, the  $\Delta$ N isotypes are 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 mouse testis.

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  $\Delta$ N 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- $\mu$ m-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  $\Delta$ Np63, reacts with all known p63 variants [15]. Horseradish 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% H<sub>2</sub>O<sub>2</sub> 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

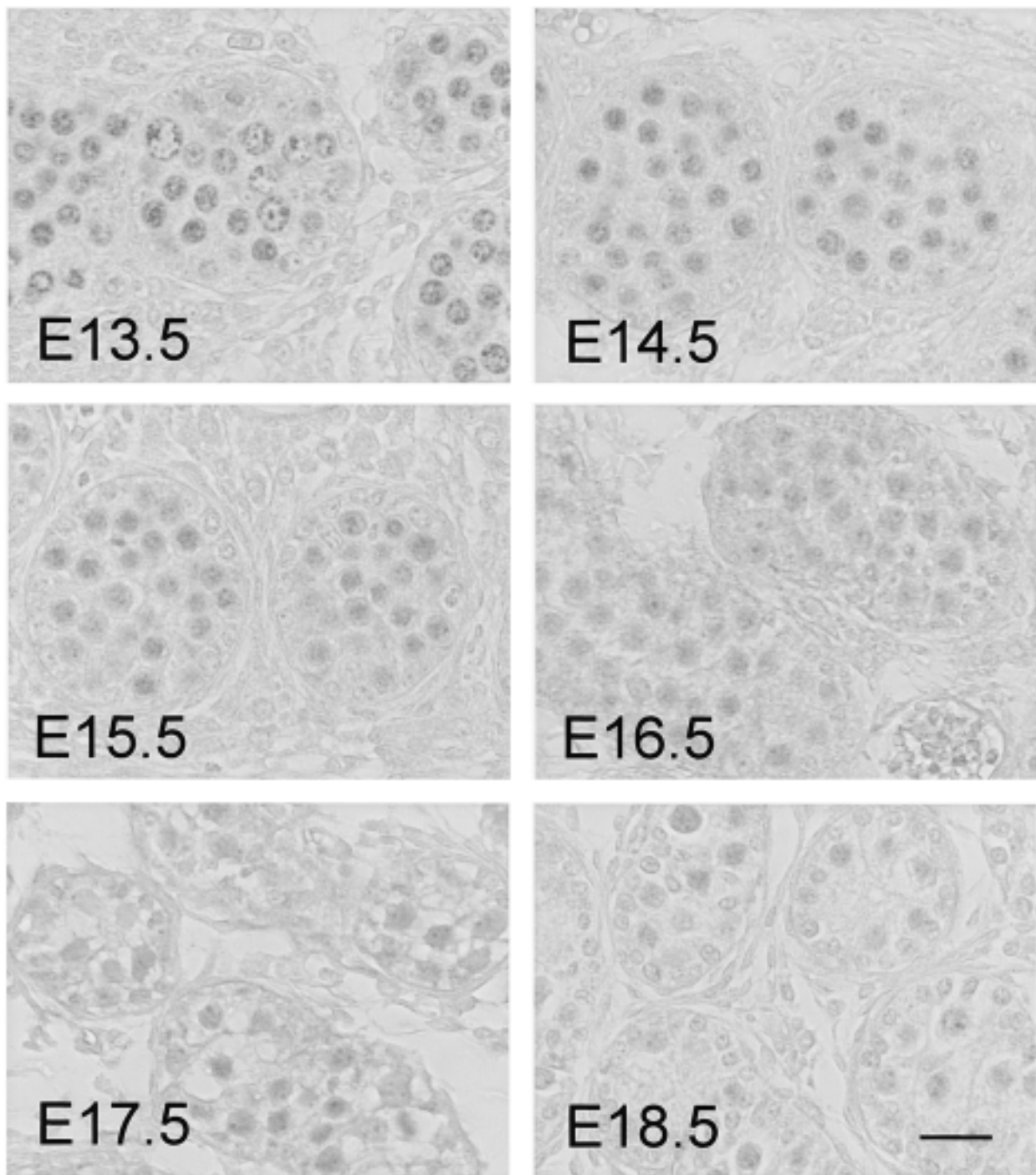


Fig. 1. Immunohistochemical localization of p63 in the embryonic mouse testis. Positive reaction for anti-p63 monoclonal antibody was observed in the nuclei of germ cells from E13.5 to E18.5. Bar = 10  $\mu$ m.

M PBS, the HRP-reaction was developed with DAB/H<sub>2</sub>O<sub>2</sub> solution diluted in 0.05 M Tris-HCl buffer (pH 7.6). Sections were observed with a light microscope Nikon BIO-PHOT and photographed using a digital CCD camera (CoolSNAP, Media Cybernetics, 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-

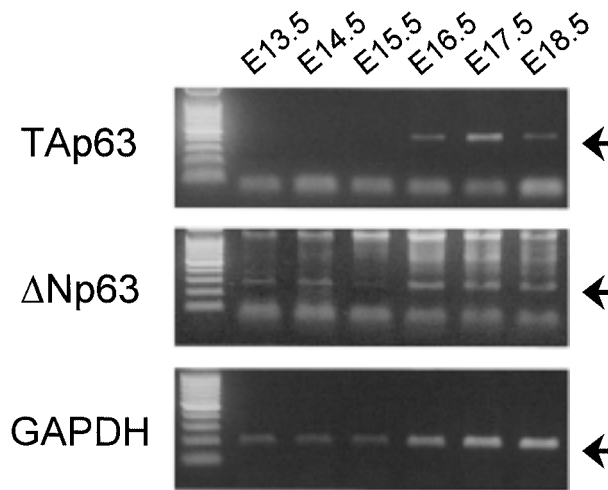


Fig. 2. RT-PCR products obtained from developing mouse gonads. Samples were amplified using specific primers designed to amplify 413 bp product for TAp63 and 248 bp product for  $\Delta$ Np63 isotypes (arrows).

