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ORIGINAL ARTICLE

Differential apoptotic activities of wild-type FOXL2 and the adult-type granulosa cell tumor-associated mutant FOXL2 (C134W)

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Some mutations in *FOXL2* result in premature ovarian failure accompanied by blepharophimosis, ptosis, epicanthus inversus syndrome type I disease, and FOXL2-null mice exhibit developmental defects in granulosa cells. Recently, *FOXL2* c.402C > G, a new somatic mutation that leads to a p.C134W change, was found in the majority of adult-type ovarian granulosa cell tumors (GCTs). In this study, we investigated the possible mechanisms by which the C134W mutation contributes to the development of GCTs. Wild-type (WT) and mutant FOXL2 displayed differential apoptotic activities. Specifically, WT FOXL2 induced significant granulosa cell death, but the mutant exhibited minimal cell death. The FOXL2-induced apoptotic response was greatly dependent on caspase 8, BID and BAK because the depletion of any of these three proteins inhibited FOXL2 from eliciting the full apoptotic response. Activation of caspase 8 and subsequent increased production of truncated BID, and oligomerization of BAK, and release of cytochrome *c* were all associated with the apoptosis induced by WT FOXL2 expression. In contrast, the mutant FOXL2 was unable to elicit the full array of apoptotic signaling responses. In addition, we found differential TNF-R1 (tumor necrosis factor-receptor 1) and Fas (CD95/APO-1) upregulation between the WT and the mutant, and the silencing of TNF-R1 or Fas and the blockage of the death signaling mediated by TNF-R1 or Fas using TNF-Fc or Fas-Fc, respectively, resulted in significant attenuations of FOXL2-induced apoptosis. Moreover, granulosa cells that expressed either WT FOXL2 or mutant exhibited distinct cell death sensitivities on activation of death receptors and deprivation of serum. Thus, the differential activities of FOXL2 and its mutant may partially account for the pathophysiology of GCT development.
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Introduction

FOXL2 is a member of the forkhead (FH) domain transcription factor family originally known to be required for the proper development of the ovary and eyelid. Mutations in FOXL2 cause blepharophimosis, ptosis, epicanthus inversus syndrome (OMIM #110100), and blepharophimosis, ptosis, epicanthus inversus syndrome type I is accompanied by premature ovarian failure of the affected female (Crisponi *et al*., 2001). FOXL2 is mainly expressed in undifferentiated granulosa cells, and blockage of ovarian follicle development because of the failure of granulosa cell differentiation was observed in $FoxL2^{lacZ}$ homozygous mutant mice (Pisarska *et al*., 2004; Uda *et al*., 2004).

Ovarian granulosa cell tumors (GCTs) originate from sex cord-stromal cells, but the pathogenesis and prognostic factors of GCTs are largely unknown. GCTs are rare tumors that affect 1 in 100 000 persons, but a prominent characteristic of these tumors is their propensity to recur (Schumer and Cannistra, 2003). A recent whole-transcriptome paired-end RNA sequencing study identified a recurrent missense point mutation, 402C → G (C134W), in the FH domain of FOXL2 from ovarian GCTs, and 97% of adult-type GCTs tested were found to possess the mutation (Shah *et al*., 2009). This somatic FOXL2 mutation has not been observed in other types of tumors, indicating a specific role for FOXL2 in the tumorigenesis of adult-type GCTs (Schrader *et al*., 2009). However, because it was only recently determined that this mutation is highly prevalent in GTC patients, there is currently no information available regarding how the C134W amino acid substitution in FOXL2 is associated with GTC formation.

We previously found that FOXL2 overexpression induces apoptosis of ovarian cells, including rat granulosa cells (Lee *et al*., 2005). The dysregulation of cellular apoptotic processes is closely associated with tumor development, and the mitochondria are the central organelles that orchestrate apoptosis (Li *et al*., 2004; Hail, 2005). The Bcl-2 family of proteins comprises subfamilies of pro-survival and pro-apoptotic members. These proteins are critical components of diverse cellular apoptotic responses and regulate mitochondrial cell death activities (Dewson *et al*., 2008). BH3 domain-only proteins of the Bcl-2 family, also known as death ligands, include BID, BIM and PUMA, which are required for the activation of the BAK and BAX

proteins through direct interaction (Jabbour *et al*., 2009). BAK and BAX are two death effector molecules that act at the mitochondrial outer membrane and are responsible for dismantling mitochondria through their oligomerization and the consequent release of cytochrome *c* into the cytosol (Chipuk *et al*., 2006).

Caspase enzymes consist of initiator caspases (caspase 1, 2, 4, 5 and 8–12) and effector caspases (caspase 3, 6 and 7). These enzymes are present in pro-forms under non-apoptotic conditions and are activated by cleavage to have protease activities (Li and Yuan, 2008). Both the mitochondrial intrinsic pathway and the death receptor-mediated extrinsic pathway converge to activate caspases. Death receptors are members of the tumor necrosis factor (TNF) superfamily, and their activation recruits adaptor proteins, such as FADD (Fas-associated death domain) and TRADD (TNF-receptor-associated death domain). The activation of death receptors and subsequent FADD and TRADD recruitment result in the autoactivation of caspase 8, which then cleaves BID to truncated BID (tBID; that is, its active form) and in turn activates BAK and/or BAX to oligomerize at the mitochondrial membrane (Muzio *et al*., 1996; Yin *et al*., 1999; Perez and White, 2000; Wei *et al*., 2000; Guicciardi and Gores, 2009). Cytochrome *c* released from the mitochondria aids in forming multi-complexed apoptosomes and leads to caspase 9 activation and cleavage of the caspase 3 zymogen, which is responsible for the proteolysis of its many substrates (Li *et al*., 1997; Zou *et al*., 1999).

In this study, we hypothesized that the mutant FOXL2 (C134W) may be defective in its ability to induce granulosa cell death, leading to GCTs. We investigated the differential apoptotic activities between wild-type (WT) and mutant FOXL2 proteins, and we also examined the molecular and cellular mechanisms by which FOXL2 induces granulosa cell death.

Results

Differential apoptotic activities of WT FOXL2 and mutant FOXL2

To assess the effects of WT and mutant FOXL2 on cell survival and death, we measured cell viability using flow cytometry after the overexpression of the two FOXL2 proteins in KGN cells (Nishi *et al*., 2001) derived from human GCTs. Overexpression of WT FOXL2 significantly increased the number of Annexin V-positive apoptotic cells, and overexpression of mutant FOXL2 clearly induced lower levels of cell death compared with the WT protein (Figure 1a). Similarly, overexpression of WT and mutant FOXL2 induced differential responses in mouse Sertoli cells (TM4) that originated from the same developmental lineage and that were a counterpart to ovarian granulosa cells present in male testis (Figure 1a). In the presence of the pan-caspase inhibitor z-VAD-fmk, FOXL2 failed to induce cell death, suggesting a caspase(s)-dependent death model (Figure 1a).

