GATA4 protects granulosa cell tumors from TRAIL-induced apoptosis

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Abstract

Disturbances in granulosa cell apoptosis have been implicated in the pathogenesis of human granulosa cell tumors (GCTs). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potent cytokine that induces apoptosis in a variety of malignancies without toxic effects on benign cells. The aim of this study was to investigate the expression and functionality of the TRAIL receptors DR4 and DR5 in human GCTs. Additionally, we examined the role of GATA4, a transcription factor expressed in normal and malignant granulosa cells, in TRAIL-induced GCT apoptosis. For this purpose, a tissue microarray of 80 primary and 12 recurrent GCTs was subjected to immunohistochemistry for DR4 and DR5, and freshly isolated primary GCT cultures were utilized to evaluate the functional effects of TRAIL on GCT cells. To clarify the role of GATA4 in the regulation of TRAIL-induced apoptosis, a human GCT-derived cell line (KGN) was transduced with lentiviral vectors expressing small hairpin RNAs targeting GATA4 or transfected with adenovirus expressing either wild-type or dominant negative mutant GATA4. We found that receptors DR4 and DR5 are expressed in a vast majority of GCTs as well as in primary GCT cultures, and that TRAIL induces apoptosis in the primary GCT cultures. Moreover, we showed that overexpressing GATA4 protects GCTs from TRAIL-induced apoptosis *in vitro*, whereas disrupting GATA4 function induces apoptosis and potentiates the apoptotic effect of TRAIL administration. Our results demonstrate that the TRAIL pathway is functional in GCT cells, and suggest that transcription factor GATA4 may function as a survival factor in this ovarian malignancy.

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Introduction

Human ovarian granulosa cell tumors (GCTs), accounting for 5% of all ovarian cancer, are the most common sex chord-derived malignancies with an incidence from 0.4 to 1.7 per 100 000 women (Schumer & Cannistra 2003, Jamieson & Fuller 2008). GCTs typically present with relatively low malignant potential. However, these tumors can relapse decades after primary diagnosis, and the treatment of recurrent disease is challenging (Schumer & Cannistra 2003). Platinum-based chemotherapy has been used in these tumors, but novel-targeted

treatments are needed especially in the recurrent cases (Colombo *et al.* 2007). Thus, unraveling the exact mechanisms leading to GCT development is needed in the search for new therapies. Recently, a mutation in the gene encoding for the transcription factor FOXL2 has been found in a vast majority of GCTs studied (Shah *et al.* 2009), but the impact of this alteration remains unknown.

Disturbances in the regulation of granulosa cell apoptosis are suggested to play a role in granulosa cell tumorigenesis (Fuller & Chu 2004). This is plausible given that the physiological fate of most normal

granulosa cells is apoptosis. Successful follicular development gives rise to typically only one oocyte-containing follicle per cycle, while majority of the initially recruited follicles degenerate through apoptosis (Tilly 1996). In general, apoptosis is regulated by two interlinked mechanisms: extrinsic and intrinsic pathways (Fulda & Debatin 2006). In ovaries, the physiological importance of Bcl-2 family in regulating the intrinsic pathway has been extensively studied (Pru & Tilly 2001). Granulosa cell apoptosis is compromised in transgenic mice overexpressing the anti-apoptotic factor Bcl-2 (Hsu et al. 1996), whereas the number of follicles is reduced in Bcl-2 null mice (Ratts et al. 1995). Recently, we have demonstrated high level expression of Bcl-2 in a majority of human GCTs, suggesting a role for Bcl-2 in the regulation of apoptosis in these tumors (Kyrönlahti et al. 2008).

