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Expression of the *p63* and Notch Signaling Systems in Rat Testes During Postnatal Development: Comparison With Their Expression Levels in the Epididymis and Vas Deferens

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ABSTRACT: The role of tubular structures that contribute to the passage of spermatozoa is not solely passive; these structures actively contribute to their own functions, although these tubules and ducts are contiguous and collaborate in the development of the male gamete along their lengths. The testis has the specific function to generate spermatozoa and spermatozoa undergo numerous changes as they pass through the epididymis. A member of the *p53* family of genes, *p63,* is highly expressed in the basal layers of epithelial tissues and plays a key role in maintaining their cell populations, whereas Notch 1 and its ligand Jagged 2 have an important role in the differentiation of germ cells and Jagged 2 is up-regulated by TA*p63*, one of the *p63* isoforms, which transactivates *p53* target genes and induces apoptosis. Although the presence of *p63* in most epithelia is established, the role of *p63* and its possible relationship with the Notch system in the seminiferous epithelium have not been examined. Therefore, we investigated the expression of *p63*, Jagged 2, and Notch 1 in the testis during postnatal development in comparison with their expression levels in the vaso-epididymal epithelium. In the testis, the expression of TAp63 mRNA increased at day 14 after birth and the expressions of Jagged 2 and Notch 1 mRNA increased at day 16 after birth, suggesting that TAp63-mediated Jagged 2 induction activates the Notch signaling system. On the other hand, the strong signal of ΔNp63 mRNA was already recognized in the vas deferens at day 0 after birth and advanced chronologically along the duct to the caput epididymis and *p63* protein was expressed in basal cells in their epithelium, whereas the mRNAs of Jagged 2 and Notch 1 were maintained at a low level. Consequently, examination of our data raises the probability that TAp63 has an important role for maintenance of germ cell numbers, triggering or balancing the development, differentiation, and apoptosis of germ cells in the testis, which is completely different from the role of ΔNp63 in other epithelial tissues.

Key words: Spermatogenesis, *p53*, differentiation.

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The tubular structures that contribute to the passage of spermatozoa and testicular fluid can be divided into intratesticular and extratesticular segments. The major part of the intratesticular segment consists of seminiferous tubules, which join together to form the duct of the epididymis, the tail of which gives rise the vas deferens (de Kretser et al, 1982). The role of all these structures is not solely passive; they actively contribute to spermatogenesis, the motility of spermatozoa, the composition of the seminal fluid, and the final step of maturation of spermatozoa.

Above all, the testis has the specific function of generating spermatozoa from precursors termed spermatogonia after a complex series of divisions (Clermont and Huckins, 1961; de Kretser and Kerr, 1994). This process takes place within the seminiferous epithelium, which is a complex structure composed of a stratified arrangement of germ cells with their cellular associations in progressive steps of differentiation and radially oriented supporting cells called Sertoli cells. Moreover, apoptosis of germ cells in the testis plays an important role in providing an intratubular environment for progression of spermatogenesis by controlling the number of each cell type (Hayashi et al, 2000, 2002).

On the other hand, the vaso-epididymal system is a single duct, which is differentiated from the Wolffian duct and conveys spermatozoa from the testis (Robaire and Hermo, 1988; Hermo et al, 1994; Kirchhoff, 1999; Atanassova et al, 2001). Spermatozoa undergo numerous changes along the epididymal duct that result in the acquisition of fertilizing ability (Hinton et al, 1995; Turner and Miller, 1997). All of these changes are controlled by the epididymal epithelium and take place progressively as the spermatozoa pass through the epididymis, which is divided into 3 major regions: caput, corpus, and cauda (Cooper, 1998; Holland and Nixon, 1998; Turner et al, 1999). This region-specific functional difference in a single Wolffian duct is accompanied by morphological differences in the luminal epithelium. Therefore, the testis and each region of vaso-epididymal system have their

