Expression of p63 in the Mouse Primordial Germ Cells

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ABSTRACT. Proteins encoded by p63 gene a have a structural similarity with tumor suppressor p53, and were thought to induce cell cycle arrest and apoptosis during development. The p63 proteins are also expressed in the basal cells of many epithelial tissues in the adult, and supposed to play important roles in maintaining the epidermal stem cells. Previously, we reported the p63 expression in the testis of mouse embryos, suggesting their involvement in the growth arrest and apoptosis of testicular germ cells (Nakamuta and Kobayashi, J. Vet. Med. Sci. 65:853–856). In this study, we investigated the timing of this p63 expression in the germ cells during migration and colonization to the gonads. Immunohistochemical analysis of mice from embryonic day (E) 7.5 to E12.5 demonstrated that p63 positive reactivity was seen as early as E8.5 when the founder cells of germ cells, primordial germ cells (PGCs), were located in the hind gut epithelium, but PGCs were negative for p63 at E7.5 when they first appeared. p63 is expressed as six isoforms, resulting from alternative splicing at C-terminus and by the use of two promoters that generate variations at N-terminal end. RT-PCR analyses suggested that at different types of p63 mRNAs were likely to be expressed in PGCs during development. These results imply that p63 may be involved in the regulation of PGC development by controlling the gene expression required for their migration and colonization to the gonads.

KEY WORDS: immunohistochemistry, PGC, p63, RT-PCR.

Primordial germ cells (PGCs) are of a founder population of germ cells which sustain the development of next generation. In the early embryonic development, they appear within endoderm of posterior extraembryonic site [20]. PGCs, recognized by their alkaline phosphatase activity, are initially found within the base of allantois at embryonic day (E) 7.5, migrate through hindgut and dorsal mesentery, and arrive at genital ridges, which will form future gonads, by E11.5. They proliferate rapidly during migration and colonization until E12.5 when sexual differentiation of the fetal gonads occurs. So, it is required for PGCs to change their cell surface molecules according to the location within embryos, through the interaction with the cells and extracellular matrices in their migration route or several inducing/repelling substances. However, precise mechanisms controlling these gene expressions are still unclear.

The p63 protein is a transcription factor encoded by a p53 family gene and, like p53 proteins, consisted of three major functional domains: N-terminal transactivation (TA) domain, central DNA-binding domain, and C-terminal oligomerization domain [11, 21]. In addition, an extended C-terminal coding region, not found in p53, is present in p63 [21]. Within this C-terminal extension, there is a sterile alpha motif (SAM) which is thought to be involved in protein-protein interactions and is found in several proteins such as cytoplasmic signaling molecules and transcription factors [18].

The p63 gene consisted of 14 exons: TA domain is encoded by exons 1 to 3, DNA-binding domain by exons 4 to 8, oligomerization domain by exons 9 and 10, secondary TA domain by exons 11 and 12, and SAM domain by exons 13 and 14. Due to the presence of two promoters, transcripts encoding two types of proteins are generated. In contrast to TAp63 with TA domain at the N-terminus, proteins lacking it are called ΔNp63. In both types, alternative splicing leads to the production of three different C-termini, designated as α, β, and γ. Transcription activating potential of TAp63 is greatly repressed by the presence of C-terminal domain of α isoform, while β and γ isoforms of p63 have a significant transactivating activity [21]. This is attributed to a C-terminal inhibitory domain which acts by binding to a region in the N-terminal TA domain [4, 16]. In addition to the role as a transcription factor, p63 proteins can regulate cellular functions by protein-protein interaction. Indeed, it has been shown that ΔNp63 acts as a positive regulator in β-catenin signaling pathway by binding to the regulatory subunit of protein phosphatase 2A and glycogen synthase kinase 3β [12].

Several lines of evidence have shown that p63 is a marker for progenitor cell population of keratinocytes [13], and is expressed in the nuclei of basal cells of many epithelial tissues including skin, oral epithelium, urothelium, prostate, and mammary gland [3, 17]. Unlike the case in p53, where mutations lead to human cancers, germ line mutations of p63 lead to congenital abnormalities characterized by abnormal limb development and ectodermal dysplasia. For example, single amino acid substitutions and frameshift mutants of p63 protein cause the human ankyloblepharon ectodermal dysplasia clefting (AEC) or ectrodactyly ectodermal dysplasia and facial clefting (EEC) syndromes [2, 6].