The ligands of the extrinsic pathway, especially tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), have emerged as potent cytokines that induce programmed cell death in a variety of human malignancies without significant toxicity for normal cells (Johnstone et al. 2008). The effects of TRAIL are mediated by two functional cell surface receptors, DR4 (also known as TRAIL-R1 and TNFRSF10A) and DR5 (TRAIL-R2 and TNFRSF10B). In humans, DR5 is expressed both in fetal and in adult granulosa cells (Jaaskelainen et al. 2009). The same study reported no DR4 expression in adult or fetal granulosa cells; however, we have detected modest DR4 staining also in adult granulosa cells (data not shown). The expression of TRAIL receptors in human GCTs is not well documented, although recombinant TRAIL is known to induce apoptosis in human granulosa cell line KGN (Woods et al. 2008a,b, Jaaskelainen et al. 2009).

We herein demonstrate that the TRAIL receptors DR4 and DR5 are expressed in a majority of human GCTs, and that TRAIL can effectively induce apoptosis in isolated primary tumor cells. Furthermore, we show that GATA4 (named after its binding to a consensus W-GATA-R motif), a transcription factor implicated in ovarian apoptosis and GCT pathobiology (Heikinheimo et al. 1997, Anttonen et al. 2005, Kyrönlahti et al. 2008), can protect tumor cells from TRAIL-induced apoptosis *in vitro*, whereas inhibiting GATA4 function sensitizes tumor cells to TRAIL-induced apoptosis. Finally, we show that disturbing GATA4 function alone is sufficient to induce apoptosis *in vitro*, suggesting that GATA4 may function as a survival factor in GCTs. Inhibition of GATA4 combined with TRAIL administration emerges as a potential means for targeted treatment of GCTs.

Materials and methods

Human tissue samples

This study was approved by the ethical committee of Helsinki University Central Hospital and the National Supervisory Authority for Welfare and Health in Finland. GCT tissue microarray of quadruple samples from 80 primary and 12 recurrent GCT samples was constructed as previously described (Kononen et al. 1998, Anttonen et al. 2005). For primary cell cultures (see below), informed patient consent was obtained before surgical operations. We obtained six fresh tumor samples, two from primary tumors and four from recurrent tumors, and all of them were verified to possess the 402C → G (C134W) mutation in FOXL2 (Shah et al. 2009). All the GCT diagnoses were re-evaluated, and tumor subtype, mitotic index, and nuclear atypia were defined by an expert pathologist (R B) as previously described (Anttonen et al. 2005); full clinical data of the patients were reviewed from hospital files.

Immunohistochemistry

Immunohistochemical staining of GCT tissue microarrays was performed as previously described (Anttonen et al. 2005, Kyrönlahti et al. 2008). The primary antibodies used were as follows: goat antihuman DR4 IgG at 1:100 dilution (sc-6823, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); goat antihuman DR5 IgG at 1:50 dilution (sc-7192, Santa Cruz Biotechnology). Tissue samples stained with secondary antibody alone served as a negative control.

The immunohistochemistry results were classified into three groups based on the intensity of staining as follows: low (–), intermediate (+), and strong (++). Two researchers (A K and M K) independently performed the evaluation, and disagreements were resolved by a joint review with a third researcher (M A).

Cell cultures and stimulations

Following intraoperative diagnosis of GCT, tumor tissue was immediately obtained for cell culture. GCT tissue was mechanically minced and treated with 0.5% collagenase (Sigma–Aldrich Corporation) in DMEM/F12 containing 0% fetal bovine serum (FBS) for 2 h. The resultant cell suspension was then filtered through 140 µm filter mesh to obtain single cells, washed with PBS, and re-suspended in DMEM/F12 containing 10% FBS. Finally, the primary tumor cells were counted and plated for experiments.

The KGN cell line (a generous gift from Dr Toshihiko Yanase, Kyushu University, Fukuoka, Japan), established from a human GCT, was cultured as previously described (Nishi *et al.* 2001). This cell line harbors the 402C→G mutation in FOXL2 found in a majority of human GCTs (Schrader *et al.* 2009). To induce apoptosis, the cells were incubated with recombinant human TRAIL (Millipore, Bedford, MA, USA) at concentrations of 10, 50, and 100 ng/ml. After 6–24 h incubation, the apoptosis was analyzed by activated caspase-3 western blotting (see below), 4',6-diamino-2-phenylindole hydrochloride (DAPI) staining (see below), and Caspase-Glo 3/7 assay (see below).