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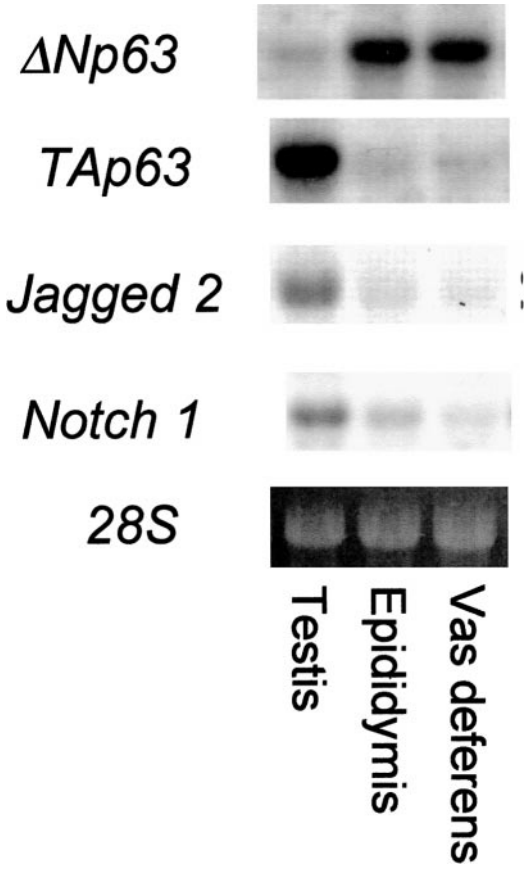

Figure 1. Northern blot analysis of ΔNp63, TAp63, Jagged 2, and Notch 1 mRNAs in adult rat testis, epididymis and vas deferens. Expression of *p63* isoforms is thought to be region specific. The TAp63, Jagged 2, and Notch 1 mRNAs were highly expressed in the testis, whereas ΔNp63 mRNA was highly expressed in the epididymis and vas deferens.

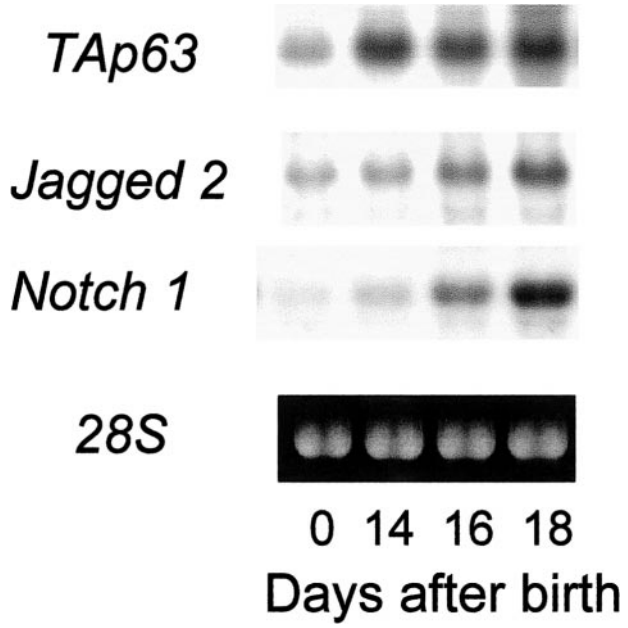

Figure 2. Northern blot analysis of TAp63, Jagged 2, and Notch 1 mRNAs in the rat testis during postnatal development. The RNA prepared from testes illustrates changing levels of TAp63, Jagged 2, and Notch 1 mRNAs in the testis at days 0, 14, 16, and 18 after birth when examined by Northern analysis. The expression of TAp63, Jagged 2, and Notch mRNAs gradually increased.

own specific structures and functions, although all these tubules and ducts are contiguous and collaborate with each other in the development of male gamete along their lengths.

A member of the tumor suppressor *p53* family of genes, *p63*, also termed *p51*, encodes *p63* with strong structural and functional similarities to *p53* (Osada et al, 1998; Yang et al, 1998). However, *p63* considerably differs from *p53* in its requirement for the developmental morphogenesis in some tissues and recent immunohistochemical studies detected *p63* expression in basal cells of mammalian tissues (Mills et al, 1999; Yang et al, 1999; Wang et al 2001). Although the function of *p63* is not fully understood, targeted studies demonstrated that this gene is highly expressed in the basal or progenitor layers of many epithelial tissues and plays a key role in maintaining their cell populations (Mills et al, 1999; Yang et al, 1999; Wang et al 2001). Moreover, the *p63* gene encodes for 2 major classes of protein. The TAp63 isoforms can transactivate *p53* target genes and induce apoptosis when overproduced, whereas ΔNp63 isoforms potentially suppress transactivation by both *p53* and TAp63 isoforms in a dominant-negative manner (Osada et al, 1998; Yang et al, 1998). On the other hand, Notch 1 and its ligand Jagged 2, critical factors in cell type specification, have an important role in the differentiation of germ cells (Hayashi et al, 2001) and Jagged 2 is up-regulated by TAp63γ, one of *p63* isoforms, which can trigger the Notch signaling system (Sasaki et al, 2002). Although

