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—Original Article—

Differential Apoptotic and Proliferative Activities of Wild-type FOXL2 and Blepharophimosis-ptosis-epicanthus Inversus Syndrome (BPES)-associated Mutant FOXL2 Proteins

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Abstract. FOXL2 is an essential transcription factor that is required for proper development of the ovary and eyelid. Mutations in FOXL2 cause an autosomal dominant genetic disorder, blepharophimosis-ptosis-epicanthus inversus syndrome (BPES). BPES type I patients have eyelid malformation and premature ovarian failure leading to infertility, whereas women with type II BPES are fertile or subfertile. In the present study, we evaluated and compared apoptotic and antiproliferative activities of wild-type (WT) and mutant FOXL2 proteins found in BPES type I and II in human granulosa cell tumor-derived KGN cells. Ectopic expression of WT FOXL2 induced apoptosis and inhibited cell cycle progression in human granulosa cells. In contrast, mutated FOXL2s found in BPES type I significantly reduced these activities, whereas mutated FOXL2s in BPES type II showed intermediate activities. Furthermore, mutant FOX L2 proteins were defective in activating transcription of target genes including *Caspase 8*, *TNF-R1*, *FAS*, *p21*, and *BMP4*, which regulate apoptosis, proliferation, and differentiation of granulosa cells. Thus, decreased apoptotic and antiproliferative activities caused by mutant forms of FOXL2 found in BPES patients may at least partially contribute to the pathophysiology of ovarian dysfunction.

Key words: Apoptosis, Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), Cell cycle, FOXL2, Granulosa cells, Premature ovarian failure, Proliferation

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FOXL2 is a member of the forkhead (FH) family of transcription factors known to be required for proper development of the ovary and eyelid [1]. *FOXL2* is a single-exon gene that encodes a protein of 376 amino acids containing a DNA-binding FH domain and an alanine-rich region. Mutations in the *FOXL2* gene cause the autosomal dominant genetic disorder blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; MIM 110100) [2]. BPES type I patients have eyelid malformation associated with premature ovarian failure (POF). In contrast, type II BPES patients show defects in eyelid development, but affected individuals are fertile [2]. Although FOXL2 mRNA is expressed in fetal, juvenile, and adult mouse ovaries, undifferentiated granulosa cells (GCs) of small follicles have prevalent FOXL2 expression [3]. Female *Foxl2* knockout mice are infertile and have defects in granulosa cell differentiation [4, 5]. Conditional knockout of *Foxl2* in adult mice results in somatic transdifferentiation of an adult ovary into a testis [4]. Also, more than 97% of adult-type ovarian GC tumors have a somatic point mutation (C134W) in the *FOXL2* gene [6]. These human and mouse studies indicate that FOXL2 is an essential protein required for proper development and maintenance of the ovary. Although the mechanism by which FOXL2 orchestrates ovarian physiology is not completely understood, its role in regulating target genes involved in apoptosis, the cell cycle, and differentiation has been reported [7–10].

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In the present study, we investigated the functional differences of FOXL2 mutants found in BPES type I and II patients and observed compromised activities of mutant FOXL2 proteins that may at least partly contribute to the pathophysiology of ovarian dysfunction induced by FOXL2 mutations.

Materials and Methods

Chemicals

The chemicals used in the experiments were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

Plasmid construction

Myc-tagged FOXL2 and mutated FOXL2s (Q53X, Q219X, Y274X, ΔN, ΔFH, ΔAla, and ΔC) were generated by PCR amplification as described previously [11]. The mutant FOXL2 I80T, I84S, N105S, N109K and G269R constructs were produced by a recombinant PCR technique using the following primers (Bioneer, Daejeon, Korea): FOXL2-F (5′-CTAGAATTCAAATGATGGCCAGCTACCCC), S33A-F (5′-CCGGCCCCAGGCAAGGGCGGTGGGGGT), I80T-F (5′-CTGTCCGGCACCTACCAGT), FOXL2-I84S-F (5′-CCAGTACAGCATCACGAA), N105S-F (5′-CATCCGCCACAGCCTCAGCC), N109K-F (5′-CCTCAAA**G**AGTGCTTCATCAAGGT), G269R-F (5′-CCCCCGGCGTAGTGAACTCGT), FOXL2 I80T-R (5′-GGTGCCGGACAGCGTGAGCCT), FOXL2-I84S-R (5′-ATGCTGTACTGGTAGAT), FOXL2-N105S-R (5′-AGGCTGTGGCGGATGCTATTTTGC), FOXL2-N109K-R (5′-AAGCACTCTTTGAGGCTGAGGTTG), FOXL2-G269R-R

(5′-ACTACGCCGGGGGGGCAGCGCCAT) and FOXL2-R (5′-CTACTCGAGTCAGAGATCGAGGCGCGAATG).

