

GATA-4 Regulates Bcl-2 Expression in Ovarian Granulosa Cell Tumors

Antti Kyrönlahti, Maarit Rämö, Maija Tamminen, Leila Unkila-Kallio, Ralf Butzow, Arto Leminen, Mona Nemer, Nafis Rahman, Ilpo Huhtaniemi, Markku Heikinheimo, and Mikko Anttonen

Children's Hospital and Institute of Biomedicine (A.K., M.R., M.T., M.H.) and Program for Women's Health (A.K., M.R., M.T., R.B., M.H., M.A.), Biomedicum Helsinki, and Departments of Obstetrics and Gynecology (L.U.-K., A.L., R.B., M.A.) and Pathology (R.B.), University of Helsinki, 00014 Helsinki, Finland; Cardiac Growth and Differentiation Unit (M.N.), Clinical Research Institute of Montreal, Montreal, Quebec, Canada H3A 2B4; Department of Physiology (N.R.), University of Turku, 20014 Turku, Finland; Department of Reproductive Biology (I.H.), Imperial College London, London W12 0NN, United Kingdom; and Department of Pediatrics (M.H.), Washington University School of Medicine, St. Louis, Missouri 63110

Excessive cell proliferation and decreased apoptosis have been implicated in the pathogenesis of ovarian granulosa cell tumors (GCTs). We hypothesized that transcription factor GATA-4 controls expression of the antiapoptotic factor Bcl-2 and the cell cycle regulator cyclin D2 in normal and neoplastic granulosa cells. To test this hypothesis, a tissue microarray based on 80 GCTs was subjected to immunohistochemistry for GATA-4, Bcl-2, and cyclin D2, and the data were correlated to clinical and histopathological parameters. In addition, quantitative RT-PCR for GATA-4, Bcl-2, and cyclin D2 was performed on 21 human GCTs. A mouse GCT model was used to complement these studies. The role of GATA-4 in the regulation of *Bcl2* and *ccnd2* (coding for cyclin D2) was studied by

transactivation assays, and by disrupting GATA-4 function with dominant negative approaches in mouse and human GCT cell lines. We found that GATA-4 expression correlated with Bcl-2 and cyclin D2 expression in human and murine GCTs. Moreover, GATA-4 enhanced Bcl-2 and cyclin D2 promoter activity in murine GCT cells. Whereas GATA-4 overexpression up-regulated and dominant negative GATA-4 suppressed Bcl-2 expression in human GCT cells, the effects on cyclin D2 were negligible. Our results reveal a previously unknown relationship between GATA-4 and Bcl-2 in mammalian granulosa cells and GCTs, and suggest that GATA-4 influences granulosa cell fate by transactivating *Bcl-2*. (*Endocrinology* 149: 5635–5642, 2008)

OVARIAN GRANULOSA cell tumors (GCTs) are hormonally active malignancies accounting for 2–5% of all ovarian cancers. They share several common features with proliferating granulosa cells of normal preovulatory follicles (1). The reported 10-yr survival varies from 50–95% for stages I and II of the disease (2). Although the overall survival is fairly favorable, these tumors are noted for their proclivity for recurrences occurring even 40 yr after the initial diagnosis (3). The recurrences are common even with stage Ia primary tumors, and they are fatal in 80% of patients with advanced primary tumors (1). GCTs are characterized by low mitotic index (MI), low nuclear atypia, and low stage (2, 4). At presentation the tumor size varies considerably, from less than 5 to over 10 cm in diameter. Measurement of serum anti-müllerian hormone (AMH) (also known as müllerian inhibiting substance) levels, together with inhibin α and estradiol, has proven to be helpful markers in the prognosis and follow-up of GCT patients (1, 2, 5, 6). Low expression of AMH in the tumor associates with larger size, suggesting that the loss of AMH expression favors GCT growth (4).

The detailed molecular pathogenesis of GCT has remained elusive. However, similar gene expression patterns suggest that the very same mechanisms are involved in the proliferation and apoptosis of normal and malignant granulosa cells (1, 7). Our recent findings suggest a role for transcription factor GATA-4 in granulosa cell tumorigenesis. Accordingly, high GATA-4 expression levels in human GCTs correlate with stage Ic or higher and with the risk of recurrence, suggesting that GATA-4 could be used as a prognostic marker (4). Interestingly, GATA-4 expression coincides with the active proliferation of both human and mouse granulosa cells, and is abruptly diminished during ovulation and follicular atresia (8–10). In addition, GATA-4 is involved in the FSH signaling cascade (10, 11), and regulates *in vitro* inhibin- α (12) and *CYP19* (13) genes, both important for normal granulosa cell function.