In the previous report, we found the p63 expression in the testicular germ cells of mouse embryos, suggesting an association with the growth arrest (around E13.5) and apoptosis (from E13.5 to E17.5) of these cells [9]. We examined here using different stages of mouse embryos to determine at which developmental stage this p63 expression starts in the germ cells. Using immunohistochemistry with a specific monoclonal antibody, p63 was demonstrated in the nuclei of...
PGCs as early as E8.5, and interestingly, it has been suggested by RT-PCR that PGCs may change their p63 isoforms during their colonization to the developing gonad.

MATERIALS AND METHODS

Animals: Timed pregnant ICR mice were obtained from CLEA Japan and kept in the Animal Research Center of Kyushu Dental College until use. Mice were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kyushu Dental College, and all procedures were approved by the Committee for the Use of Laboratory Animals at Kyushu Dental College.

Immunohistochemistry: At 7.5, 8.5, 9.5, 10.5, 11.5, and 12.5 days of gestation, animals were sacrificed by cervical dislocation under diethyl ether anesthesia. Embryos were collected in phosphate-buffered saline (PBS), and fixed in Bouin’s fixative at room temperature (RT). After dehydrogenase (GAPDH).

RESULTS

Immunohistochemical localization of p63 in the nuclei of mouse PGCs: Initially, we investigated the expression pattern of p63 proteins in the germ cells of mouse embryos from E7.5 to E12.5 to clarify at which developmental stage p63 expression can be seen for the first time in PGCs. Using an antibody which reacts with all known p63 variants [21], nuclear staining was demonstrated in mouse PGCs as described below in detail. Migrating PGCs were identified by their distribution and characteristic cell shape. During the period investigated here, p63 protein was localized in mouse PGCs from E8.5 to E12.5 (Figs. 1 to 7). We examined six serially sectioned E7.5 mouse embryos, but failed to detect any immunopositivity at the base of allantochorion (Fig. 1). As shown in Fig. 2, PGCs migrating along hindgut epithelium expressed p63 at E8.5. In the E9.5 embryo, p63-positive cells were seen in hindgut and dorsal mesentery (Fig. 3). At E10.5, positive cells were observed within dorsal mesentery and urogenital ridges (Fig. 4). In the E11.5 embryo, p63-positive cells were present in genital ridges (Fig. 5). By E12.5, sexual differentiation of gonads occurred, and it was possible to distinguish testes from ovaries morphologically. However, germ cells showed p63-positive staining in both testis (Fig. 6) and ovary (Fig. 7). In contrast to the p63-positive germ cells of the testis integrated in the forming testicular cords, PGCs were scattered over the entire gonad in female.

To confirm whether the p63-positive reactivity was specific to PGCs, we also performed a double staining with Sox9, a Sertoli cell marker, and p63. The transcription factor encoded by Sox9 gene, which is expressed at sites of chondrogenesis in mouse embryo, is also detected in Sertoli cell precursors at E11.5, and testicular expression of Sox9 gene can be seen from embryonic period to adult [5, 8]. Figure 8 shows the result of immunofluorescence using anti-p63 and anti-Sox9 antibodies in a section of E12.5 mouse testis. Within the testicular cord, the nuclear localization of p63 protein in PGCs and Sox9 protein in Sertoli cells was evident.

RT-PCR analysis for p63 mRNAs in the PGCs of mice: Subsequently, to investigate the p63 isoforms expressed in PGCs, we performed RT-PCR using RNAs prepared from embryonic tissues including genital ridges at E10.5 and E11.5, or male and female gonadal primordia (E12.5). As}
an internal control for RNA extraction and cDNA synthesis, PCR amplification was first performed on all cDNA samples using primers for the housekeeping gene, GAPDH. The amplified products were resolved in 1.5% agarose gels, stained with ethidium bromide, and photographed under ultraviolet transillumination light. As shown in Fig. 9b, single bands corresponding to GAPDH were detected in each lane from E10.5 to E12.5, while samples run without reverse transcriptase did not yield significant amplification (data not shown).

As mentioned above, p63 proteins are divided into two groups with respect to their N-termini. While the TA isoforms are generated by the use of an upstream promoter, the ΔN isoforms, produced from an intronic promoter, lack the TA domain encoded by exons 1 to 3 (Fig. 9a). Therefore, using forward primers at exon 3 (encoding a part of TA domain) for reverse transcription did not yield significant amplification (data not shown).
domain) or at exon 3’ (where ΔN variants start transcription) in combination with a reverse primer at exon 4 (shared by TA and ΔN isoforms), it is possible to distinguish them by RT-PCR as described previously [10].