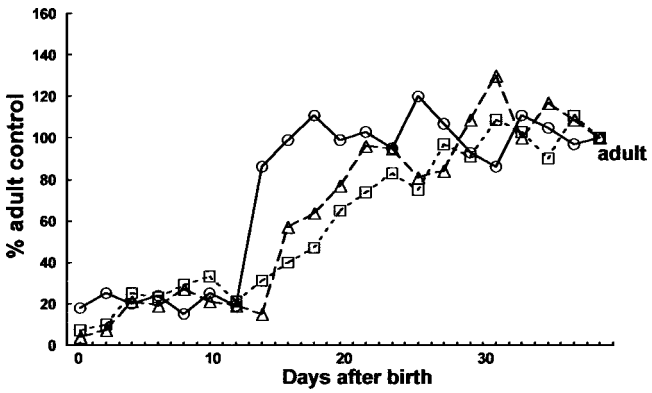

Figure 3. Changes of TAp63 (circles), Jagged 2 (squares), and Notch 1 (triangles) mRNA signal levels in the rat testis during postnatal development. Relative signal intensity levels are defined as the ratio to adult control levels of Northern blot analysis after they are normalized against the abundance of 28S RNA. Values are means of 4 experiments. The TAp63 mRNA expression began to increase markedly at day 14 after birth. Jagged 2 and Notch mRNAs began to increase at day 16 after birth.

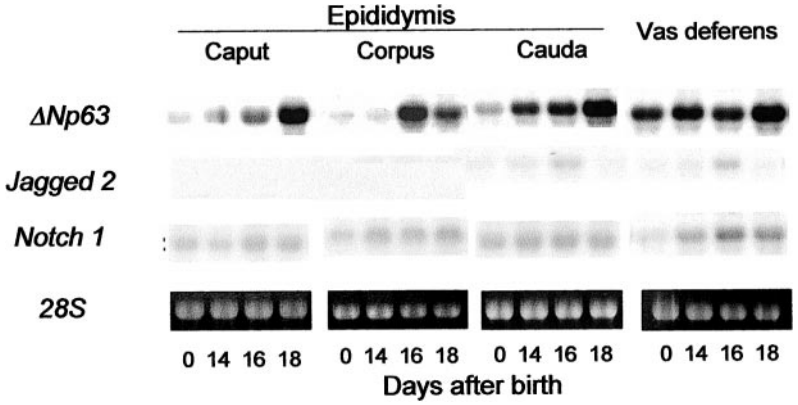

Figure 4. Northern blot analysis of ΔNp63, Jagged 2, and Notch 1 mRNAs in the rat epididymis and vas deferens during postnatal development. The RNA prepared from epididymis and vas deferens illustrates changing levels of ΔNp63 mRNA at days 0, 14, 16, and 18 after birth. The signal of ΔNp63 mRNA was maintained at a high level in the vas deferens, whereas its strong expression began after birth in the caput, corpus, and cauda epididymis. Jagged 2 and Notch 1 mRNAs were maintained at a low level.

expression patterns of p63 in most epithelia are established, the role of *p63* and its possible relationship with the Notch signaling system in the seminiferous epithelium has not been examined yet.

In the following study, we examined the expression of *p63* isoforms and Notch 1 and Jagged 2 transcripts in the developing rat testis and also examined those in the epididymis and vas deferens, the representative epithelium of the male reproductive tract, as an initial step to elucidate the physiological role of *p63* and the Notch signaling system and the possible relationship between them in the regulation of the proliferation and differentiation of germ cells in comparison with the epithelial cells in the male reproductive tract. Examination of the data provides new insights into the role of these proteins in the biology of the testis.