Differential caspase activation and cytochrome c *release induced by WT and mutant FOXL2*

As WT and mutant FOXL2 displayed disparate apoptotic activities and the caspase inhibitor blocked the apoptotic response, we assessed the activation of caspase proteases. Western blot analyses confirmed that cells expressing WT FOXL2 had significant cleavage of caspases 8, 9 and 3, and cells expressing mutant FOXL2 displayed minimal caspase cleavage (Figure 1b). To determine whether the differential effects of the two FOXL2 proteins were related to their ability to modulate the mitochondrial cell death pathway, we analyzed cytochrome *c* release. Both WT and mutant FOXL2 were primarily present in the heavy membrane fraction, and efficient separation of the cytosolic and heavy membrane fractions was determined by immunoblotting with anti-β actin and anti-Cox IV antibodies, respectively. Cells overexpressing FOXL2 had significantly increased release of cytochrome *c* into the cytosolic fraction relative to cells transfected to the empty vector, whereas cells overexpressing the mutant FOXL2 released less cytochrome *c* (Figure 1c).

Enhanced tBID production as a mediator of FOXL2-induced apoptosis

On death receptor activation, pro-caspase 8 is processed to active caspase 8, which then cleaves BID to tBID and further activates BAK and/or BAX at the outer mitochondrial membrane (Wei *et al*., 2000). As we found that the expression of FOXL2 promotes caspase 8 cleavage (Figure 1b), we determined whether the C-terminal fragment of BID could be produced following FOXL2 expression. As shown in Figure 2a, the overexpression of FOXL2 increased BID cleavage in a concentration-dependent manner, and the control cells had no apparent tBID. In contrast, overexpression of mutant FOXL2 resulted in much weaker formation of tBID compared with that caused by WT FOXL2 (Figure 2a). As FOXL2 stimulated BID cleavage, we generated BID-knockdown cells (Figure 2b) and determined the role of BID in FOXL2-induced apoptosis. In BID-silenced cells, the apoptotic activity of WT FOXL2 was significantly reduced, and mutant FOXL2-mediated cell death activity was also attenuated (Figure 2b). In contrast, the silencing of BIM, another BH3-only death ligand, did not affect the apoptosis-inducing capability of either FOXL2 isoform (Figure 2b), suggesting a specific action of BID in the mechanism of FOXL2-mediated cell death.

Critical role of BAK in FOXL2-induced apoptosis

tBID (p15) translocates into the mitochondria, in which it induces oligomerization of BAK and/or BAX (Wei *et al*., 2000). Thus, we investigated whether the overexpression of WT and mutant FOXL2 induces differential formation of BAK and/or BAX oligomers. The formation of BAK dimers was readily detectable in cells overexpressing WT FOXL2, but the formation of these dimers was much weaker in cells overexpressing the mutant (Figure 2c). In contrast, we detected no

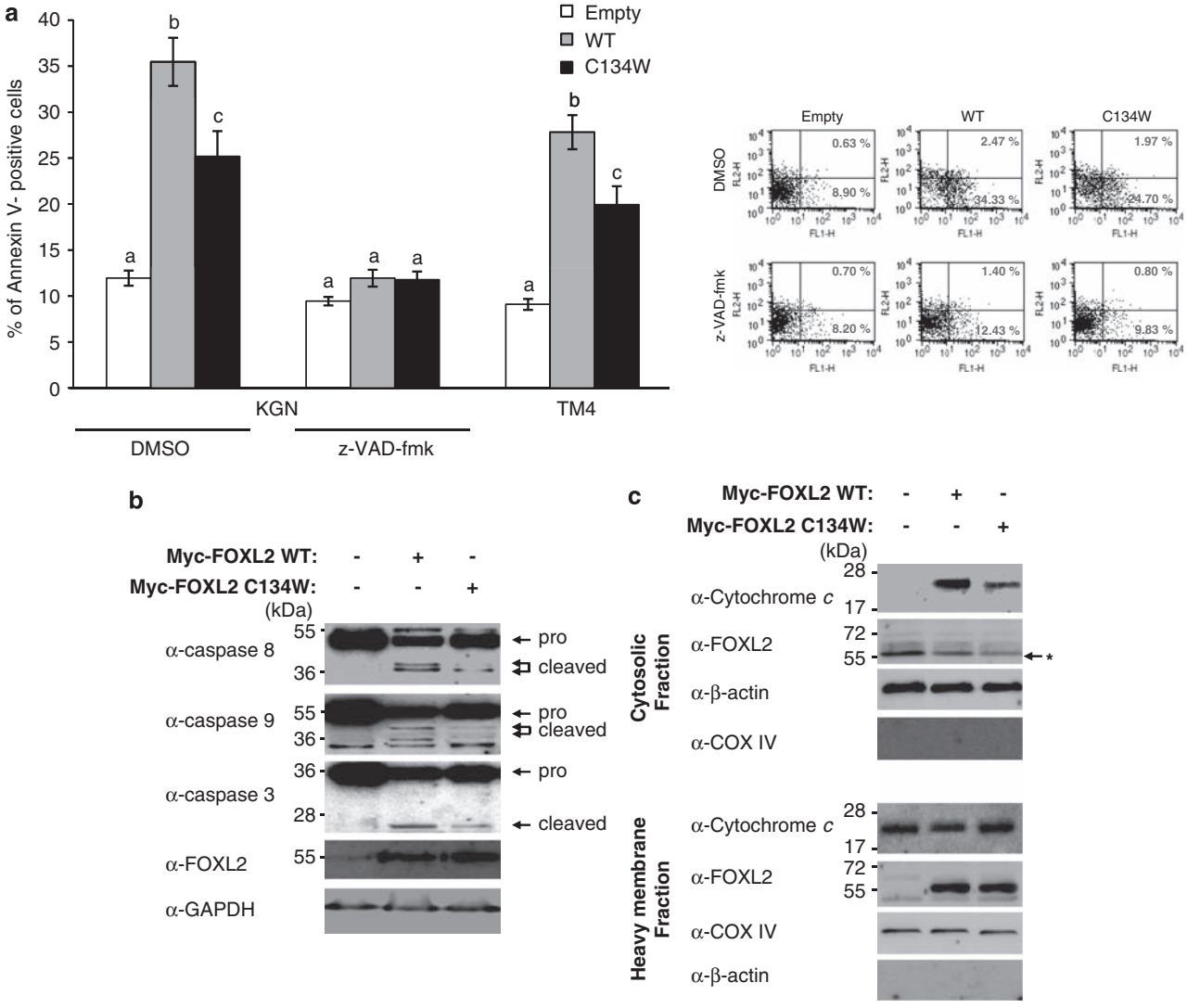

Figure 1 Distinct apoptotic activities between WT and mutant (C134W) FOXL2. (**a**) For quantitative analysis of granulosa cell apoptosis, KGN cells (1×10^6) were transfected with 3 μg of pCMV Myc-empty vector, FOXL2 or FOXL2 C134W constructs (either with or without 10 μM of z-VAD-fmk), and the cells were analyzed after 24 h. TM4 cells were transfected with 4 μg of DNA constructs followed by apoptosis analysis at 24 h post-transfection. Annexin V-positive apoptotic cells were detected by fluorescence-activated cell sorting (FACS) analysis, and data (mean ± s.e.m.) are presented as absolute percents of Annexin V-positive cells (left panel). Three independent experiments were performed in triplicate, and statistically significant differences between groups are denoted by **a**–**c** ($P < 0.05$). The representative dot plots of KGN cells overexpressing the WT or mutant FOXL2 analyzed for apoptotic cells are shown in the right panel. (**b**) KGN cells (2×10^6) were transfected with DNA-encoding Myc-FOXL2 or FOXL2 C134W (6 μg), and the cell lysates were prepared after 24 h of transfection. Activation of the caspases was assessed by immunoblot analyses with anti-caspase 8, caspase 9 and caspase 3 antibodies. The anti-FOXL2 antibody was used to detect both types of FOXL2. The same membrane was stripped and analyzed for the detection of different caspases and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Equal loadings of the lysates were confirmed by western blot analysis using an anti-GAPDH antibody. (**c**) Release of mitochondrial cytochrome *c* into the cytosol was assessed in KGN cells transfected with each of the three plasmids. The cytosolic and heavy membrane fractions were separated and analyzed by western blotting with appropriate antibodies. *A nonspecific band detected by the anti-FOXL2 antibody.