mentary DNA (cDNA) was synthesized from total RNA samples using the SuperScript Preamplification System for First-Strand cDNA Synthesis (Invitrogen, Carlsbad, California, U.S.A.) according to the manufacturer's protocol. cDNA samples were then subjected to PCR with specific primers for the two different p63 isotypes, TA and  $\Delta$ N, as described by Yang *et al.* [15]. As an internal control for RNA extraction and cDNA synthesis, PCR amplification was also performed on all cDNA samples using primers specific for the constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplified products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and then photographed under ultraviolet transillumination light.

## 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 nucleus 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  $\Delta$ N. 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  $\Delta$ Np63 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  $\Delta$ Np63 isotypes 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  $\Delta$ Np63 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  $\Delta$ Np63 acts as a positive regulator in the  $\beta$ -catenin signaling pathway [12].  $\beta$ -catenin is one of the targets of Wnt signaling cascade, which plays an important role in many cancers. Intracellular accumulation of  $\beta$ -catenin leads to the activation of  $\beta$ -catenin-responsive transcription resulting in the cell proliferation and differentiation [1, 8].  $\Delta$ Np63 associates with the B56 $\alpha$  regulatory subunit of protein phosphatase 2A (PP2A) and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), leading to a dramatic inhibition of PP2A mediated GSK3 $\beta$  reactivation [12]. The inhibitory effect of  $\Delta$ Np63 on GSK3 $\beta$  mediates a decrease in phosphorylation levels of  $\beta$ -catenin, which induces intranuclear accumulation of  $\beta$ -catenin and activates  $\beta$ -catenin-dependent transcription [12]. In the nucleus,  $\beta$ -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  $\Delta$ Np63 expressed in the embryonic mouse testes. In support of this hypothesis, testicular germ cells of mouse embryos express  $\beta$ -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 testis of mouse embryos through the regulation of  $\beta$ -catenin signaling pathway.

## REFERENCES

1. Barker, N. and Clevers, H. 2000. Catenins. Wnt signaling and cancer. *Bioessays* **22**: 961–965.
2. Behrens, J., von Kries J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature (Lond.)* **382**: 638–642.
3. Boulogne, B., Olaso, R., Levacher, C., Durand, P. and Habert, R. 1999. Apoptosis and mitosis in gonocytes of the rat testis during foetal and neonatal development. *Int. J. Androl.* **22**: 356–365.
4. Coucouvanis, E. C., Sherwood, S. W., Crumpton, C. C., Spack, E. G. and Jones, P. P. 1993. Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. *Exp. Cell Res.* **209**: 238–247.
5. Di Carlo, A. and De Felici, M. 2000. A role for E-cadherin in mouse primordial germ cell development. *Dev. Biol.* **226**: 209–219.
6. Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F. and Jacks, T. 2002. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature (Lond.)* **416**: 560–564.
7. Hamer, G., Gademan, I. S., Kal, H.B. and de Rooij, D. G. 2001. Role for c-abl and p73 in the radiation response of male germ cells. *Oncogene* **20**: 4298–4304.
8. He, T., Sparks, A., Rago, C., Hermeking, H., Zawel, L., da Costa, L., Morin, P., Vogelstein, B. and Kinzler, K. 1998. Identification of c-MYC as a target of the APC pathway. *Science* **281**: 1509–1512.
9. Matsui, Y., Nagano, R. and Obinata, M. 2000. Apoptosis of fetal testicular cells is regulated by both p53-dependent and independent mechanisms. *Mol. Reprod. Dev.* **55**: 399–405.
10. Mills, A. A., Zheng, B., Wang, X.J., Vogel, H., Roop, D. R. and Bradley, A. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature (Lond.)* **398**: 708–713.
11. Osada, M., Ohba, M., Kawahara, C., Ishioka, C., Kanamaru, R., Katoh, V., Ikawa, Y., Nimura, Y., Nakagawara, A., Obinata, M. and Ikawa, S. 1998. Cloning and functional analysis of human p51, which structurally and functionally resembles p53. *Nat. Med.* **4**: 839–843.
12. Patturajan, M., Nomoto, S., Sommer, M., Fomenkov, A., Hibi, K., Zangen, R., Poliak, N., Califano, J., Trink, B., Ratovitski, E. and Sidransky, D. 2002.  $\Delta$ Np63 induces  $\beta$ -catenin nuclear accumulation and signaling. *Cancer Cell* **1**: 369–379.
13. Sheikh, M. S. and Fornace, A. J. Jr. 2000. Role of p53 family members in apoptosis. *J. Cell. Physiol.* **182**: 171–181.
14. Wang, R. A., Nakane, P. K. and Koji, T. 1998. Autonomous cell death of mouse male germ cells during fetal and postnatal period. *Biol. Reprod.* **58**: 1250–1256.
15. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dotsch, V., Andrews, N.C., Caput, D. and McKeon, F. 1998. p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell* **2**: 305–316.
16. Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C. and McKeon, F. 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature (Lond.)* **398**: 714–718.