Immunocytochemistry and western blotting

Primary GCT and KGN cells were plated on 8-well glass chambers, and then confluent washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Immunocytochemical staining was performed using Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA, USA). Primary antibodies were as follows: mouse antihuman α -inhibin IgG (MCA 9515, AbD Serotec, Oxford, UK) at dilution of 1:100, goat antihuman anti-Müllerian hormone (AMH) IgG (sc-6886, Santa Cruz Biotechnology, Inc.) at dilution of 1:100, and goat antihuman GATA4 IgG (sc-1237, Santa Cruz Biotechnology) at dilution of 1:200; goat antihuman DR4 IgG at 1:100 dilution (sc-7863, Santa Cruz Biotechnology), goat antihuman DR5 IgG at 1:50 dilution (sc-7192, Santa Cruz Biotechnology), rabbit antihuman DR4 IgG at 1:400 dilution (ab8414, Abcam, Cambridge, UK), and DR5 IgG at 1:400 dilution (ab8416, Abcam).

Western blotting was performed as previously described (Kyronlahti *et al.* 2008). Antibodies used were as follows: rabbit antihuman cleaved caspase-3 IgG (#9661L, Cell Signaling Technology, Danvers, MA, USA) at dilution of 1:1000, goat antihuman GATA4 IgG (sc-1237, Santa Cruz Biotechnology) at dilution of 1:1000, and goat antihuman β -actin IgG (sc-1616, Santa Cruz Biotechnology, Inc.) at dilution of 1:10 000.

Caspase activation assay and DAPI analysis

Caspase-3/7 activation was measured using Caspase-Glo 3/7 assays (Promega Corporation) as previously described (Jaaskelainen *et al.* 2009). For DAPI staining, cells were washed twice with PBS and fixed with formalin for 10 min at room temperature. After rinsed with PBS, cells were incubated with cold

methanol for 10 min at -20°C , washed with PBS, and incubated for 30 min at room temperature with 0.4 $\mu\text{g/ml}$ of DAPI in PBS. Finally, the cells were dehydrated through graded alcohols and Ultra Clear (J T Baker, Deventer, Holland), and mounted with UltraKitt (J T Baker).

Adenoviral transfection

Adenoviral transfections were performed as previously described (Kyronlahti *et al.* 2008). The replication-deficient adenoviral construct expressing wild-type rat GATA4 (G4wt) and dominant negative GATA4 (G4dn, GATA4 producing engrailed repressor domain fusion protein tagged with flag epitope) have been previously described (Charron *et al.* 2001, Liang *et al.* 2001).

Generation of GATA4 small hairpin RNA expressing stable cell lines

pLKO.1 small hairpin RNA (shRNA) targeting GATA4 (shGATA4)-containing constructs were obtained from Sigma (TRC shRNA lentivirus library) and were as follows: shGATA4-1 (TRCN0000020426) 5'-GAGGAGATGCGTCCCATCAA-3' and shGATA4-2 (TRCN0000020427) 5'-CTGAATAAATCTAAGACACCA-3'. Lentiviruses were produced as previously described (Partanen *et al.* 2007) using packaging constructs and protocols provided by the Biomedicum Helsinki Virus Core Facility.

Subconfluent 6-well plates of KGN cells were transduced with 750 μl of lentiviral particles in a total volume of 1.5 ml in DMEM/F12 containing 10% FBS. Polybrene was added to enhance transduction at 1:1000 dilution. Cells were incubated with lentiviral supernatant for 10 min at $+37^{\circ}\text{C}$, centrifuged at 2500 r.p.m (500 g) for 30 min, and then incubated at $+37^{\circ}\text{C}$ for 2 h, after which the lentiviral supernatant was replaced with normal growth media. After 72 h, 1:2500 transduced cells were selected with puromycin (1 $\mu\text{g/ml}$).