KGN cell culture and transfection

Human granulosa cell tumor-derived KGN cells were cultured and transfected accordingly to a previous study [11]. In brief, KGN cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (GIBCO, Gaithersburg, MD, USA). Media containing 10% fetal bovine serum (FBS) (PAA, Etobicoke, ON, Canada) and 1% penicillin-streptomycin (Welgene). KGN cells (1×10^6) were suspended in Resuspension Buffer R (Invitrogen, Carlsbad, CA, USA), electroporated with plasmid DNA using an MP-100 MicroPorator (Invitrogen), and incubated on plates containing fresh media for 24 h.

Isolation and transfection of mouse granulosa cells

Immature female ICR mouse (5 weeks) were purchased from Samtako (Osan, Korea), housed at Chung-Ang University animal facilities, and maintained in accordance with guidelines provided and protocols approved by the Institutional Animal Care and Use Committee of Chung-Ang University. The isolated mouse ovaries were incubated in warm DMEM-F12 medium. Ovaries were transferred to a new dish and punctured with a 1-ml syringe needle gently (Kovax, Ansan, Korea). Granulosa cells were collected by centrifugation (180 × g) for 3 min and washed with DMEM-F12. Isolated granulosa cells (1×10^5) were plated onto 24-well plates (Nunc, Copenhagen, Denmark) and incubated for 24 h to allow attachment. The medium was replaced with new medium to remove oocytes, and then the granulosa cells were transfected with 300 ng of plasmids coding WT or mutated FOXL2 using Metafectene (Biontex, Martinsried, Germany) as suggested by the manufacturer.

Cell viability assay

Cell viability was measured 24 h after transfection of KGN cells (1×10^6) using a CellTiter-Glo assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Flow cytometry analysis of Annexin V-positive cells

To detect apoptotic cells, KGN cells were stained 24 h post-transfection with a FITC-conjugated Annexin V-FITC kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Flow cytometry analyses were then carried out using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Analysis of cell cycle

Twenty-four hours after transfection of KGN cells (1×10^6), the cells were harvested, resuspended in PBS, and fixed with cold ethanol (70%) followed by incubation for 1 h on ice. After centrifugation and washing, the cell pellets were resuspended in PBS containing 0.2 mg/ml RNase A and incubated at 37 C for 1 h. Propidium iodide (10 μg/ml) was added to the cell mixture, and the cells were kept in the dark at 4 C until analysis using the a FACSCalibur flow cytometer.

Western blot analysis

Cell lysates were prepared 24 h after transfection, subjected to electrophoresis, and immunoblotted with antibodies. The antibodies used were as follows: anti-Myc (Clontech, Mountain View, CA, USA), anti-Fas (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (Santa Cruz Biotechnology), and anti-GAPDH (AbFrontier, Seoul, Korea).

Reverse transcription (RT) and real-time PCR analysis

Reverse transcription of total RNA extracted and subsequent real-time PCR were conducted as previously reported [11]. Quantification of transcripts was normalized to GAPDH. The nucleotide sequences of the primers were as follows: 5′-ACGACGCGTATGAGCCGAGGAAGGCACTGA and 5′-CTACTCGAGACCAAAAACTCAGAGCACATG *(Caspase 8)*, (5′-CCAAATGGGGGAGTGAGAGG and 5′-AAAGGCAAA GACCAAAGAAAATGA (*TNF-R1*), 5′-TGAAGGACATGG CTTAGAAGTG and 5′-GGTGCAAGGGTCACAGTGTT *(FAS)*, and 5′-GGCAGACCAGCATGACAGATT and 5′-GCGGATTAG GGCTTCCTCT *(p21)*, 5′-TTCCTGGTAACCGAATGCTGA and 5′-CCCTGAATCTCGGCGACTTTT *(BMP4)*, 5′-GGGCACCTTTAC GGCTTCC and 5′- GGTTCTCCTTACAGCCACACA (mouse *TNF-R1*), 5′-GTCTCGGTGACAAAGTCGAAGTT and 5′-GTCTCGGTGACAAAGTCGAAGTT (mouse *p21*).