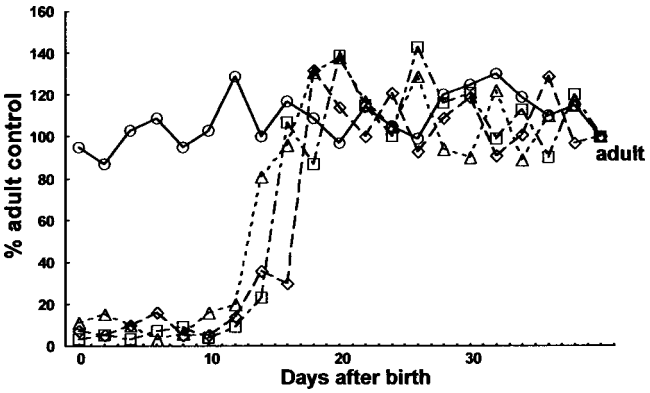

Figure 5. Changes of ΔNp63 mRNA signal levels in the rat caput (triangles), corpus (squares), cauda epididymis (diamonds), and vas deferens (circles) during postnatal development. Relative signal intensity levels are defined as the ratio to adult control levels after they are normalized against the abundance of 28S RNA. Values are means of 4 experiments. The signal of ΔNp63 mRNA increased markedly at days 18, 16, and 14 after birth in the caput, corpus, and cauda epididymis, respectively, whereas it was already high at day 0 after birth in the vas deferens.

Materials and Methods

Animals and Tissue Preparation

Male Sprague-Dawley rats ranging from day 0 to day 40 after birth and at day 180 after birth were obtained. Five to 12 rats at the same age were killed by decapitation before removal of the testis, epididymis, and vas deferens. The epididymis was divided into caput, corpus, and cauda. Rats were handled in the laboratory according to institutional guidelines as well as the *Guide for Care and Use of Laboratory Animals* of the National Research Council and with the approval of the institutional animal care and use committee.

Each tissue was cut in half. Then half of each tissue at the same age was snap frozen and pooled upon collection for RNA extractions with Isogen (WAKO Pure Chemical Industry, Tokyo, Japan), an RNA isolation reagent. The remaining half of each tissue was fixed in Bouin's fluid for 5–8 hours immediately upon collection, dehydrated, and embedded in paraffin for immunohistochemistry.

Northern Blot Analysis

The cDNA was synthesized from 2 μg of the total RNA samples by using the SuperScript Preamplification System for First-Strand cDNA Synthesis (Life Technologies, Gaithersburg, Md). Two microliters of the synthesized cDNA samples were subjected to polymerase chain reaction (PCR) with 2 μL of 10× reaction buffer (100 mM Tris-HCl pH 8.3 containing 500 mM KCl), 2 μL of deoxynucleoside triphosphate mixture (1.25 mM each of deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate), 2 μL of an upper-strand primer (10 μM), 2 μL of a lower-strand primer (10 μM), 0.1 μL of AmpliTaq DNA polymerases (5 U/μL, TAKARA Biomedicals, Tokyo, Japan), and 12 μL of H_2O. The reaction was performed by denaturing for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extending for 60 seconds at 72°C. Twenty-four cycles of PCR were performed for Notch 1 and 30 cycles were used for Jagged 2, ΔNp63, and TAp63. The sequences of the primers and expected fragment sizes are as follows: Notch 1, CTGTGAGCCCACAT-