Statistical analysis of the data

For tissue microarray scoring results and clinicopathological data, contingency tabling (2×2) was performed for the nominal variables, followed by the χ^2 or Fisher's exact test. Statistical analysis of caspase assay experiments was performed using the Student's *t*-test (for G4dn and G4wt experiments) or one-way ANOVA, followed by Dunnett's test (for shGATA4 experiments) using JMP software (JMP, Cary, NC, USA). $P < 0.05$ was considered significant in all experiments.

Results

TRAIL receptors are expressed in human GCTs

To study the expression of the TRAIL receptors in GCTs, a tissue microarray based on 80 primary and 12 recurrent GCT samples was subjected to immunohistochemistry for DR4 and DR5. Of the GCTs analyzed, 91% exhibited strong (18%) or intermediate (73%) immunostaining for DR4 (using sc-6823 antibody); only 9% exhibited low DR4 expression (Fig. 1A and C). Similarly, strong or intermediate DR5 expression was detected in 92% of the GCTs (strong 17% and intermediate 75%), and only 8% of the tumors exhibited low DR5 expression (Fig. 1B and D).

To validate DR4 staining results, we utilized a second commercially available antibody (ab8414). These results significantly correlated ($P < 0.001$) with those of the sc-6823 DR4 antibody. However, we were unable to validate DR5 stainings (sc-7192) with another antibody (ab8416); these stainings remained unreliable given that we found nuclear staining in addition to membrane staining when using this antibody.

Figure 1 Immunohistochemical analysis of death receptors DR4 (A, C, and E) and DR5 (B, D, and F) from tissue microarray of GCTs. Examples of strong, intermediate, and low expression are depicted in upper, middle, and lower rows respectively. Higher magnifications are shown in insets. Brown color indicates positive staining. Scale bar is 50 μ m.

Figure 2 Immunohistochemical analysis of TRAIL receptors DR4 (A), DR5 (B), and GCT markers AMH (C), α -inhibin (inh- α) (D), and GATA4 (E) in cultured GCT cells. Brown color indicates positive staining. (F) A negative control using secondary antibody only. Higher magnifications are shown in insets. Scale bar is 50 μ m.

Importantly, 11 of 12 recurrent GCTs exhibited strong or intermediate DR4 expression, and 12 of 12 tumors exhibited strong or intermediate DR5 expression. The DR4 and DR5 expression patterns did not correlate with clinical stage of primary tumor, size of the tumor, atypia, mitotic index, or with recurrence tendency (data not shown).

TRAIL receptor expression in primary GCT cell cultures

To further assess the functional role of TRAIL-induced apoptosis in human GCTs, we characterized the expression of DR4 and DR5 in primary GCT cell cultures that we had subsequently treated with TRAIL (see below). For *in vitro* experiments, we obtained six tumor samples, two from primary tumors and four from recurrent tumors. All the samples of primary GCTs studied expressed the TRAIL receptors DR4 (Fig. 2A) and DR5 (Fig. 2B).

The successful isolation of the tumor cells was confirmed by immunostaining for GCT markers as follows: AMH, inhibin- α , and GATA4 (Fig. 2C–E;

Rey *et al.* 2000, La Marca & Volpe 2007, Jamieson & Fuller 2008). Immunocytochemistry results were verified using reverse transcription-PCR analysis of primary GCT cell lysates (data not shown).