Luciferase assay

Luciferase assays were conducted in mouse granulosa cells isolated using the reporter constructs with *TNF-R1 and p21* promoters, as previously described [9, 10].

ChIP-qPCR analysis

Chromatin immunoprecipitation (ChIP) was conducted as previously reported [11]. Briefly, KGN cells were cross-linked with 1% formaldehyde at room temperature for 10 min, washed with PBS, and lysed followed by centrifugation at 2400 × g. The pellet was resuspended in shearing buffer and sonicated. Ten percent of the mixture containing protein/DNA complexes was used for "input DNA" analysis. An equal amount of the protein/DNA complex mixture was then incubated at 4 C with protein G agarose beads (Millipore, Milford, MA, USA), and overnight with control IgG (Santa Cruz Biotechnology) or anti-Myc antibody (Clontech). The precipitated DNA fragments were amplified using specific primers for *TNF-R1* and *p21* that encompass the putative FOXL2-binding sequences (–751 to –532 of *TNF-R1* and –805 to –554 of *p21*) via real-time PCRs, and the data were analyzed as provided by the manufacturer (Qiagen, Valencia, CA, USA).

Statistical analysis

Multiple comparison analyses of values were performed with the Student-Newman-Keuls test (SAS). The data represent means ± SEM, and $P<0.05$ was considered to be statistically significant.

Results

Generation of mutant FOXL2 proteins

In order to evaluate changes in the cellular activities of mutant FOXL2 proteins found in BPES patients, constructs encoding the mutants were cloned. For mutants found in type I BPES, various truncated forms of FOXL2 at the carboxy terminus due to frame