oligomerization of BAX in cells overexpressing either of the FOXL2 proteins (Figure 2c), indicating that FOXL2 discriminates between the BAK and BAX proteins. As positive controls, KGN cells were overexpressed with either BAK or BAX, and we observed oligomer formation of BAK or BAX (Figure 2c). Next, we assessed the death response of *bax*$^{-/-}$, *bak*$^{-/-}$ and *bax*$^{-/-}$ *bak*$^{-/-}$ mouse embryo fibroblast cells. Overexpression of WT FOXL2 induced significant cell death in the WT mouse embryo fibroblast cells, but overexpression of mutant FOXL2 induced a lower level of cell death (Figure 2d), similar to what we observed in human granulosa cells. These effects on cell death were not altered in *bax*-deficient cells (Figure 2d). However, in the *bak*$^{-/-}$ knockout and *bax*$^{-/-}$*bak*$^{-/-}$ double-knockout cells, overexpression of FOXL2 failed to induce any significant cell death (Figure 2d), indicating that FOXL2-mediated cell death requires the presence of Bak.

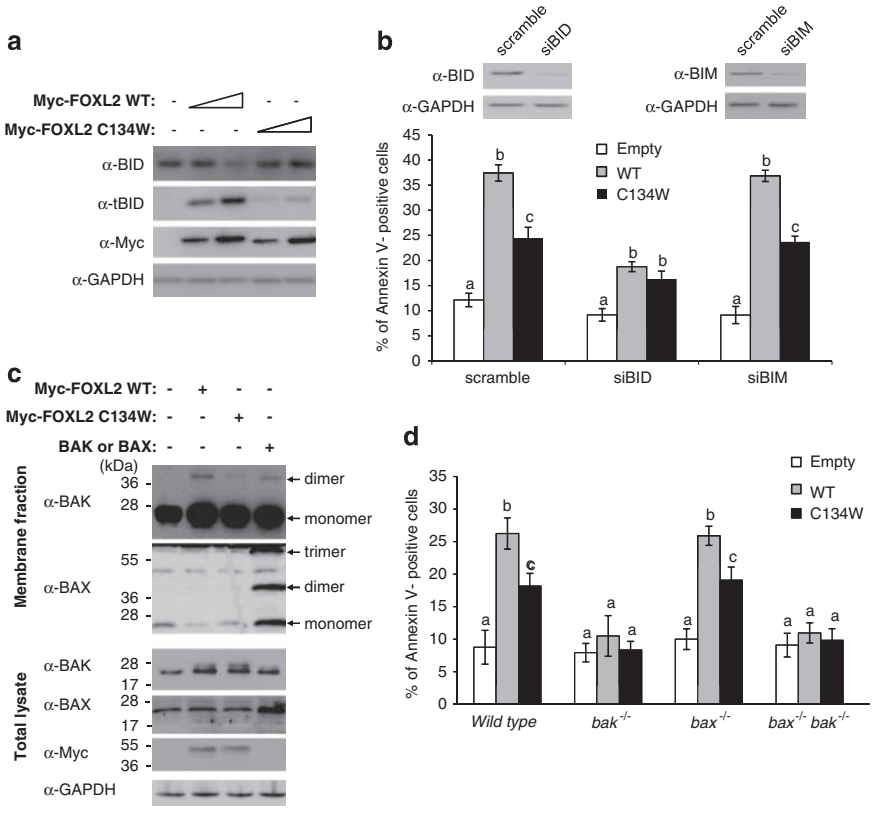

Figure 2 BAK dependence and BID involvement in FOXL2-induced cell death. (**a**) Cell lysates were prepared 24 h after transfection of KGN cells (2×10^6) with an empty vector (6 μg), Myc-FOXL2 (3 or 6 μg) or Myc-FOXL2 C134W (3 or 6 μg) plasmids. Expression of the parent BID and production of its cleaved form were assessed by immunoblot analyses with respective BID antibodies. (**b**) KGN-knockdown cells were prepared by transfection with scrambled sequences or siRNAs for BID or BIM, and reduced BID and BIM expression was demonstrated by western blotting. Subsequently, the WT or mutant FOXL2 was overexpressed in the knockdown cells for 24 h, and fluorescence-activated cell sorting (FACS) analysis was performed. Three independent experiments were performed in triplicate, and data are expressed as the percentage (mean ± s.e.m.) of Annexin V-positive cells. Statistically significant differences between groups are denoted by different letters ($P < 0.05$). (**c**) After the overexpression of WT or mutant FOXL2 proteins in KGN cells (as described above), the heavy membrane fraction was obtained, and glutaraldehyde was used as a crosslinker for BAK or BAX. As a positive control, cells were transfected with one-tenth the amount of the BAK- or BAX-coding plasmids. The formation of BAK or BAX oligomers was determined by western blot analysis using the appropriate antibodies. (**d**) WT, *bax*$^{-/-}$, *bak*$^{-/-}$ and *bax*$^{-/-}$*bak*$^{-/-}$ mouse embryo fibroblast (MEF) cells (1×10^6) were transfected with 1 μg of empty vector, WT FOXL2 or mutant FOXL2, and cellular FACS analysis was employed as described in **a**. Three independent experiments were performed in duplicate, and data are expressed as the percentage (mean ± s.e.m.) of Annexin V-positive cells.

Involvement of caspase 8 in FOXL2-induced apoptosis

As we found that FOXL2 promotes the cleavage of caspase 8 (Figure 1b) and BID (Figure 2a), we next investigated whether FOXL2-induced apoptosis is dependent on caspase 8-mediated BID activation. In the presence of the caspase 8 inhibitor z-IETD-fmk (10 μM), FOXL2-mediated tBID production was greatly diminished (Figure 3a). In addition, FOXL2-induced apoptosis was significantly attenuated in cells incubated with z-IETD-fmk (Figure 3b). To further confirm the critical role of caspase 8 in FOXL2-induced apoptotic events, KGN cells were transfected with small interfering RNA (siRNA) specific for caspase 8, and efficient knockdown of caspase 8 was demonstrated by immunoblot analysis (Figure 3c). In caspase 8-depleted cells, FOXL2-induced tBID production was prominently hindered (Figure 3c). Moreover, FOXL2-induced apoptosis was significantly blocked in caspase 8-knockdown cells (Figure 3d).