Several studies have revealed that GATA-4 regulates genes suggested to be important for GCT pathogenesis, albeit these actions have also been demonstrated in other than ovarian cells. *Ccnd2*, encoding cyclin D2, is strongly expressed in human GCTs and has been found pivotal for FSH stimulated granulosa cell proliferation (1, 14). The physiological function of cyclin D2 is to facilitate the G₁ to S phase transition in a coordinated fashion together with its antagonist p27^{Kip1} (15). *Ccnd2* $-/-$ female mice are infertile due to FSH unresponsive, hypoplastic granulosa cells (16). In pulmonary artery smooth muscle cells, the overexpression of GATA-4 up-regulates cyclin D2 expression, whereas disturbing the endogenous GATA-4 with dominant negative (DN)

First Published Online July 24, 2008

Abbreviations: AMH, Anti-Müllerian hormone; DN, dominant negative; DnG4, dominant negative GATA-4 activity; FBS, fetal bovine serum; FOXO1, fork-head box protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCT, granulosa cell tumor; MI, mitotic index; WT, wild type.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

variants suppresses it (17). In granulosa cells, cyclin D2 up-regulation requires FSH stimulated relief of transcription factor forkhead box O1 (FOXO1) mediated repression of transcription as well as functional activin signaling. Activin executes its action via the intracellular mediators Smad2 and Smad3 (18). Interestingly, induction of inhibin- α expression in granulosa cells requires an interplay between Smad3 and GATA-4 (12). However, the possible functional connection between cyclin D2 and GATA-4 in the ovary has not been investigated to our knowledge.

Besides inappropriate cell proliferation, a decreased rate of apoptosis may also contribute to granulosa cell tumorigenesis. During the normal menstrual cycle, the majority of developing follicles undergo atresia by the mechanism of granulosa cell apoptosis (19). Genes of the *Bcl-2* family are known to be important in regulating follicular apoptosis (20), and one of the key factors in this process is the antiapoptotic Bcl-2 (21). Transgenic mice overexpressing Bcl-2 demonstrate decreased granulosa cell apoptosis (22), whereas Bcl-2 knockout mice show a decreased number of follicles (23). The temporospatial expression pattern of GATA-4 suggests its involvement in the regulation of granulosa cell apoptosis (10, 24). Interestingly, GATA-4 regulates the expression of Bcl-2 in cardiomyocytes by binding directly to the promoter of its gene (25). However, in the context of granulosa cell function and tumorigenesis, the possible role of GATA-4 in the regulation of Bcl-2 has not been studied.

Based on these facts, we hypothesized a role for GATA-4 in granulosa cell tumorigenesis, and in the regulation of Bcl-2 and cyclin D2 genes in mammalian granulosa cells. We analyzed the expression pattern of GATA-4, Bcl-2, and cyclin D2 in normal human proliferating granulosa cells, in a human GCT tissue microarray of 80 primary tumors, in frozen samples of 21 primary human GCTs, as well as in a mouse GCT model. We also correlated the expression patterns of GATA-4, Bcl-2, and cyclin D2 with each other and with clinicopathological parameters of the human GCTs. Finally, we studied the role of GATA-4 in the regulation of Bcl-2 and cyclin D2 genes *in vitro*.

Materials and Methods

Patients

The study was approved by the hospital ethics committee and the National Authority for Medicolegal Affairs in Finland. The GCT patient series is described in detail previously (4). In brief, 80 GCT patients, diagnosed as having GCT at Helsinki University Central Hospital between 1971 and 2003, were included in the study, and the clinicopathological data of the patients were retrospectively collected from patient files. The mean age of the patients was 52 yr (range 19–87), and 54% were postmenopausal.

Human and mouse tissue samples

The human tumor tissue samples were critically reevaluated by a gynecological pathologist (R.B.) with the help of immunohistochemical markers. Tumors were classified to differentiated and sarcomatoid subtypes, and evaluated for the degree of nuclear atypia, MI, and Ki67 as previously described (4). Tissue microarray of the human GCT samples was constructed as previously described (26). Quadruple samples from the 80 primary GCTs were arrayed in one recipient paraffin block. In addition to the formalin-fixed samples, 21 tissue samples, snap frozen upon pathological diagnosis, were used in the analysis. Three normal human ovaries, removed because of cervical cancer from women

younger than 35 yr of age, were used as normal reference tissues. Mouse GCT samples originated from transgenic mice, expressing the SV40 T antigen driven by the inhibin- α promoter (27). Given that these mice spontaneously develop typical GCTs by 4–5 months of age, samples from a 5-month-old mouse were used in this study.