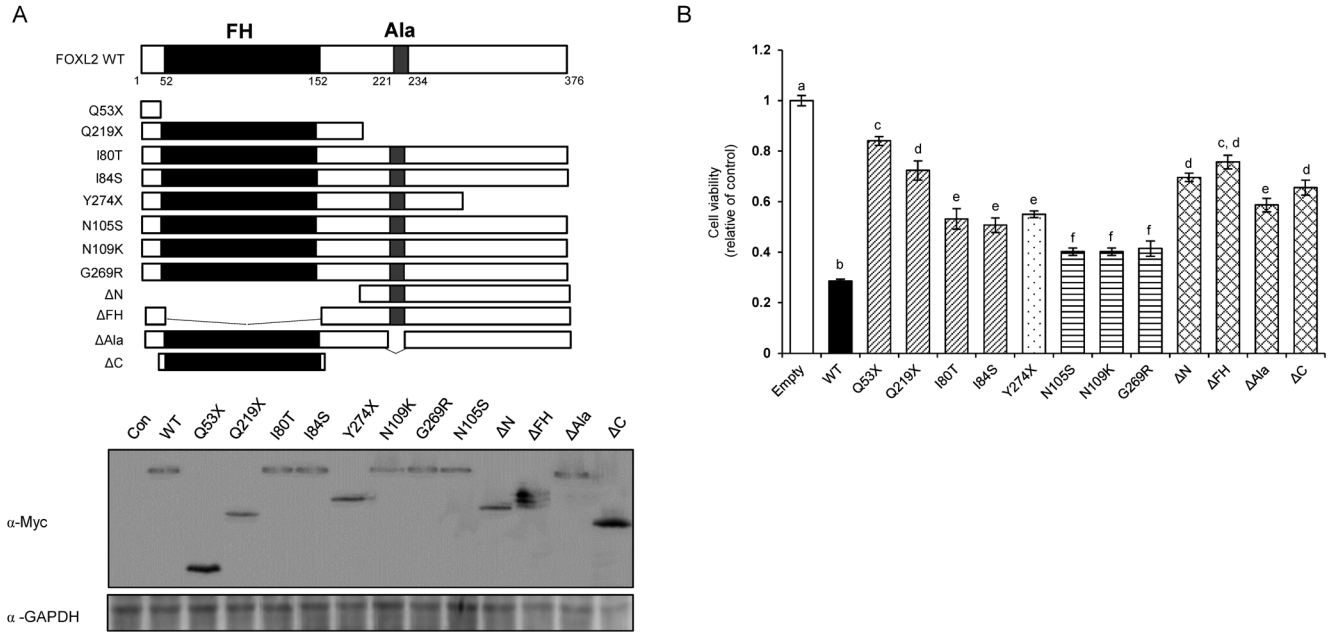

Fig. 1. Differential apoptotic activities of wild-type and mutated FOXL2 proteins. A: The secondary structure of FOXL2 and its mutants are shown: endogenous mutants found in BPES type I patients include Q53X, Q219X, I80T, I84S, and Y274X, while Y274X, N105S, N109K, and G269R were found in BPES type II. Artificial FOXL2 mutants cloned include ΔN, ΔFH, ΔAla, and ΔC (upper panel). Each plasmid was ectopically expressed in KGN cells, and the lysates were used for immunoblot analysis. FOXL2 proteins were detected with the anti-Myc antibody (lower panel). Equal loading of lysates was ensured by GAPDH expression. B: The viability of KGN cells was analyzed as described in the Materials and Methods. For all experiments, equivalent levels of protein expression were confirmed, and the results are from three independent experiments performed in triplicate. Different letters denote statistically significant values ($P<0.05$).

shift mutations leading to premature stop codons were cloned: Q53X, Q219X, I80T, I84S, and Y274X, the latter was also reported to manifest type II BPES [12–15] (Fig. 1A). Amino acid-substituted mutants found in BPES type II that were cloned included: Y274X, N105S, N109K, and G269R [16–18] (Fig. 1A). In addition, artificial FOXL2 mutants that could allow mapping of the functional region critical for its activities were cloned: ΔN, ΔFH, ΔAla, and ΔC (Fig. 1A). Each FOXL2 protein was overexpressed in the human GC line KGN, and their appropriate expression was confirmed by Western blot analysis (Fig. 1A).

Defective apoptotic activity of mutant FOXL2 proteins