Differential upregulation of death receptors by WT and mutant FOXL2

As FOXL2-mediated cell death was found to be dependent on caspase 8 and the mutant was found to be a considerably weaker inducer of caspase 8 cleavage than WT, we tested the possibility that overexpression of WT and mutant FOXL2 leads to differential transcriptional activities of *caspase 8*. A luciferase reporter assay demonstrated that both types of FOXL2 proteins greatly stimulated *caspase 8* promoter activation, and no significant difference in transcriptional regulation was observed between the two FOXL2 proteins (Figure 4a). Thus, these results suggest that the differential apoptotic activity between the two FOXL2 proteins is not likely the result of distinct transcriptional regulation of *caspase 8*. Therefore, we next investigated whether WT and mutant FOXL2 differentially regulate the expression of death receptors,

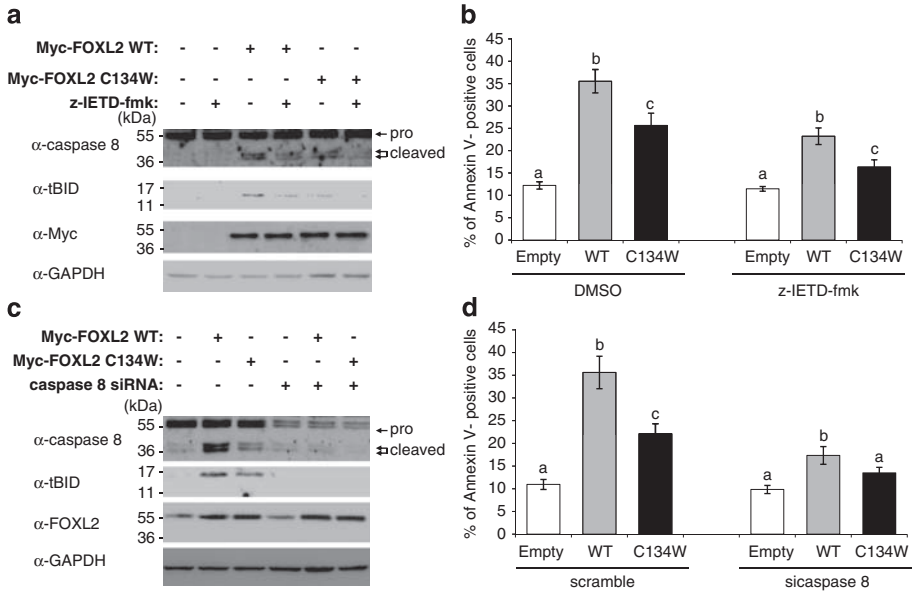

Figure 3 Critical roles of caspase 8 in FOXL2-mediated apoptotic responses. (**a**) After transfection of KGN cells (2×10^6) with 6 μg of an empty vector, Myc-FOXL2 WT or Myc-FOXL2 C134W, the cells were incubated with z-IETD-fmk (10 μM) or 0.1% dimethylsulphoxide (DMSO) (solvent control) for 24 h. Cell lysates were prepared, subjected to electrophoresis, and immunoblotted with the appropriate antibodies. (**b**) The transfected cells described in **a** were analyzed for Annexin V-positive populations by flow cytometry. The cells were treated with 0.1% DMSO and 10 μM z-IETD-fmk for 24 h. Data (mean ± s.e.m.) were from three independent experiments conducted in triplicate, and statistically significant values between groups are indicated by different letters ($P<0.05$). (**c**) Caspase 8 expression was reduced in KGN cells (2×10^6) using caspase 8-specific siRNA, and western blot analysis of caspase 8 knockdown was confirmed at 24 h post-transfection. The silenced cells were further transfected with 6 μg of an empty vector, WT FOXL2 or mutant FOXL2 (C134W). (**d**) The KGN cells described in **c** were used to determine proportions of Annexin V-positive cells by fluorescence-activated cell sorting (FACS) analysis. Data analysis was conducted as described for **b**.

which may lead to greater activation of the caspase 8 that we observed (Figure 1b). Real-time PCR was conducted using KGN cells transfected with either WT or the mutant FOXL2. As shown in Figure 4b, cells transfected with WT FOXL2 displayed a significant increase in the transcription of TNF-R1 (TNF-receptor 1) and Fas (CD95/APO-1), while cells transfected with the mutant FOXL2 showed no statistically significant increase in death receptors. To further determine the differential upregulation of death receptor transcription induced by overexpression of WT and mutant FOXL2, we cloned the *TNF-R1*, *Fas* and *TRAIL-R1* (TNF-related apoptosis-inducing ligand receptor 1/death receptor 4) promoters and performed reporter assays. WT FOXL2-transfected KGN cells showed a significant transactivation of death receptors including *TNF-R1*, *Fas* and *TRAIL-R1* compared with control cells transfected with an empty vector (Figure 4c). In contrast, mutant FOXL2 transactivated the promoters of *TNF-R1* and *Fas* to a lesser degree (Figure 4c). To verify the WT FOXL2-mediated upregulation of *TNF-R1* and *Fas*, we performed western blot analyses and confirmed the increased expression of both TNF-R1 and Fas at the protein level (Figure 4d). Mutant FOXL2 showed compromised upregulation compared with WT FOXL2 (Figure 4d). However, the levels of TRAIL mRNA and protein were not significantly altered by FOXL2 (Figures 4b and d), suggesting the selective actions of TNF-R1 and Fas. The FH domain-deleted (ΔFH) FOXL2 protein was unable to affect the expression of the death receptors (Figure 4d), indicating a critical role for the FH region in FOXL2-induced apoptosis.

Implication of TNF-R1 and Fas signaling in FOXL2-mediated apoptosis

As TNF-R1 and Fas were preferentially induced by WT FOXL2 rather than the mutant, we assessed changes in the apoptotic responses of the WT and mutant FOXL2 in KGN cells in which each of the death receptors was knocked down. As shown in Figure 5a, silencing either TNF-R1 or Fas resulted in a significant attenuation of the apoptosis induced by WT FOXL2 compared with mutated FOXL2. In contrast, the depletion of TRAIL-R1 did not significantly affect the apoptotic activities of either WT or mutant FOXL2 (Figure 5a). To further confirm the observation in Figure 5a, we employed death receptor blockers of TNF or Fas in a subsequent experiment. Treatment of KGN cells transfected with WT FOXL2 with TNF-Fc (Enbrel; etanercept), a competitive inhibitor of TNFα that prevents its association with the TNF receptor, greatly prevented WT FOXL2-induced death, although treatment of mutant-transfected KGN cells resulted in less apoptosis blockage (Figure 5b). In accordance, a similar response was observed in cells incubated with Fas-Fc, a neutralizer of FasL (Figure 5b). Thus, these results together suggest that the death signaling of TNF-R1 and Fas are crucial components in the mechanism of FOXL2-induced cell death.