Figure 9b shows the presence of p63 transcripts in the samples, possibly reflecting the p63 expression in mouse PGCs from E10.5 to E12.5. Both mRNAs encoding TA and ΔNp63 were detected in the 10.5-day-old embryo. However, TA/p63 but not ΔNp63 could be amplified at E11.5. At E12.5, TA/p63 was also present in the male and female gonads, while ΔNp63 was not. These data suggest that p63 is transcribed mainly from TA promoter when PGCs are colonized in the genital ridges, and both TA and ΔN promoters are used when PGCs are still in the dorsal mesentery of developing mouse embryos.

Finally, RT-PCR analyses were undertaken with regard to 3’-end splicing events of p63. Alternative splicing gives rise to three different C-termini designated as α, β, and γ. In contrast to full-length α isoform, β lacks exon 13, and γ lacks exons 11 to 14, instead terminates in exon 10’. Therefore, we designed primers to distinguish these splicing variants by choosing forward primer at exon 10 shared by three isoforms and reverse primers at exon 13 for α isoform, across exon 12 and exon 14 for β, and at exon 10’ for γ isoforms. Using these primers, PCR products of 432 bp can be obtained for p63α, 387 bp for p63β, and 189 bp for p63γ.

As shown in Fig. 9c, β isoform was detected in the samples of E10.5, while α and γ isoforms were amplified from all samples between E10.5 to E12.5. The result was confirmed by PCR using another primer pair specific to β isoform (data not shown). Therefore, among the three variants produced by 3’ alternative splicing, β isoform is expressed only at the period when PGCs are migrating in the dorsal mesentery, whereas α and γ isoforms are continuously present during migration and colonization to the gonads.
In this paper, we described the pattern of p63 expression in the PGCs of mouse embryos during migration and colonization to the gonads using immunohistochemical analysis. At E7.5 when PGCs were located at the base of allantois, no p63 immunopositive reaction could be observed. From E8.5 to E11.5, PGCs were p63-positive during their migration through hindgut epithelium and dorsal mesentery. Immunoreactivity for p63 was also observed in the germ cells of both testis and ovary at E12.5 when testes became distinguishable from ovaries. While p53 protein behaves as a canonical tumor suppressor, p63 and p73 have been shown to play important roles in ectodermal differentiation and neural development, respectively [7, 22, 23]. Particularly, the observation that mice deficient for p63 gene develop with limb truncation and lack of stratified epithelia suggest that p63 proteins are required for limb and epidermal morphogenesis. However, the roles of p63 in the germ cells are still unknown. p63 proteins are not only expressed in PGCs as shown in the present study, but also localized in the nuclei of testicular germ cells of mouse during embryonic development, as well as in the postnatal period [9, 10]. Furthermore, p63 proteins are present in the meiotic germ cells of mouse ovaries (our unpublished observation). Consequently, these data suggest that p63 proteins play important roles in the process of germ cell development.

In an attempt to determine the p63 isoforms expressed in mouse PGCs, we analyzed embryonic tissues by RT-PCR and obtained the results which could be considered to reflect the p63 expression in PGCs. From our data, it can be deduced that different promoters are used by PGCs before and after their colonization to the genital ridges, and that β isoform is expressed during the period when PGCs are located in the dorsal mesentery (E10.5), in addition to α and γ isoforms continuously detected from E10.5 to E12.5. An analysis of RNAs extracted from E12.5 mouse gonads yielded the same results in the testis and ovary with regard to p63 expression. Moreover, no differences were found between both sexes as to p63 expression in the urogenital ridges of E10.5 and E11.5 mice, judging from Ube1 gene PCR (data not shown). These results imply that there are no differences between males and females as for the roles of p63 in PGCs before the germ cells are determined their different developmental fates to the testis or ovary. Interestingly, the data obtained here suggest that all six p63 isoforms are likely to be expressed in PGCs, and therefore p63 proteins exhibit wide varieties of transactivation activity by their tetramer formation [16].
In the mouse embryos, germ cells are formed during gastrulation and incorporated into the developing hindgut. Then, they actively migrate toward the sites where the gonad will be formed and combined with somatic cells. During migration, it is required for PGCs to change their gene expression in response to their environmental signals, and to express cell surface molecules to interact with neighboring cells or extracellular matrices at different parts of the embryos. Notch is a transmembrane receptor which is activated by ligand binding and implicated in the cell fate determination throughout development [1]. Interestingly, it has been shown that TAp63 upregulates genes encoding two Notch ligands, Jagged1 and Jagged2, while ΔNp63 down-regulates Jagged2 gene [15, 19]. Therefore, the Notch signaling system might be involved in the cellular interactions required for PGC development, and the regulation of their expression may be one of the roles of p63 in PGCs.

In conclusion, these expression data presented here demonstrate that PGCs express p63 and are likely to change their isoforms by alternative splicing and promoter usage during development. p63 expression may be one of the roles of p63 in PGCs.

REFERENCES


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