As we reported previously, WT FOXL2 decreased the viability of KGN cells [10] (Fig. 1B). In contrast, this cell death effect was significantly diminished upon ectopic expression of Q53X, Q219X, I80T, I84S, and Y274X, with the more truncated form showing less activity (Fig. 1B). Decreased cell death was also observed for N105S, N109K, and G269R, but the effect was less severe compared with BPES type I mutants (Fig. 1B). All of the FOXL2 peptides lacking the FH DNA binding domain (ΔN and ΔFH), alanine repeats (ΔAla), and *C*-terminus (ΔC) showed decreased cell death activity (Fig. 1B), implying that each region plays a role in proper functioning of FOXL2. Flow cytometry analysis of Annexin V-positive apoptotic cells also showed a similar trend (Fig. 2A).

Differential activation of genes regulating apoptosis by mutant FOXL2 proteins

We previously showed that *Caspase 8,* tumor necrosis factor-receptor 1 (*TNF-R1*), and *FAS* (CD95/APO-1), crucial genes involved in apoptosis signaling, are transcriptionally regulated by FOXL2 [10]. In this study, we compared activation of these target genes by WT and mutant FOXL2 proteins by quantitative RT-PCR. Forced expression of WT FOXL2 stimulated *Caspase 8* expression; however, the activation was abolished or markedly reduced by mutated FOXL2s found in BPES type I including Q53X, Q219X, I80T, I84S, and Y274X (Fig. 2B). In contrast, mutations of FOXL2 involved in type II BPES including Y274X, N105S, N109K, and G269R showed intermediate transcriptional activities (Fig. 2B). In addition, *Caspase 8* transcriptional analysis with the artificial FOXL2 mutants showed that both the FH domain and alanine repeats are necessary for complete transcriptional activity of FOXL2 (Fig. 2B). Similarly, compromised transcriptional regulation of *TNF-R1* and *FAS* was observed with mutant FOXL2 proteins (Fig. 2C and D). As a negative control, differential expression of Apolipoprotein A1 (APOA1) by WT and FOXL2 mutants was not observed (Supplementary Fig. 1: on-line only). In addition, Western blot analysis confirmed that the differential mRNA expression of *FAS* resulted in changes in FAS protein expression (Fig. 2D).