Immunohistochemistry

Immunoperoxidase staining of human and mouse samples for GATA-4 and cyclin D2, and mouse samples for Bcl-2 followed the protocol previously described (4, 9). The primary antibodies, goat antimouse GATA-4 IgG at dilution 1:200 (sc-1237; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit antimouse cyclin D2 IgG at dilution 1:1000 (sc-593; Santa Cruz Biotechnology), and rabbit antihuman Bcl-2 IgG at dilution 1:100 (sc-783, used for mouse tissue; Santa Cruz Biotechnology), were incubated overnight at +4 C. Immunostaining of human samples for Bcl-2 (anti-Bcl-2 antibody clone 124, no. M0887; Dako Denmark A/S, Glostrup, Denmark) was performed in a Dako TechMate 500 automated staining machine. In control experiments, nonimmune serum or PBS replaced the primary antibody.

Scoring of the immunohistochemistry results

The stained tissue sections were scored as previously described (4). Scoring of the antigens represents the proportion of positive cells, *i.e.* low for 0–20% positive cells or 1–80% positive cells with weak immunoreactivity, intermediate for 20–100% positive cells with at least moderate immunoreactivity, and high for 80–100% positive cells. Because of the loss of tissue from the sections or artifact staining, we were unable to analyze cyclin D2 expression in two tumors and Bcl-2 expression in the same two tumors and one other.

Cell lines

The KGN cell line (a generous gift from Dr. Toshihiko Yanase, Kyushu University, Fukuoka, Japan), established from a human GCT and expressing typical granulosa cell markers, was cultured as previously described (28). The KK-1 cell line, expressing typical granulosa cell markers, obtained from a mouse GCT of inhibin- α -driven T-antigen transgenic mice was cultured as previously described (27). COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin.

Plasmids and luciferase assays

The plasmids used have been previously described: pMT2-GATA-4 [wild-type (WT) GATA-4] (29), 1281 bp-*Bcl-2*-luciferase (25), and pGL3-680 bp-*Ccnd2*-luciferase (18). In luciferase assays, 1.8×10^5 (COS-7) or 1.3×10^5 (KK-1) cells were distributed on 12-well plates and grown for 24 h before transfection. Cells were transfected with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The total amount of DNA transfected was kept constant by addition of empty vectors to controls when needed. Cells were harvested 48 h later, and relative luciferase levels to cotransfected pCMV β -galactosidase were calculated as previously described (12).

Adenoviral infections

For adenoviral infections, 5×10^5 KGN cells were plated in six-well plates 24 h before transfection. The KGN cells were infected by incubating them with viruses in 1 ml DMEM containing 0% FBS. Multiplicity of infection used varied from two to 10. After 1 h, 2 ml DMEM containing 10% FBS was added to stop the infection. Cells were harvested 48 h (DN) or 96 h (WT) after transfection. The replication-deficient adenoviral constructs expressing WT rat GATA-4 and DN GATA-4 (engrailed repressor domain, GATA-4 fusion with the flag epitope) have been previously described (30, 31).

RNA extraction and quantitative real-time PCR

Total RNA and protein were extracted from the KGN cells and human tissue samples, and contaminating genomic DNA was eliminated using the NucleoSpin RNA/Protein kit (MACHERY-NAGEL GmbH & Co.