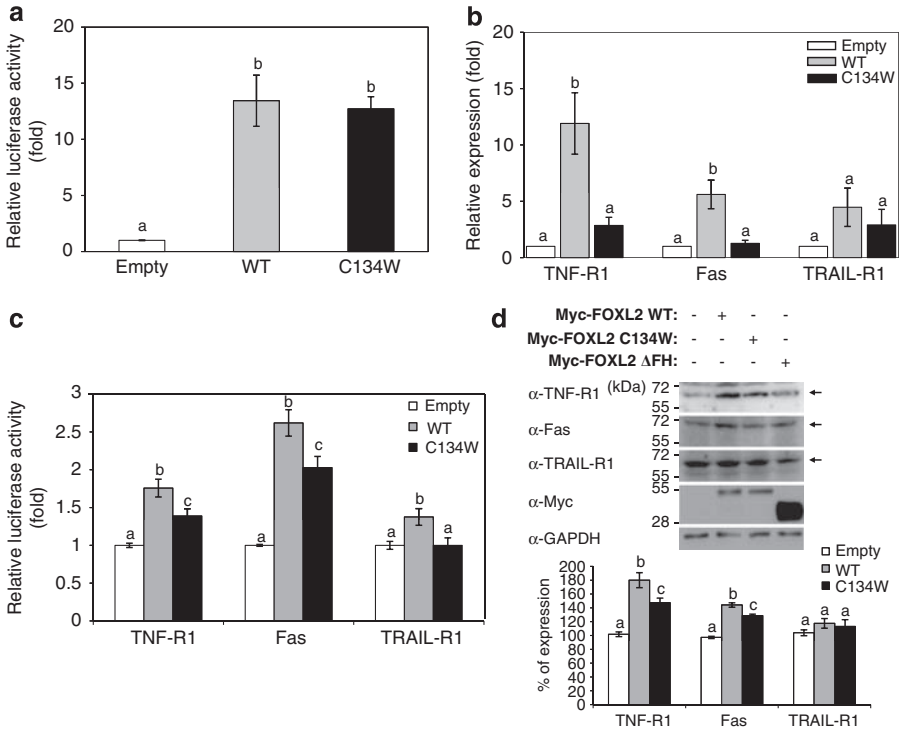

Figure 4 Differential upregulation of death receptors in response to WT and mutant FOXL2. (**a**) Promoter activation of caspase 8 by overexpression of either WT or mutant FOXL2 was determined by luciferase assays. Data are from three independent experiments conducted in triplicate and presented as a relative luciferase activity in fold. Statistically significant values between groups are denoted by **a**–**c** ($P<0.05$). (**b**) Real-time PCR was performed using a complementary DNA (cDNA) library synthesized from RNA isolated from KGN cells (1×10^6) transfected with 3 μg of an empty vector, WT or mutant FOXL2 for 24 h, and results are shown as relative expression in fold after normalization by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Data analysis was performed as described for **a**. (**c**) Transcriptional activities of FOXL2 WT and FOXL2 C134W on death receptors promoters were investigated using a luciferase reporter system containing *TNF-R1, Fas* or *TRAIL-R1* promoters in KGN cells. As a control, cells were transfected with empty vector together with pGL3 *TNF-R1, Fas* or *TRAIL-R1* promoters. Cells were collected 12 h after transfection. Five independent experiments conducted in triplicate and presented as a relative luciferase activity in fold. (**d**) KGN cells were transfected with an empty vector, WT FOXL2, C134W FOXL2 or ΔFH FOXL2, and lysates were prepared 24 h post-transfection for western blot analysis using antibodies against the death receptors. Three independent western blotting were performed, and the expression of the receptors were quantified, normalized to the level of GAPGH, and presented as % of expression.

The FOXL2 mutant is defective in eliciting the cell death response induced by some apoptotic stimulants

On the basis of the fact that the majority of FOXL2 mutations found in GCTs were heterozygotes of the 402C *FOXL2* and 402G *FOXL2* alleles (Shah *et al*., 2009), we attempted to test the possibility that the mutant may behave as a dominant-negative mutation. As KGN cells are also heterozygous for *FOXL2* (Benayoun *et al*., 2010), FOXL2 was first knocked down using siRNA targeting of the 3′ untranslated region to avoid any compounding effects of being heterozygotic, and its efficient depletion was confirmed by immunoblot analysis using an anti-FOXL2 antibody (Figure 5c). Subsequently, the knocked-down cells were transfected with increasing amounts (1, 2 and 3 μg) of either WT- or mutated-FOXL2-coding plasmids, and changes in cellular apoptosis were assessed. As shown in Figure 5c, WT FOXL2 transfection increased apoptosis of FOXL2-silenced cells significantly compared with the scrambled control cells transfected with WT. In contrast, transfection of the mutant FOXL2 led to decreased apoptosis of FOXL2-silenced cells relative to the scrambled control overexpressed with the mutant (Figure 5c). Moreover, co-expression of WT and mutant FOXL2 resulted in a reduction of the apoptotic activity elicited by WT FOXL2 (Figure 5c). Thus, these results imply that mutant FOXL2 may act to inhibit the cell death activity of WT, but more information is required to conclude. In addition, the overexpression of ΔFH did not induce any cell death (Figure 5c), suggesting a critical role for the FH domain in the apoptotic activity of FOXL2.

To further investigate the possible role of mutant FOXL2 in mediating apoptotic signals, we overexpressed either WT or mutant FOXL2 in FOXL2-knocked-down KGN cells and then incubated these cells with increasing concentrations of death receptors ligands. We chose concentrations of the ligands at which no obvious cell death was detected after exposure to any individual ligand. Treatment of WT FOXL2-transfected KGN cells with increasing amounts of TNFα (15 and 30 ng/ml) further augmented apoptosis in a concentration-dependent manner, whereas mutant FOXL2-transfected cells failed to potentiate the apoptotic response elicited by TNFα (Figure 5d). We observed a similar trend in cells overexpressing either WT or mutant