TRAIL induces apoptosis in primary GCT cultures but does not affect GATA4 levels

Recombinant TRAIL has been shown to induce apoptosis in the human GCT cell line KGN (Woods *et al.* 2008a, Jaaskelainen *et al.* 2009). To clarify the significance of the TRAIL pathway in GCT pathobiology and apoptosis, we compared the effects of TRAIL in primary human GCT cells and in the KGN cell line. Freshly isolated primary GCT cells and KGN cells were stimulated with increasing doses of TRAIL for 24 h before the measurement of caspase activity. Analysis of nuclear morphology (based on DAPI staining) was utilized to confirm the induction of apoptosis. In these experiments, TRAIL dose dependently activated caspase-3 and induced apoptosis in all the six GCTs studied (Fig. 3A and B). Activation of caspase-3 was also evident in KGN cells (Fig. 3A),

Figure 3 (A) Western blotting showing the activation of caspase-3 in both primary GCT and KGN cells 24 h after TRAIL administration. Expression of GATA4 remains unaltered. β -Actin served as a loading control. (B) DAPI staining of apoptotic primary GCT cells 24 h after TRAIL administration. Arrows indicate apoptotic cells. (C) A representative histogram of dose-dependent activation of GCT apoptosis by TRAIL.

confirming the similar behavior between KGN cell line and primary human GCT cells. However, the primary GCT cells were more sensitive to TRAIL-induced apoptosis as activated caspase-3 was detected in TRAIL concentrations as low as 10 ng/ml. This difference may reflect the more robust nature of established cell line, KGN, or the greater cellular stress involved in obtaining the GCT cells from tumor samples.

We next studied the effect of TRAIL administration on GATA4 expression in GCT-derived cells. In human granulosa cells, rapid downregulation of GATA4 precedes the physiological apoptosis of ovulating follicles suggesting its role in the regulation of granulosa cell apoptosis (Heikinheimo *et al.* 1997). Furthermore, high GATA4 expression is known to correlate with the aggressive behavior of GCT tumors (Anttonen *et al.* 2005). However, in our experiments, GATA4 protein levels after TRAIL administration changed in neither the primary tumor cells nor the KGN cells (Fig. 3A).

Taken together, these results indicate the presence of functional extrinsic apoptosis pathway in primary GCT cells, and the ability of TRAIL to induce apoptosis in GCTs similar to other ovarian cancer types (Cuello *et al.* 2001).

Overexpression of GATA4 protects GCT cells from TRAIL-induced apoptosis

Next, we studied the effect of GATA4 on TRAIL-induced GCT apoptosis by assessing the effect of GATA4 overexpression on TRAIL-induced apoptosis in KGN cells using an adenoviral GATA4 construct. KGN cells were chosen instead of primary tumor cells to allow enough experimental material for replicated assays. Consistent with prior studies (Jaaskelainen *et al.* 2009), TRAIL induced apoptosis in KGN cells in a similar way to primary GCT cell cultures (Figs 3 and 4, $P < 0.05$).

GATA4 overexpression efficiently protected KGN cells from TRAIL-induced apoptosis (Fig. 4). Of note, GATA4 was able to counteract even high doses of TRAIL (100 ng/ml) after both 6 h (Fig. 4B; $P < 0.05$) and 24 h (Fig. 4C; $P < 0.05$) of stimulation. Overexpression of GATA4 did not affect the baseline caspase activity of KGN cells (Fig. 4B and C). The results are shown in relative caspase activity levels to maintain uniformity between individual adenoviral transfection since individual transfections showed some fluctuation (7000 fluorescence units $\pm$ 2031). Importantly, however, normalization to baseline apoptosis rates yielded similar results.

Figure 4 GATA4 protects KGN cells against TRAIL-induced apoptosis. (A) Western blotting showing overexpression of GATA4. The KGN cells were transfected with GATA4 adenovirus (G4wt) or lacZ control adenovirus (lacZ), and treated with vehicle or indicated doses of TRAIL 48 h later. Caspase activity was measured 6 h (B) and 24 h (C) after TRAIL administration. Caspase activity is presented relative to control transfection as the mean $\pm$ s.e.m. of three independent experiments performed in triplicate. * $P < 0.05$ as compared to lacZ controls with the same TRAIL concentration. $^{\S}P < 0.05$ as compared to lacZ control with no TRAIL.