KG, Düren, Germany). First-strand cDNA synthesis was performed from 0.8 μ g total RNA using SYBR Green RT-PCR reagents and random hexamers (Applied Biosystems, Foster City, CA). The primers were designed using the ABI Primer Express software (Applied Biosystems) applied to cDNA sequences derived from the UCSC Genome Bioinformatics web site (<http://genome.ucsc.edu/>) and were as follows: Bcl-2 sense, 5'-GTG GAT GAC TGA GTA CCT GAA CC-3'; Bcl-2 antisense, 5'-AGC CAG GAG AAA TCA AAC AGA G-3'; Ccnd2 sense, 5'-AAT GGT CAT TTC AGG CAC AA-3'; Ccnd2 antisense, 5'-TGC TGA TGG CTT CTC AAA AC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-TCA TTT CCT GGT ATG ACA ACG-3'; GAPDH antisense, 5'-TTA CTC CTT GGA GGC CAT GT-3'; GATA-4 sense, 5'-CTC CTC TGC ACA TTG CTG TT-3'; and GATA-4 antisense, 5'-GTG TGG GGA GGC GTA GTA AT-3'. Analysis of Bcl-2, Ccnd2, GAPDH, and GATA-4 expressions was performed with SYBR Green RT-PCR reagents and an ABI PRISM 7700 sequence detection system (Applied Biosystems) using the relative standard curve method according to the manufacturer's instructions. GAPDH was used as an internal loading control. All measurements were performed in triplicate.

Western blotting

Protein from KGN cells was extracted with the NucleoSpin RNA/Protein kit according to the manufacturer's instructions, and 10 μ g protein was separated by 7.5% SDS-PAGE and transferred onto Immobilon-P membrane (Millipore, Bedford, MA). Nonspecific binding was blocked with 5% nonfat milk in 0.1% Tris-buffered Tween-saline buffer for 1 h. Primary antibodies against GATA-4, Bcl-2, cyclin D2, β -actin, and flag epitope in DN adenovirus were incubated for 1 h at room temperature. After incubation with secondary antibody for 1 h at room temperature, proteins were visualized by the Enhanced Chemiluminescence Plus Kit (Amersham Biosciences Inc., Piscataway, NJ). β -Actin was used as a loading control.

Statistical analysis of the expression data

For correlations, the three categorical scoring results of the tissue microarray immunohistochemistry were combined into two: 1) expression reflecting the immunoreactivity level in normal granulosa cells, and 2) expression that is reduced from the normal immunoreactivity level (see *Results*). Contingency tabling (2×2) was performed for the resulting nominal variables, followed by the χ^2 or Fisher's exact test, if χ^2 was not appropriate. The results of quantitative mRNA analysis of tumor tissue

samples were analyzed with a simple regression model using Statview 5.0.1 software (SAS Institute Inc., Cary, NC) for Macintosh (Apple Computer, Inc., Cupertino, CA). Statistical comparison of luciferase assay experiments was performed using one-way ANOVA, followed by Dunnett's test, and the data from adenoviral experiments were analyzed using the Student's *t* test. $P < 0.05$ was considered significant in all experiments.

Results

GATA-4, Bcl-2, and cyclin D2 expression coincide in human granulosa cells from primary to the antral follicle stage

We first characterized the expression patterns in normal proliferating granulosa cells. As previously reported, GATA-4 immunoreactivity was constantly high in human granulosa cells from primary follicles through the antral stage (Fig. 1, A and B) (4). Similarly to GATA-4, immunoreactivity for Bcl-2 (Fig. 1, C and D) and cyclin D2 (Fig. 1, E and F) was high in both primary and small antral follicles. Based on these results, the cutoff levels for GATA-4, Bcl-2, and cyclin D2 expression were set between high and intermediate/low (*i.e.* groups of high and reduced expression

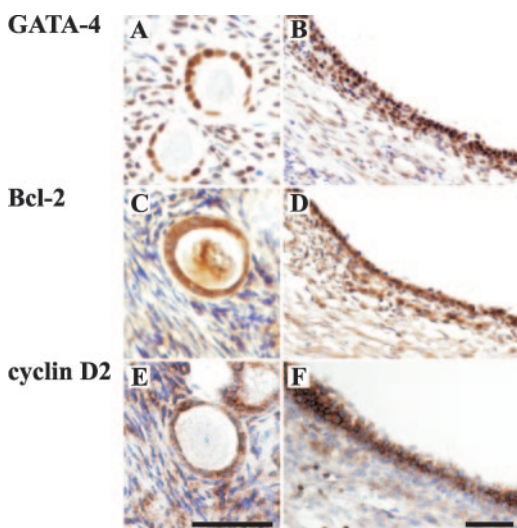


FIG. 1. Immunohistochemical analysis of GATA-4 (A and B), Bcl-2 (C and D), and cyclin D2 (E and F) in normal human proliferating granulosa cells. Expression of each antigen in primary follicles is shown in the left panels, and those in small antral follicles in the right panel. Brown color indicates positive staining. Note that all the examined antigens are expressed in the granulosa cells of developing follicles in a similar manner. Bars, 50 μ m.