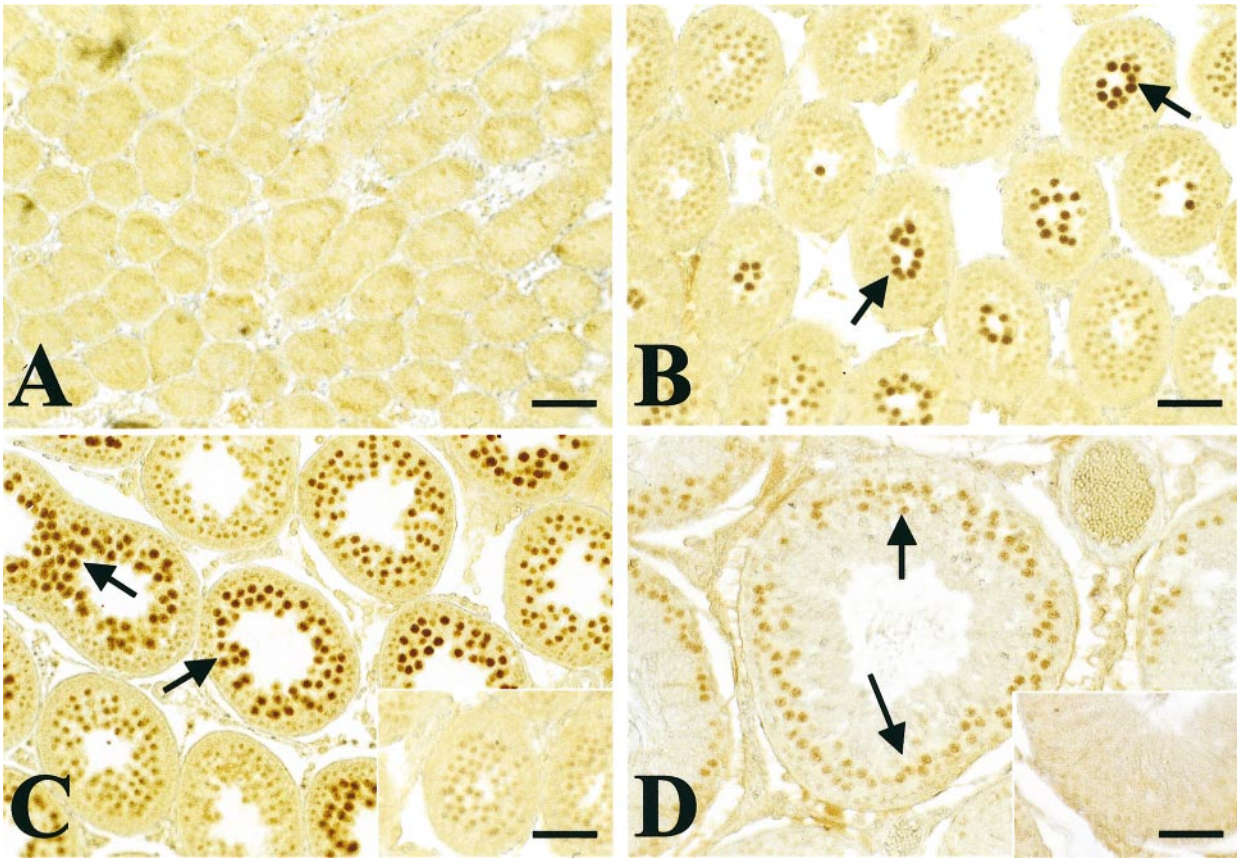

Figure 6. Localization of *p63* protein in the developing rat testis. Immunohistochemistry was used to localize *p63* protein in the testis at day 0 **(A)**, day 14 **(B)**, day 19 **(C)**, and day 180 after birth **(D)**. The negative controls of **C** and **D** are at the lower right corner of each panel. **A**: No positive staining was recognized at day 0 after birth. **B**, **C**, and **D**: Positive staining of *p63* expression (arrows) was recognized at days 14, 19, and 180, respectively, after birth and was confined to nuclear regions of spermatocytes. No significant staining was observed in the negative controls. Scale bar = 40 μm **(A–D)**.