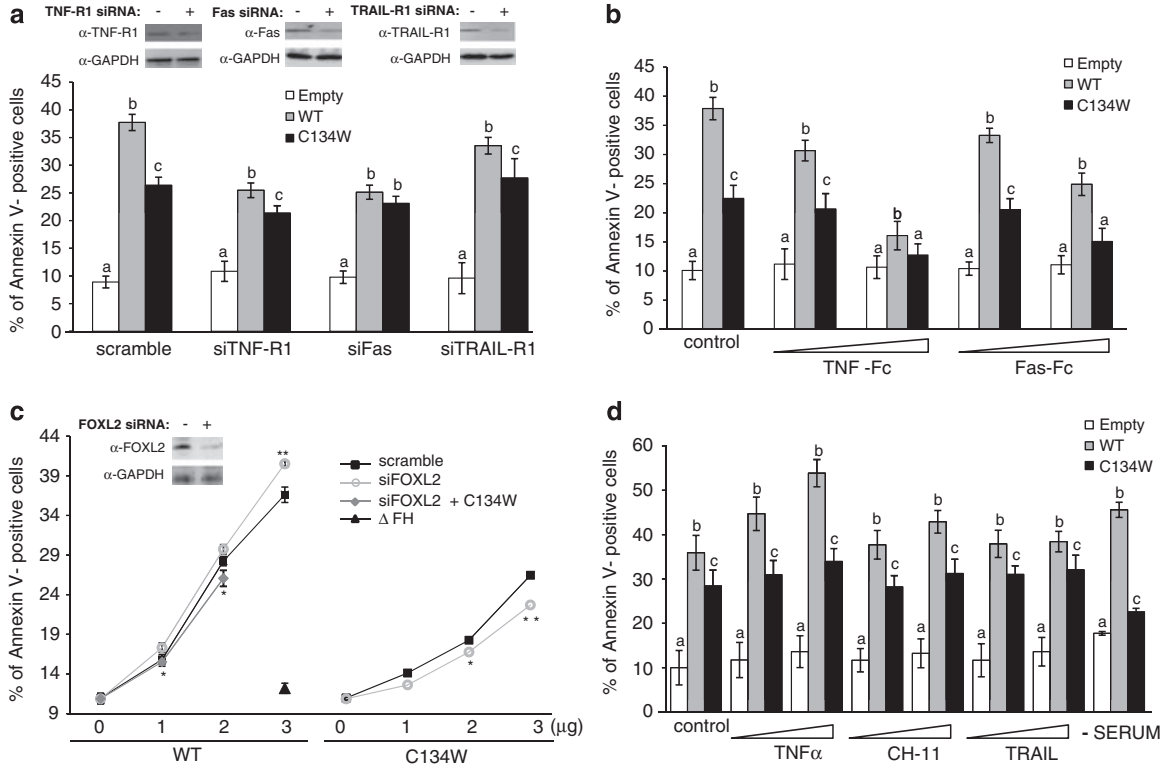

Figure 5 Involvement of TNF-R1 and Fas in FOXL2-mediated apoptosis and the defective role of C134W FOXL2 in mediating the death signals of these death receptors. (**a**) Reduced expression of death receptors in KGN cells after transfection of specific siRNA was shown by western blot analysis using appropriate antibodies (upper panel). At 6 h after transfection with siRNA, the cells were further transfected with 3 μg of either WT FOXL2- or C134W FOXL2-encoding plasmids and incubated for an additional 18 h. Annexin V-positive apoptotic cells were detected by fluorescence-activated cell sorting (FACS) analysis, and the data (mean ± s.e.m.) are from three independent experiments conducted in triplicate. (**b**) KGN cells were transfected with 3 μg of either plasmids encoding WT FOXL2 or C134W FOXL2, and the cells were incubated with 0.4 and 4 μg/ml of TNF-R1 or Fas blocking agents, TNF-Fc or Fas-Fc, respectively, for 24 h. Data analysis was identical to that described for **a**. (**c**) Endogenous expression of both WT FOXL2 and C134W FOXL2 was reduced by transfection with siRNA oligonucleotides specific for FOXL2, and the reduction in expression was confirmed by immunoblot analysis. At 6 h after transfection with siRNA, the FOXL2-depleted KGN cells were additionally transfected with plasmids encoding WT FOXL2 or mutant (0, 1, 2 or 3 μg) in which 3 μg of empty vector was used for 0 μg. For co-transfection of WT and mutant FOXL2, 1 μg of the mutant DNA was used. The KGN cells were also transfected with 3 μg of ΔFH FOXL2. The cells were analyzed at 18 h post-transfection of DNAs. Annexin V-positive apoptotic cells were detected by FACS analysis, and data (mean ± s.e.m.) are from three independent experiments conducted in duplicate. *Significant values compared with the scrambled control KGN cells overexpressed with respective amounts of either WT or mutant (*$P<0.05$; **$P<0.005$). (**d**) The FOXL2-depleted KGN cells were transfected with either WT FOXL2- or C134W FOXL2-encoding plasmids, and the cells were further incubated with TNFα (15 and 30 ng/ml), CH-11 (30 and 60 ng/ml) or TRAIL (50 and 100 ng/ml) for 18 h. For the serum deprivation experiment, media was displaced with serum-free media 6 h after transfection, and the cells were cultured for an additional 18 h before harvesting. Data analysis was identical to that described for **a**.

FOXL2 that were incubated with CH-11, a Fas-activating antibody (30 and 60 ng/ml) (Figure 5d). In contrast, WT FOXL2 failed to potentiate the cell death response induced by TRAIL (Figure 5d). Furthermore, WT FOXL2 significantly augmented the cell death effect by serum deprivation, while mutant FOXL2 was unable to induce the same effect and even showed attenuated apoptosis compared with the FOXL2 mutant-transfected control without any apoptotic stimulation (Figure 5d).

Discussion

The recently identified recurrent 402C→G point mutation in *FOXL2* has emerged as a plausible pathogenic factor for GCTs (Shah *et al*., 2009). On the basis of our previous study demonstrating the induction of death in rat granulosa cells after FOXL2 expression (Lee *et al*., 2005), we determined the cell death activity of mutant FOXL2 in human GCT-derived cells relative to that of WT FOXL2, and we found significant differences between the WT and the mutant proteins in their abilities to induced granulosa cell death. The ectopic expression of FOXL2 stimulated the cleavage of BID (Figure 2a), oligomerization of BAK (Figure 2c) and release of cytochrome *c* into the cytosol (Figure 1c), indicating that FOXL2 initiates a mitochondria-mediated apoptotic cell death mechanism involving the Bcl-2 family. In contrast, mutant FOXL2 exhibited minimal effects according to a battery of cell death assays, revealing that the mutant is a much weaker inducer of cell death than WT FOXL2.

To address how FOXL2 stimulates granulosa cell apoptosis and how the WT and mutant FOXL2 proteins exhibit differential apoptosis-related activities, we investigated the underlying molecular and cellular mechanisms of the FOXL2 signaling network. On the basis of our current data, caspase 8 is likely to have an important role in FOXL2-dependent apoptotic events because FOXL2 stimulated the activation of caspase 8. Further, FOXL-2-induced apoptosis was significantly compromised when caspase 8 was either inhibited or depleted from the granulosa cells (Figure 3). In sharp contrast, a minimal amount of activated caspase 8 was produced by the mutant FOXL2 compared with WT (Figures 1b, 3a and c). This differential caspase 8 cleavage by WT and variant FOXL2 likely did not result from disparate transcriptional regulation of the *caspase 8* gene because both proteins transactivated *caspase 8* to comparable levels (Figure 4a). Thus, the distinctive abilities of WT and mutant FOXL2 to cleave pro-caspase 8 *per se* to its active form seem to be more accounted for in its distinctive apoptotic activities. It is not clear why the upregulation of caspase 8 protein was not readily observable despite increases in FOXL2 transcript levels. As FOXL2 stimulated cleavage of caspase 8, it may possible that the upregulated caspase 8 protein level is not readily detectable. At the same time, we cannot exclude a possibility that the transcriptional activation of *caspase 8* induced by FOXL2 does not lead to its stimulated translation.

In the absence of an activation signal, caspase 8 exists as the inactive pro-caspase 8. Following the activation of death receptors, such as TNF-R1, Fas and TRAIL-R1, pro-caspase 8 is then autoactivated on recruitment to form death-inducing signaling complexes (Muzio, 1998; Chang *et al*., 2002). Both real-time PCR and western blot analyses demonstrated that WT FOXL2 significantly upregulated death receptors, especially TNF-R1 and Fas, whereas mutant FOXL2 showed marginal effects (Figures 4b and d). Thus, the upregulation of death receptors by WT FOXL2 would be expected to augment death-inducing signaling complex recruitment and subsequent cleavage of caspases. However, because it is a transcription factor, FOXL2 may also have other unidentified target genes that have a role in the apoptotic signaling of FOXL2, and additional studies are needed to address this possibility. The cysteine-134 residue is located on the surface of the FH domain involved in DNA binding, and thus, it is reasonable to assume that WT and mutant FOXL2 may exhibit disparate transcriptional activities toward their targets. Such an effect may contribute to the differential apoptotic responses and tumorigenesis observed for the two FOXL2 isoforms.