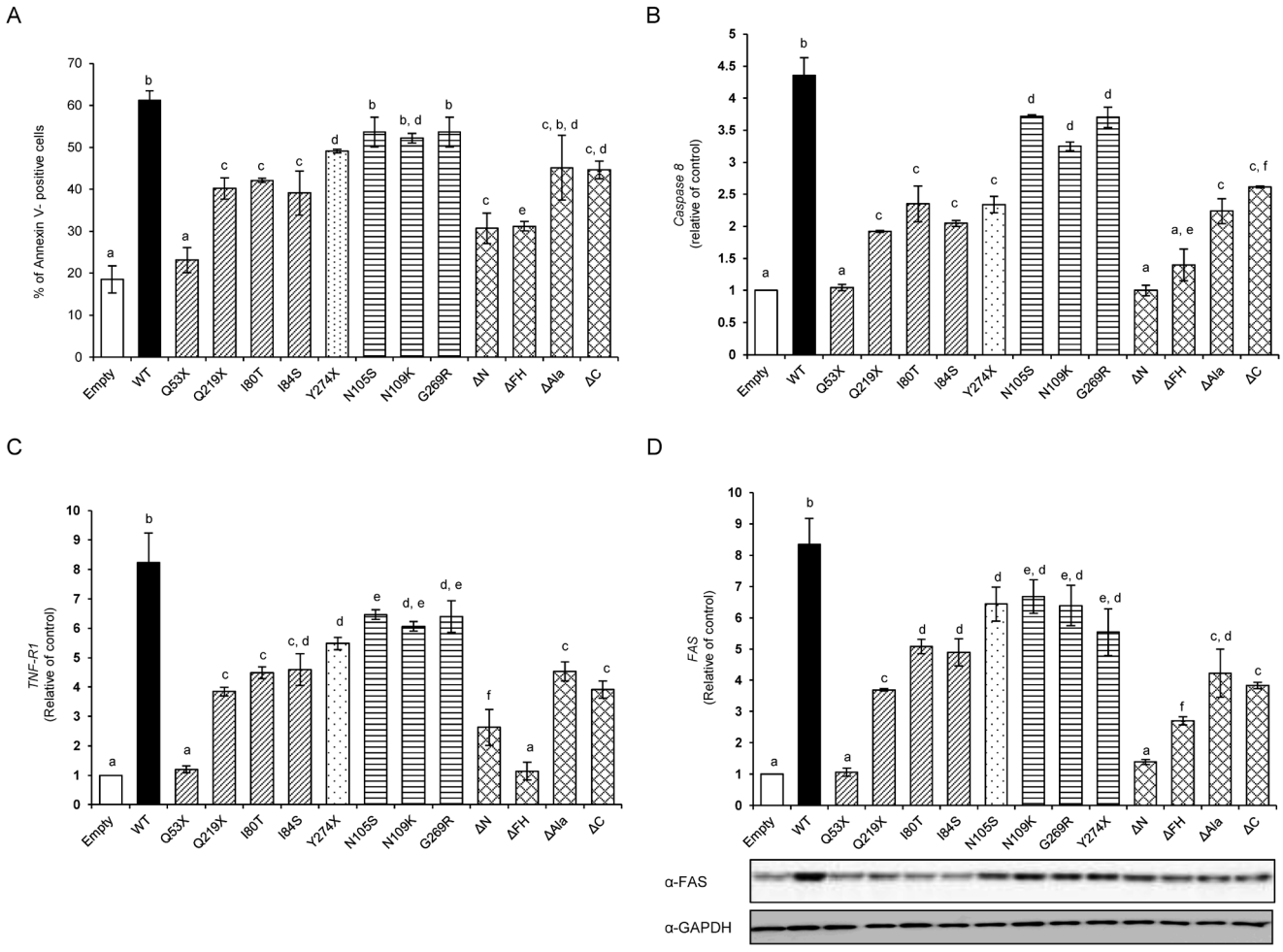

Fig. 2. Defective activation of genes regulating apoptosis by mutant FOXL2 proteins. (A) The results of flow cytometry analysis of Annexin V-positive apoptotic cells are shown. The transcriptional activity of FOXL2 WT and its mutants described in Fig. 1A on *Caspase 8* (B), *TNF-R1* (C), and *FAS* (D), was determined by real-time PCR analysis. Regulation of FAS protein expression by mutant FOXL2 proteins was determined by Western blot analysis using the anti-FAS antibody (D).

Defective cell cycle and differentiation activities of mutant FOXL2 proteins

We overexpressed WT and mutant FOXL2 proteins in KGN cells and analyzed cell populations in different phases of the cell cycle by flow cytometry. As shown in Fig. 3A and B, WT FOXL2 increased cell populations in the G0 and G1 phases, while it decreased cells in the S, M, and G2 phases. In contrast, mutant FOXL2 proteins associated with BPES type I lost this effect on the cell cycle, whereas those involved in BPES type II generally showed an intermediate response (Fig. 3A and B). In addition, we also examined the transcriptional activity of *p21*, a crucial cyclin-dependent kinase (CDK) inhibitor that inhibits cell cycle progression by FOXL2 proteins. WT FOXL2 stimulated transcriptional activation of *p21*; however, a significant defect in transactivation by type I mutants and a moderate defect by type II mutants were observed (Fig. 3C). Western blot analysis confirmed the respective changes in p21 protein level (Fig. 3C). In addition, mRNA expression levels of bone morphogenetic protein 4 (*BMP4*), which controls the initial differentiation of GCs [18], was significantly increased by overexpression of WT FOXL2 (Fig. 3D).

Differential recruitment of target genes by FOXL2 mutants

To understand the molecular mechanism associated with the defective transcriptional activities of FOXL2 mutants, ChIP-qPCR analysis was performed. Overexpression of FOXL2 showed significant enrichment of both *TNF-R1* and *p21* promoter DNAs (Fig. 4A and B). In contrast to WT FOXL2, a BPES type I mutant (Q219X) recruited significantly less DNA of both *TNF-R1* and *p21* promoters, whereas the BPES type II mutant N109K showed intermediate enrichment of the DNAs (Fig. 4A and B).

Discussion

The ovary is a distinctive organ that retains follicles composed of oocytes and surrounding somatic cells including GCs, theca cells,