Disrupting GATA4 function induces apoptosis in GCT cells and potentiates the effect of TRAIL administration

To further evaluate the role of GATA4 in TRAIL-induced GCT apoptosis, we investigated the effect of GATA4 downregulation on GCT cell apoptosis. For this purpose, we transduced KGN cells with lentiviral vectors expressing two different shGATA4 (Fig. 5A). The silencing of GATA4 by either of these shRNAs enhanced the effect of TRAIL-induced apoptosis (Fig. 5B). Despite a relatively modest downregulation of GATA4, these experiments showed increased baseline apoptosis of KGN cells.

To gain better insight into this process, we transfected KGN cells with GATA4-enrained fusion protein (G4dn), which is known to have a G4dn activity (Liang *et al.* 2001). Disrupting GATA4 function with the dominant negative mutant markedly increased baseline apoptosis in KGN cells and sensitized these cells to TRAIL-induced apoptosis (Fig. 5D).

Figure 5 Effect of shGATA4 and dominant negative GATA4 on KGN apoptosis. (A) Western blotting showing silencing of GATA4 in KGN cells expressing two different shGATA4 constructs (sh1 and sh2). (B) The KGN cells stably expressing shGATA4 were treated with vehicle or indicated doses of TRAIL. Caspase activity was measured 6 h later. (C) Western blotting showing expression of dominant negative GATA4 (G4dn) detected by anti-flag antibody. (D) The KGN cells were transfected with G4dn or control adenovirus, and treated with vehicle or indicated doses of TRAIL. Caspase activity and protein levels were measured 6 h later. All caspase activities are presented relative to control transfection as the mean $\pm$ s.e.m. of three independent experiments performed in triplicate. * $P < 0.05$.

The increase in apoptosis with G4dn exceeded that obtained by shGATA4, probably because of a relatively modest downregulation effect of shGATA4, 42% (for Sh1) and 35% (for Sh2) as compared to non-target controls. Another potential explanation for a larger effect seen with G4dn transfections could be the effect of this construct on nuclear factor κ B (NF- κ B) levels. We did not, however, detect any effects of G4dn transfection on NF- κ B protein levels when evaluated by western blot analysis (data not shown). Taken together, these results suggest that GATA4 functions as an anti-apoptotic factor in GCTs.

Discussion

Recombinant human TRAIL and antibodies mimicking its apoptosis-inducing effect have emerged as promising drugs to treat variety of malignant tumors. Currently, phase II clinical studies are being conducted to study this approach in the treatment of both advanced colorectal and non-small cell lung cancer as well as relapsed non-Hodgkin's lymphoma (Ashkenazi 2008).

A prerequisite for TRAIL-based therapy is the expression of death receptors on the cancer cell surface (Ashkenazi 2008). We now show that both TRAIL death receptors DR4 and DR5 are present in a majority of human GCTs. We also report that TRAIL receptors, and the other components of TRAIL pathway, are functional in primary isolated GCT cells. Taken together, these preclinical studies provide a basis to test the use of TRAIL as an anti-cancer drug also in human GCTs. Since TRAIL receptors are present in normal human granulosa cells, further studies are needed to assess the sensitivity of these cells to TRAIL-induced apoptosis.

Besides the extrinsic pathway via death receptors, apoptosis is also regulated by the intrinsic pathway. Indeed, promising combination therapies targeting both these pathways are being studied in different malignancies (Johnstone *et al.* 2008). In physiological granulosa cells, genes of Bcl-2 family are known to play key role in the regulation of the intrinsic apoptosis pathway (Pru & Tilly 2001). In animal studies, Bcl-2 knockout mice demonstrate decreased number of follicles (Ratts *et al.* 1995), whereas granulosa cell apoptosis is compromised in transgenic mice overexpressing Bcl-2 (Hsu *et al.* 1996). Intriguingly, the transcription factor GATA4 is known to regulate the expression of Bcl-2 in human GCTs (Kyronlahti *et al.* 2008).