Dysregulation of apoptosis contributes to cancer development and chemo-resistance. As a major component of the extrinsic apoptosis signaling machinery, the death receptor family has an important role in maintaining homeostasis, and death receptor dysfunctions have been observed in various cancer types (Kang *et al*., 2003; Guicciardi and Gores, 2009; Mahmood and Shukla, 2010). The activation of death receptors on binding their cognate ligands initiates cytotoxic and non-cytotoxic signaling cascades (Guicciardi and Gores, 2009). The death receptors and their ligands are also targets for the development of various cancer therapeutics (Green and Kroemer, 2005; Mahmood and Shukla, 2010). In this report, we found that granulosa cells expressing WT or variant FOXL2 displayed distinct responses to the activation of death receptors, including TNF-R1 and Fas. Cells expressing WT FOXL2 showed

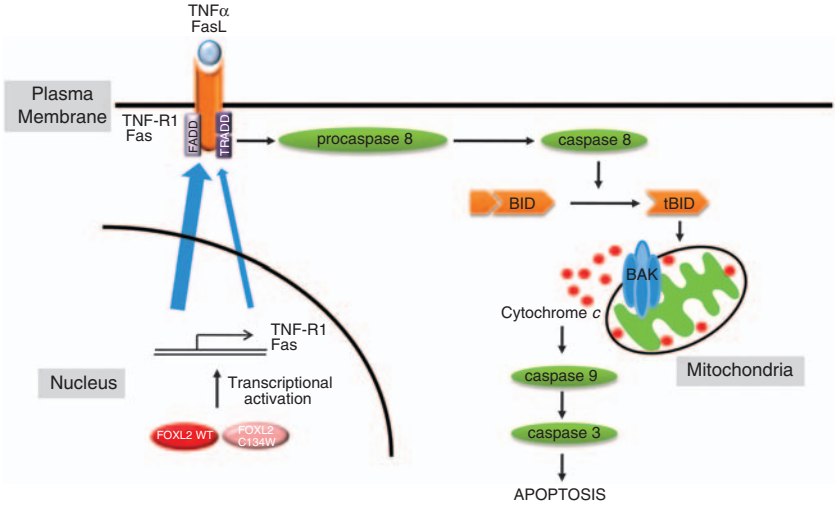

Figure 6 A proposed model of FOXL2-induced apoptosis in ovarian granulosa cells. The expression of death receptors (TNF-R1 and Fas) is upregulated by FOXL2, which may subsequently stimulate the activation of caspase 8 to its cleaved forms. The activated caspase 8 protease further cleaves the BH3-only death ligand BID to its active form tBID, which subsequently activates BAK to oligomerize. Cytochrome *c* is then released from the mitochondria, which leads to the activation of caspase 9. Caspase 8 and caspase 9 may be the initiator proteases that lead to the cleavage of the effector caspase 3 in cells. The mutant FOXL2 C134W exhibits marginal effects on the series of apoptotic events compared with the WT protein, which may partly account for the development of GCTs by the mutant FOXL2.

significantly enhanced susceptibility to death receptor signaling and serum deprivation, whereas cells expressing the mutant FOXL2 were resistant to death ligand-induced apoptosis (Figure 4d). Moreover, mutant FOXL2 seemed to behave as an inhibitory mutant because co-transfection with both the WT and mutant resulted in hindered apoptotic activity of the WT protein (Figure 5c). Together, these differential sensitivities may help to explain why the mutation of FOXL2 (C134W) in granulosa cells is associated with the development of granulosa tumors.

FOXO3a (also known as FKHRL1), another member of the FH family, induces the apoptosis of various cells. Activation of FOXO3a promotes cytochrome *c* release and caspase-mediated cell death by induction of TRAIL, BIM and NOXA in neuroblastoma cells (Obexer *et al*., 2007). FOXO family members, including FOXO3a, are known tumor suppressors implicated in various cancers (Paik *et al*., 2007; Kornblau *et al*., 2010; Naka *et al*., 2010). Therefore, FOXL2 and FOXO3a seem to utilize common and distinctive signaling pathways to control cellular apoptosis. The present report provides insight into how FOXL2 induces cell death, as summarized in Figure 6, whereby the TNF-R1, Fas, caspase 8, BID and BAK proteins seem to be critical and serve as specific apoptotic components of the FOXL2 response.

In summary, this study is the first to report the differential apoptotic activities between WT and mutant FOXL2. As the FOXL2 mutation is extremely well conserved in most cases of adult-type GCTs and is also GCT-specific over other types of ovarian tumors (Schrader *et al*., 2009; Shah *et al*., 2009; Kim *et al*., 2010), WT FOXL2 may function as a tumor suppressor in normal granulosa cells by efficiently modulating both extrinsic and intrinsic apoptosis signaling pathways. In addition, the disparate apoptotic activities of the two proteins may at least partially account for the pathophysiology of GCT development that occurs by somatic mutation (C134W) of FOXL2.

Materials and methods

Chemicals

All chemicals used in this study were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated. Embrel was obtained from Wyeth Korea (Seoul, Korea), and Fas-Fc was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Plasmid constructions

The Myc-tagged WT FOXL2- and ΔFH FOXL2-encoding plasmids were cloned as previously described (Park *et al*., 2010). The mutant FOXL2 C134W construct was produced using a recombinant PCR technique using the following primers (Bioneer, Daejeon, Korea): FOXL2-F (5′-CTAGAAT TCAAATGATGGCCAGCTACCCC-3′), FOXL2 C134W-F (5′-GCCTGGGAAGACATGTTCGA-3′), FOXL2 C134W-R (5′-ATGTCTTCCCAGGCCGGGTC-3′) and FOXL2-R (5′-CT ACTCGAGTCAGAGATCGAGGCGCGAATG-3′). The PCR products were digested with *Eco*RI and *Xho*I (Enzynomics, Daejeon, Korea) and ligated into the pCMV-Myc (Clontech, Mountain View, CA, USA). The human promoters were produced by PCR amplification from human genomic DNA using the *caspase 8* primers 5′-ACGACGCGTATGAG CCGAGGAAGGCACTGA-3′ and 5′-CTACTCGAGACCA AAAACTCAGAGCACATG-3′, the *TNF-R1* primers 5′-AC GACGCGTTGTACCAAGAAGATAGTTGGG-3′ and 5′-CT ACTCGAGTGCCAGACAGCTATGGCCTCT-3′, the *Fas* primers 5′-ACGACGCGTCCAAGTTGCTGAATCAATGG AAGC-3′ and 5′-CTAAGATCTTGGTTGTTGAGCAATCC TCCG-3′, the *TRAIL-R1* primers 5′-ACGACGCGTATAGT AACAAAAATAGTTAGA-3′ and 5′-CTAAGATCTTCCTG ACAGGTCAATCCAAGA-3′. The PCR products were digested with *Mlu*I and *Xho*I or *Bgl*II (Dakara, Otsu, Japan) and ligated into the pGL3 basic vector (Promega, Madison, WI, USA).