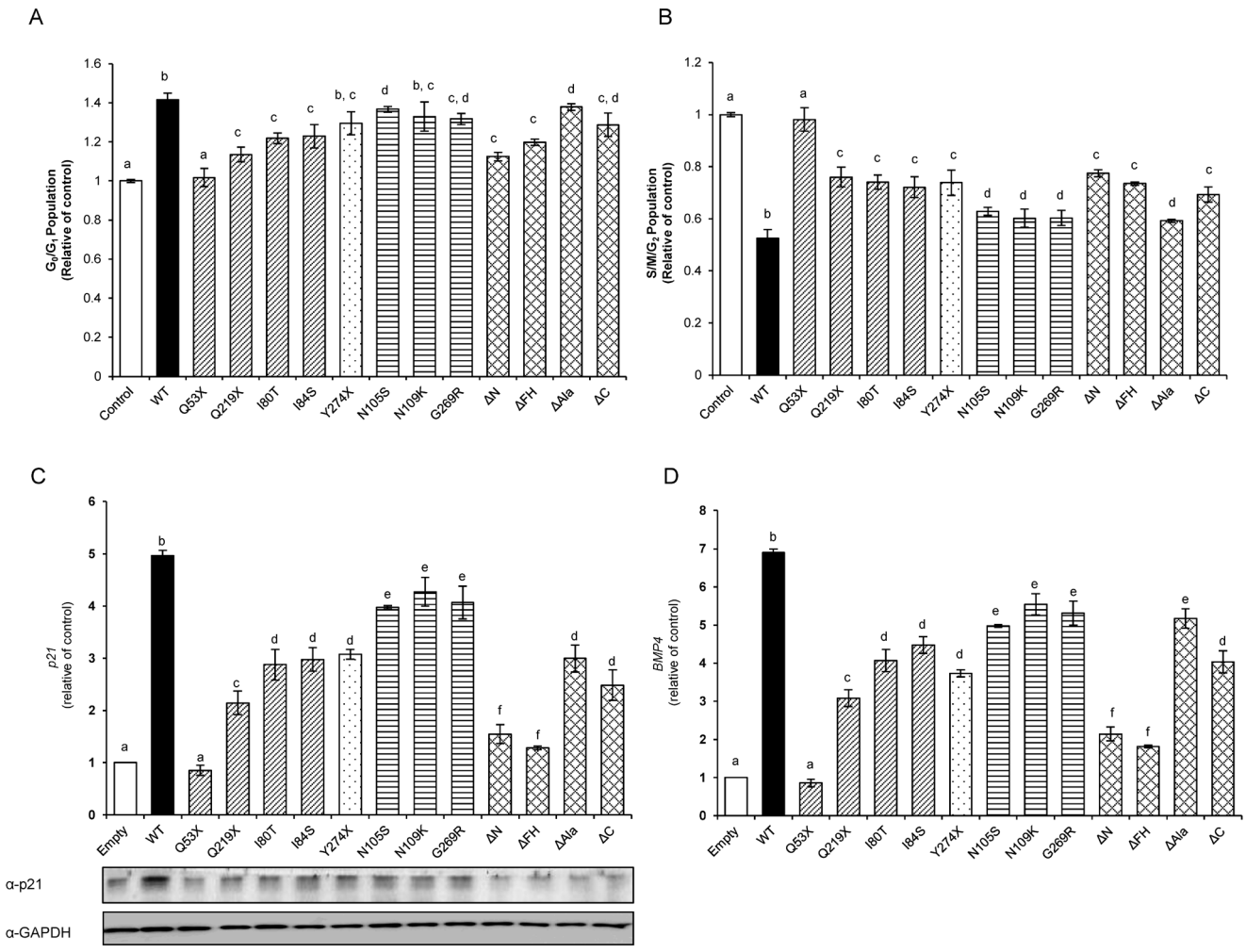

Fig. 3. Disparate effects of WT FOXL2 and its mutants on cell cycle arrest. Twenty-four hours after transfection of KGN cells with the respective plasmids, cell cycle stages G1/G0 (A) and S/M/G2 (B) were analyzed as described in the Materials and Methods. (C) The transcriptional activity of FOXL2 WT and its mutants on the *p21* was determined by real-time PCR analysis. Regulation of p21 protein expression by mutant FOXL2 proteins was determined by Western blot analysis using the anti-p21 antibody. (D) The effect of FOXL2 proteins on *BMP4* mRNA expression was determined by real-time PCR.

and stromal cells. Growth of ovarian follicle from primordial follicles to preovulatory follicles is a complex process involving multiple factors that is not completely understood [19]. Proper proliferation and differentiation of GCs are mandatory for normal folliculogenesis and fertility [20]. FOXL2 is an essential transcriptional factor required for normal development of GCs. Failure of GCs to complete the squamous to cuboidal transition leads to oocyte atresia in *Foxl2* knockout mice [21]. The present study demonstrated that endogenous mutant forms of FOXL2 found in BPES type I patients manifesting POF and infertility were severely defective in terms of the induction of apoptosis and cell cycle arrest in KGN cells. In contrast, mutants found in type II BPES patients manifesting the craniofacial defect without severe ovarian dysfunction showed modest malfunction compared with WT FOXL2. Moreover, similar results were observed from mouse granulosa cells isolated (Supplementary Fig. 2: on-line only). More recently, findings of intra- and interfamilial phenotypic variability associated with the same mutants have been reported [22, 23]. However, conventionally, FOXL2 mutations accompanying POF are predicted to produce truncated FOXL2 proteins that lack the alanine-rich region leading to haploinsufficiency, whereas point mutations, frameshifts, or duplications further downstream represent hypomorphic alleles and result in BPES type II [15, 22, 24]. The differential apoptotic and proliferative activities of FOXL2 and its mutants that we observed are correlated with the degree of ovarian dysfunction induced by FOXL2 mutations. In addition, our ChIP-qPCR analysis of FOXL2 proteins suggests that only the DNA binding domain but also other regions of FOXL2 are likely involved in FOXL2-induced gene regulation.