CCGAGA and AGTTGCACTGGCTGTCACAG (330 bp); Jagged 2, ATCAACGCCGAGCCTGACCA and GCCAATCAGGTTTTTGCAAG (360 bp); ΔNp63, GGAAAACAATGCCCAGACTC and GAAGGACACGTCGAAACTGTG (252 bp); and TAp63, GGTGCGACAAACAAGATTGAG and GAAGGACACGTCGAAACTGTG (296 bp). The amplified products were electrophoresed in 1.5% agarose gels stained with ethidium bromide. The PCR products were sequenced to verify their identity as described previously (Hayashi et al, 2001). The PCR for ΔNp63, TAp63, Jagged 2, and Notch 1 was performed with a cDNA sample of appropriate pools. The amplified products of expected size were cut out from agarose gels, purified by using a Geneclean II kit (BIO 101 Inc, La Jolla, Calif), and then cloned into a pGEM-T Easy plasmid vector (Promega Corporation, Madison, Wis) with T4 DNA ligase. The DNA sequences of inserts were determined in both directions by using T7 (TAKARA Biomedicals) and SP6 promoter primer (Stratagene, La Jolla, Calif). Each set of primers used for reverse transcription–PCR also was used for the sequencing reaction. We employed dideoxy chain reaction methods by using a Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham Life Science, Inc, Cleveland, Ohio). For Northern blot analysis, total RNA (10 μg) was electrophoretically separated on a 1% agarose gel containing 2.2 M formaldehyde and blotted on a nitrocellulose membrane. The RNA was visualized with ethidium bromide to ensure that it was intact and loaded in similar amounts and to confirm proper transfer. Hybridization was performed with alpha ^{32}P-labeled DNA probes prepared from PCR clones as described previously (Hayashi et al, 2001). Quantitative analysis of mRNA transcript was defined as the ratio to adult control level after normalization against the abundance of 28S RNA. Experiments were repeated 4 times for each mRNA to establish the reproducibility of these results and data are shown as the mean of 4 experiments.

Immunohistochemistry for the Detection of p63 Protein

Immunohistochemistry was employed to localize the *p63* in rat sections by following standard protocols with minor modifications as previously described (Hayashi et al, 2001). Briefly, 5-μm sections were floated on diethyl pyrocarbonate–treated MilliQ (Millipore, Bedford, Mass) water and dried onto slides. Then sections were dewaxed, rehydrated, treated with 0.3% hydrogen peroxide for 5 minutes, and heated to 100°C in 50 mM glycine, pH 3.5, for 10 minutes. Slides were washed twice in Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.6)