Mammalian cell culture

Human granulosa cell-derived KGN cells were cultured in Dulbecco's modified Eagle's medium/F12 (Welgene, Seoul, Korea). Mouse embryo fibroblasts were a generous gift from Dr CB Thompson (University of Pennsylvania), and mouse Sertoli TM4 cells (Korean Cell Line Bank, Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (Welgene). All media contained 10% fetal bovine serum (PAA, Etobicoke, Canada) and 1% penicillin-streptomycin (Welgene).

Transfection of cells

One or two million KGN cells were resuspended in Resuspension R Buffer (Digital Bio Technology, Seoul, Korea), electroporated with 3 or 6 μg of plasmid DNA, respectively, using a MicroPorator MP-100 (Digital Bio Technology), and incubated on plates containing fresh media unless otherwise indicated. For the electroporation of TM4 (1×10^6) and mouse embryo fibroblast (1×10^6) cells, 4 and 1 μg of DNA constructs were used, respectively.

RNA interference

Endogenous FOXL2 was silenced using specific oligonucleotides (Bioneer): 5′-GCUCCUGUCGCUCCUCUUU-3′ and 5′-AAAGAGGAGCGACAGGAGC-3′. For knockdown of caspase 8, the siRNAs 5′-CAGCCAGUGCCAGACACAGU CUGUA-3′ and 5′-UACAGACUGUGUCUGGCACUGGC UG-3′ were synthesized. The sense and antisense sequences of siRNAs used to target BID or BIM were 5′-GGGAUGAGU GCAUCACAAA-3′ and 5′-UUGUGAUGCACUCAUCC C-3′ or 5′-CUACCUCCCUACAGACAGA-3′ and 5′-UCUG UCUGUAGGGAGGUAG-3′, respectively. Endogenous death receptors were silenced using the following siRNAs: 5′-GCU GUGGACUUUUGUACAU-3′ and 5′-AUGUACAAAAGU CCACAGC-3′ for TNF-R1; 5′-GAGAGUAUUACUAGAG CUU-3′ and 5′-AAGCUCUAGUAAUACUCUC-3′ for Fas; and 5′-CUGGAAAGUUCAUCUACUU-3′ and 5′-AAGUA GAUGAACUUUCCAG-3′ for TRAIL-R1. The oligonucleotide sequences of scrambled siRNAs were 5′-CCUACGCC ACCAAUUUCGU-3′ and 5′-ACGAAAUUGGUGGCGUA GG-3′. The sense and antisense oligonucleotides were annealed in the presence of Annealing Buffer (Bioneer). KGN cells were transfected with the appropriate siRNAs, and knockdown was assessed by western blotting approximately 24 h after transfection.

Flow cytometry analysis of Annexin V-positive cells

To detect apoptotic cells, KGN cells were stained with fluorescein isothiocyanate-conjugated Annexin-V using ApoScan (BioBud, Seoul, Korea) at 24 h post-transfection. Then,

the cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Data are expressed as the absolute percent of Annexin V-positive cells that were either propidium iodide-positive or negative.

Western blot analysis
Cell lysates were prepared 24 h after transfection, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Amersham, Little Chalfont, Buckinghamshire, UK). Polyclonal FOXL2 antibodies were raised as previously reported (Park *et al*., 2010). Antibodies purchased include anti-caspase 9 (Cell Signaling, Danvers, MA, USA), anti-caspase 8 (Cell Signaling), anti-caspase 3 (Cell Signaling), anti-BID (Cell Signaling), anti-tBID (Invitrogen, Carlsbad, CA, USA), anti-BAK (Calbiochem, Gibbstown, NJ, USA), anti-BAX (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TNF-R1 (Santa Cruz), CH-11 (MBL, Woburn, MA, USA), anti-TRAIL-R1 (Santa Cruz), anti-β-actin (Santa Cruz), anti-cytochrome *c* (Santa Cruz), anti-COX IV (Invitrogen) and anti-glyceraldehyde 3-phosphate dehydrogenase (AbFrontier, Seoul, Korea).

Cytochrome c *release*
Digitonin-based cytochrome *c* release assays were performed (Arnoult, 2008). Briefly, KGN cells were harvested and resuspended in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, at pH 7.2) containing 300 μg/ml digitonin and 10% protein inhibitor cocktail (Sigma), and the cells were incubated for 5 min on ice. After centrifugation at 2000 *g* for 5 min, the pellets were solubilized in mitochondrial lysis buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM ethyleneglycol tetraacetic acid, 0.2% Triton-X 100 and 0.3% NP-40) containing 10% protease inhibitor cocktail. Following centrifugation at 10 000 *g* for 10 min at 4 °C, the fractions were analyzed by western blotting.

BAK and BAX oligomerization
The digitonin permeabilization method (Ganju and Eastman, 2003) was employed. KGN cells were harvested, and a 5% glutaraldehyde solution was added to a final concentration of 0.3%. The samples were then incubated for 30 min at room temperature, and the reaction was quenched by the addition of Laemmli sample buffer supplemented with 10% β-mercaptoethanol. Oligomers were detected by immunoblotting.

Reverse transcription and real-time PCR analysis
Reverse transcription of total RNA and subsequent real-time PCR were conducted as previously reported (Park *et al*., 2010) after transfection of KGN cells (1×10^6) with 3 μg of either WT or mutant FOXL2 DNA. Quantification of transcripts was normalized to 18s ribosomal RNA. The nucleotide sequences of the primers used are as follows: 18s ribosomal RNA, 5′-TACCTACCTGGTTGATCCTG-3′ and 5′-GGGTTGGTTTTGATCTGATA-3′; TNF-R1, 5′-CCAAATGGGGGAGTGAGAGG-3′ and 5′-AAAGGCAAAGACCAAAGAAAATGA-3′; Fas, 5′-TGAAGGACATGGCTTAGAAGTG-3′ and 5′-GGTGCAAGGGTCACAGTGTT-3′; and TRAIL-R1, 5′-GCGCAGCGAGTGGGACAGAG-3′ and 5′-GGCACTGGGTCCGTGCTGTC-3′.

Luciferase assay
KGN cells (4×10^5) were transfected with 170 ng of pCMV β-galactosidase plasmid (Clontech), 300 ng of *TNF-R1*-, *Fas*- and *TRAIL-R1*-responsive luciferase reporter constructs, and 300 ng of plasmids encoding WT or mutant FOXL2. At 12 h after transfection, the cells were harvested, and their luciferase activities were measured using the Luciferase Assay System Kit (Promega). Data were normalized to the β-galactosidase activities. The absorbance was measured using a Perkin-Elmer 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA).

Statistical analyses
Multiple comparison analyses of values were performed using the Student–Newman–Keuls test and Tukey's test, and the Student's *t*-test was conducted for paired data (SAS version 8.0, Cary, NC, USA).

Conflict of interest

The authors declare no conflict of interest.

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