Ovarian POF, also called primary ovarian insufficiency (POI), affects approximately 1% of women and results in cessation of

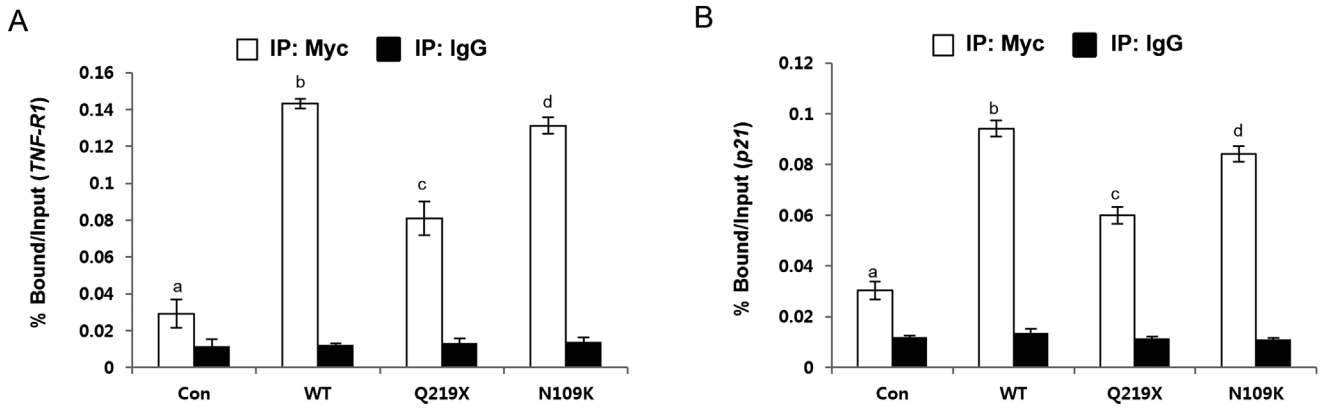

Fig. 4. Differential recruitment of target genes by FOXL2 mutants. ChIP-qPCR analysis was performed to compare enrichment of (A) *TNF-R1* and (B) *p21* by WT, Q219X, and N109K FOXL2 in KGN cells. Using the chromatin fragment precipitated by the anti-Myc antibody, PCR was conducted with a set of *TNF-R1*- and *p21*-specific primers that encompassed the FOXL2-binding elements. As a negative control, normal IgG was used. Data shown are from two independent experiments performed in quadruplet. Different letters denote statistically significant values ($P<0.05$).

ovulation and ovarian endocrine function before the age of 40 [25, 26]. POF patients experience infertility and have an increased risk of developing cardiovascular disease and osteoporosis later in life [27, 28]. Although the pathophysiology of POF is largely unknown for the majority of cases, it is characterized by oligomenorrhea or amenorrhea, elevated serum FSH and LH levels, and decreased estrogen [1, 26]. BPES patients with heterologous FOXL2 mutations were reported to have elevated gonadotropin levels and amenorrhea associated with infertility at a young age [27, 28]. Histological analysis of ovaries from these patients showed accelerated small-follicle recruitment with abnormal small follicles [28]. Specifically, GCs of primordial and transition follicles of patients possessing FOXL2 mutations expressed the proliferation marker Ki67 from late G1 to M, which is uncommon for normal GCs at these follicle stages [28–30]. This finding is in accordance with observations in *Foxl2*$^{-/-}$ mice, in which all Foxl2-expressing cells were shown to be Ki67-negative nondividing cells [5]. These reports suggest that FOXL2 may play a key role in maintaining primordial follicles in a quiescent state by regulating GC proliferation. This view is in accordance with our results showing defective apoptotic and cell cycle arrest activities caused by mutations of FOXL2 found in BPES patients. Thus, although a complete understanding of POF caused by FOXL2 mutations awaits further studies, the present study suggests that the disparate activities of FOXL2 mutants may possibly contribute to the pathophysiology of ovarian dysfunction.

Acknowledgments

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