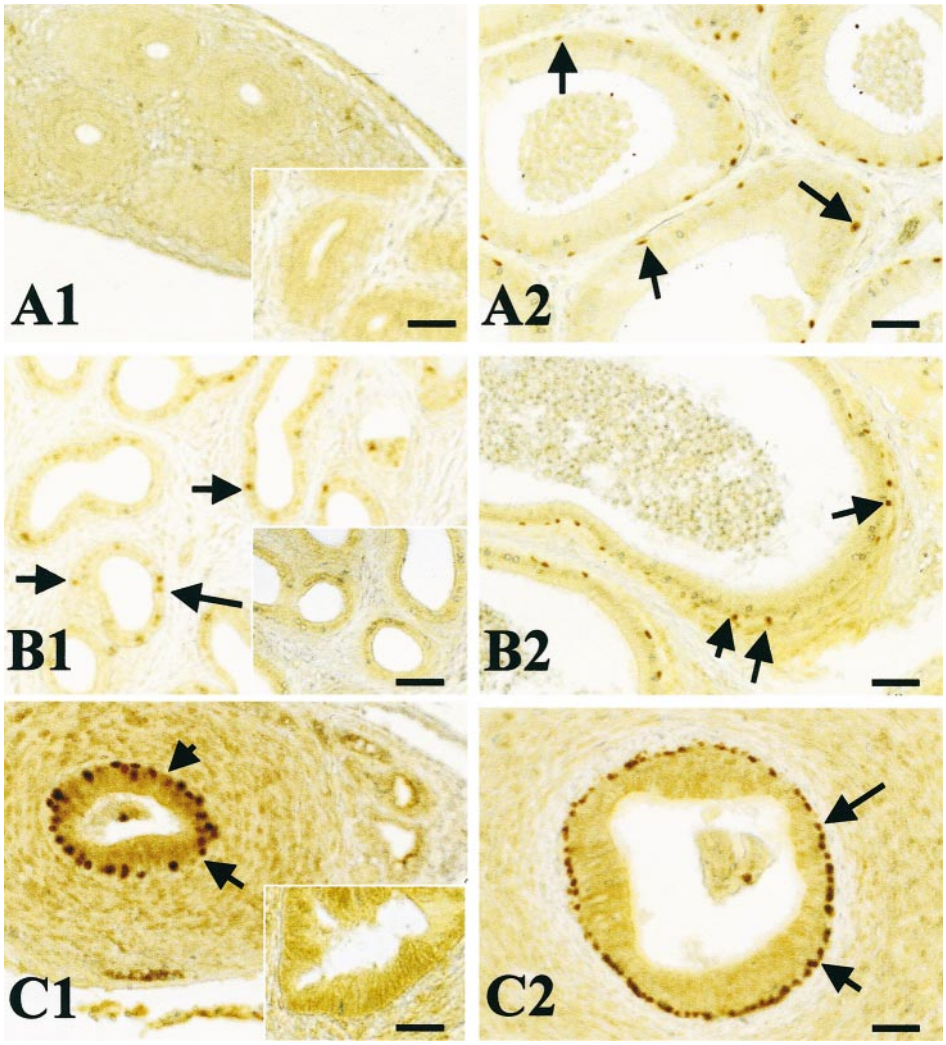

Figure 7. Localization of *p63* protein in the developing rat epididymis and vas deferens. Immunohistochemistry was used to localize *p63* protein in the caput **(A1** and **A2)**, cauda epididymis **(B1** and **B2)**, and vas deferens **(C1** and **C2)**. The negative controls of **A1**, **B1**, and **C1** are at the lower right corner of each panel. **A1**: No positive staining was recognized in the caput epididymis at day 14 after birth. **A2**: Positive staining (arrows) was recognized in the caput epididymis at day 18 after birth. **B1**: Positive staining (arrows) was recognized in the cauda epididymis at day 14 after birth. **B2**: Positive staining (arrows) was recognized in the cauda epididymis at day 18 after birth. **C1**: Positive staining (arrows) was recognized in the vas deferens at day 0 after birth. **C2**: Positive staining (arrows) was recognized in the vas deferens at day 18 after birth. Positive staining in every section was confined to basal cells of their epithelium. No significant staining was observed in the negative controls. Scale bar = 20 μm.

for 5 minutes between all subsequent incubations, which were performed at room temperature in a humid chamber. A blocking solution was added for 20 minutes, consisting of 5% normal serum diluted in TBS with 0.1% bovine serum albumin (TB). Primary antibodies were diluted in TB and incubated for 60 min. A mouse monoclonal antibody 4A4 (sc-8431; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) raised specifically against all *p63* isoforms was used at a dilution of 1:200. Incubation with biotinylated secondary rabbit anti-mouse immunoglobulin G diluted in TB proceeded for 30 minutes, followed by incubation with streptavidin–horseradish peroxidase (Silenus, Tokyo, Japan) at 2.68 μg/ml for 30 minutes. Antibody binding was visualized by using hydrogen peroxide–activated diaminobenzidine tetrahydrochloride (2.13 mg/ml in TBS; Sigma). Sections were cleared in xylene and mounted with Permount (Fisher Scientific Corp, Fairlawn, NJ). As negative controls, sections were processed together in an identical manner, except for substitution of either mouse anti-p63 primary antibody with an equivalent dilution of nonimmune serum. In all cases, no significant staining was observed for control serum in any tissues. Experiments were repeated 5 times to establish reproducibility.

Results

Expression of ΔNp63, TAp63, Jagged 2, and Notch 1 mRNAs in the Testis, Epididymis, and Vas Deferens

In the adult rat testis, TAp63, Jagged 2, and Notch 1 mRNAs were highly expressed, whereas the expression

of ΔNp63 mRNA was at a low level (Figure 1). In the adult epididymis and vas deferens, on the other hand, ΔNp63 mRNA was highly expressed, whereas the expressions of TAp63, Jagged 2, and Notch 1 mRNAs were at a low level (Figure 1).

During postnatal development, TAp63, Jagged 2, and Notch 1 mRNAs were at low levels in the testis at day 0 after birth. The expression of TAp63 mRNA began to increase markedly at day 14 after birth and reached the adult level at day 16 after birth, and then the expression of Jagged 2 and Notch 1 mRNAs began to increase progressively in the testis (Figures 2 and 3). On the other hand, in the caput, corpus, and cauda epididymis, the expression of ΔNp63 mRNA was at low level at day 0 after birth and then increased markedly at days 18, 16, and 14 after birth, respectively, whereas its high expression was already recognized at day 0 after birth and continued during postnatal development in the vas deferens. However, Jagged 2 and Notch 1 mRNAs were expressed at a low level at all the ages examined during postnatal development in the vaso-epididymal system (Figures 4 and 5).