Transcription factor GATA4 is best known for its role in heart muscle development and function (Heikinheimo *et al.* 1994). Mice devoid of

GATA4 die *in utero* due to disturbances in cardiac morphogenesis (Kuo *et al.* 1997, Molkentin *et al.* 1997). Recently, GATA4 has emerged as an important factor in the development and function of endocrine tissues (Viger *et al.* 2008). In adult cardiomyocytes, GATA4 acts as a pivotal anti-apoptotic factor during different apoptosis inducing stimuli such as hypoxemia (Heineke *et al.* 2007) or treatment with anti-cancer drug doxorubicin (Kobayashi *et al.* 2006, 2009).

In this study, we have tested the ability of GATA4 to modify TRAIL-induced apoptosis in human GCTs. Treating KGN cells with TRAIL did not have any effect on endogenous GATA4 levels in GCT cells. Thus, the apoptosis-inducing effect of TRAIL does not require the downregulation of GATA4. Overexpression of GATA4 partially protects KGN cells from TRAIL-induced apoptosis. The anti-apoptotic effect of GATA4 in GCT cells is of interest given that the tumors with high GATA4 expression are aggressive and relapse more often and earlier than tumors with low GATA4 expression (Anttonen *et al.* 2005). In tumors of high expression, elevated levels of GATA4 may therefore protect malignant cells from the apoptotic effects of endogenous factors such as TRAIL. The role of GATA4 in GCT pathobiology is strengthened by our findings that disturbing GATA4 by either dominant negative adenovirus or shRNA-producing constructs sensitizes KGN cells to TRAIL-induced apoptosis. As a next step, injection of cell lines with increased or silenced GATA4 expression to SCID mouse models could be utilized to assess the interplay between recombinant TRAIL and sensitizing treatments. Combining the stimulation of the extrinsic TRAIL-dependent pathway to sensitizing treatments may be useful in treating GCTs.

Interestingly, disrupting GATA4 function with either dominant negative adenovirus or shGATA4 lentivirus induced apoptosis in GCTs even in the absence of exogenous TRAIL supporting the premise that GATA4 functions as an anti-apoptotic factor in GCTs. The induction of apoptosis after GATA4 downregulation was of the same extent with all the TRAIL concentrations tested and even without TRAIL stimulation, indicating that the effect of GATA4 knockdown is not specific for TRAIL stimulations. GATA4 may thus modify multiple apoptosis-inducing pathways in granulosa cells. This observation is in accordance with the findings in normal ovary, in which downregulation of GATA4 precedes the physiological apoptosis of ovulating follicles (Heikinheimo *et al.* 1997). It is not known how GATA4 exerts its anti-apoptotic effects in the ovary, but it is known to regulate the two anti-apoptotic Bcl-2 family members,

namely *Bcl-2* and *Bcl-XL*, in GCT cells as well as in cardiomyocytes by directly binding to their promoters (Kitta et al. 2003, Kobayashi et al. 2006, Kyrönlähti et al. 2008). Thus, proteins in the Bcl-2 family appear as putative candidates to mediate the GATA4 effects in human GCTs.

In summary, our studies document the expression of functional TRAIL receptors in GCTs. Furthermore, we show that GATA4 protects GCT cells from TRAIL-induced apoptosis *in vitro*, whereas inhibiting the normal GATA4 function predisposes GCT cells to TRAIL-induced apoptosis. Finally, we show that disturbing GATA4 function alone is sufficient to induce GCT apoptosis *in vitro*, suggesting that GATA4 may function as a survival factor in GCTs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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