Tissue Localization of p63 Protein in the Testis, Epididymis, and Vas Deferens

The expression of *p63* protein was not detectable at day 0 after birth in the rat testis (Figure 6A), whereas it was detectable at first in spermatocytes in the rat seminiferous tubules at day 14 after birth (Figure 6B). Positive staining was confined to nuclear regions of spermatocytes. The p63-positive spermatocytes increased in number as the diameter of seminiferous tubules increased in size (Figure 6C and D). However, no positive staining was present in spermatogonia, spermatids, or spermatozoa in the adult testis (Figure 6D).

On the other hand, the expression of *p63* protein was detectable from day 0 after birth only in the vas deferens (Figure 7C1 and C2), whereas it was not detectable in cauda epididymis and caput epididymis until day 14 or day 18 after birth, respectively (Figure 7A1 through B2), and positive staining was confined to nuclear regions of basal cells of their epithelium.

Discussion

This is the first study demonstrating that the increase of TAp63 mRNA, which is expressed in the testis, is followed by that of the Notch signaling system during postnatal development and *p63* protein is expressed in spermatocytes in the testis. Taken together with the result of our previous study showing an expression of Notch signaling system in the testis (Hayashi et al, 2001), it seems likely that the expression of *p63* protein in spermatocytes is followed by that of Jagged 2 and Notch 1 protein in spermatids during spermatogenesis as well as expression of their mRNAs. Consequently, the expression of *p63* protein precedes the expression of the Notch signaling system during germ cell development. Jagged 2 also has been recently found to be up-regulated by TAp63γ, one of the *p63* isoforms, which can trigger the Notch signaling system (Sasaki et al, 2002). Therefore, examination of our results first shows the probability that p63-mediated Jagged 2 induction activates the Notch signaling system in the testis, which plays an important role for germ cell differentiation. Moreover, evidence exists to indicate that TAp63 isoforms can transactivate *p53* target genes and induce apoptosis when overproduced (Osada et al, 1998; Yang et al, 1998). Therefore, it is possible that TAp63 has an important role for maintenance of the number of germ cells of each type by triggering or balancing the development, differentiation, and apoptosis of germ cells in the testis.

On the other hand, the current study is also the first to demonstrate that the beginning of the marked increase of ΔNp63 mRNA expression advanced chronologically along the duct from vas deferens to caput epididymis in the highly region- and age-specific manner and that the convolution of the tubule was recognized in the part of mesonephric tubule with a low level of ΔNp63 expression at birth, suggesting the probability that the convolution of the tubule occurs while ΔNp63 expression is at a low level and ceases after the expression increased to the adult level. There is evidence to indicate that ΔNp63 isoforms play a critical role in regulating differentiation of the normal epithelium and maintaining normal epithelium structure (Mills et al, 1999; Yang et al, 1999; Yang and McKeon, 2000). Therefore, it seems most likely that the chronological differences of ΔNp63 expression play an important role in the regional differences in the morphology and function of the mesonephric tubule, which differentiates into various parts of epididymis and vas deferens.

In conclusion, it seems most likely that the role of TAp63 in the testis is completely different from the role of the ΔNp63 in the epithelial tissues of the vaso-epididymal system. Examination of the data in this study now clearly raises the probability that TAp63 governs the balance between development, differentiation, and apoptosis of germ cells in the testis through the Notch signaling system and *p53* target genes, facilitating an understanding of the action of these proteins in the testis, and that the chronological differences of ΔNp63 expression have an important role for the regional differences in the development of the epithelial cells of mesonephric tubule, which result in their morphological and functional differences. Clearly, further studies are required to determine how TAp63 controls the number of germ cells by modulating the balance between development, differentiation,

and apoptosis of germ cells and how ΔNp63 mediates the differentiation of epithelial cells in the mesonephric tubule.

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