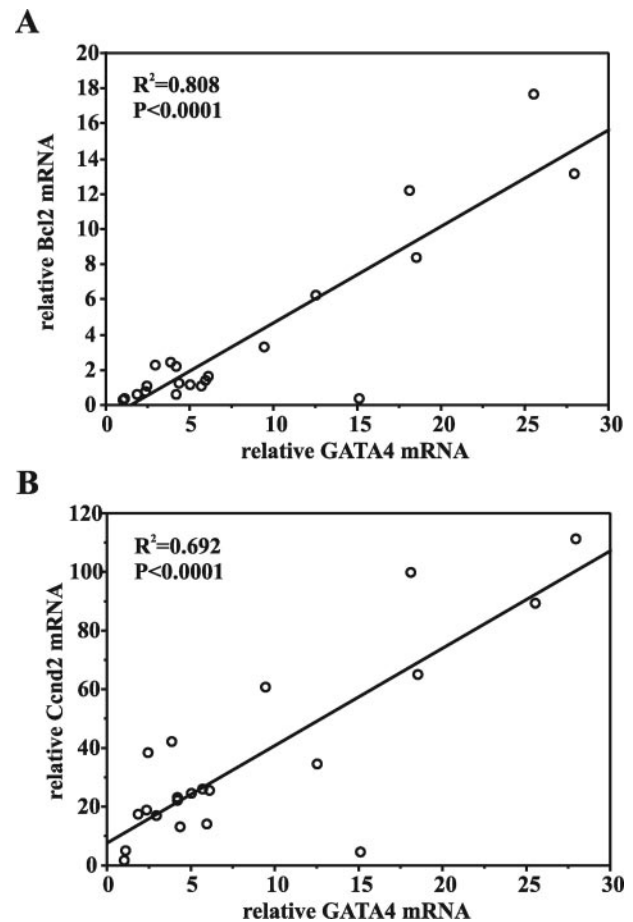


FIG. 2. Correlations of mRNA expression levels of GATA-4, Bcl-2, and cyclin D2 in 21 human GCTs. The relative levels of GATA-4 expression are depicted on the x-axis, and those of Bcl-2 (A) and cyclin D2 (B) on the y-axis of the regression plots. Relative mRNA levels were determined using quantitative real-time RT-PCR; the level of expression was normalized to GAPDH and presented relative to control normal ovary (defined as 1.0). The results were analyzed according to a simple regression model.

were formed) for subsequent immunohistochemical analyses of GCTs.

Expression profiles of Bcl-2 and cyclin D2 associate with GATA-4 in GCTs

Human GCTs have previously expressed GATA-4 and cyclin D2 mRNAs (8, 14, 16). We now confirmed these findings using quantitative real-time PCR on 21 primary GCTs, and found that the Bcl-2 gene was also expressed in human GCTs (Fig. 2). Relative to the mRNA levels in the reference normal ovary (defined as 1.0), cyclin D2 (mean 36.1, median 24.9, range 1.8–111.3) was strongly expressed, whereas the mRNA levels of GATA-4 (mean 8.5, median 5.0, range 1.0–27.9) and Bcl-2 (mean 3.8, median 1.4, range 0.3–17.7) were somewhat closer to the normal ovary. GATA-4 mRNA expression correlated with those of Bcl-2 and cyclin D2 ($P < 0.0001$ for both; Fig. 2, A and B).

Our previous studies have shown that 44% of the primary human GCTs maintain high GATA-4 protein expression, whereas 56% exhibit intermediate/low (*i.e.* reduced compared with normal) expression (4). We next analyzed GCTs of transgenic mice and found strong expression of GATA-4 (Fig. 3A), Bcl-2 (Fig. 3B), and cyclin D2 (Fig. 3C) proteins. In analyses on human GCT tissue array, Bcl-2 protein was found to be expressed at high levels in 52% of the tumors, 42% showed intermediate expression, and only 6% showed low expression (Fig. 3, E and H). Correspondingly, cyclin D2

protein expression was high in 42%, intermediate in 44%, and low in 14% of the tumors analyzed (Fig. 3, F and I). Similarly to mRNA expression, the protein expression of GATA-4 and Bcl-2, as well as GATA-4 and cyclin D2 associated with each other in human GCTs ($P = 0.014$ and $P < 0.001$, respectively) (Fig. 4, A and B). High GATA-4 expression was found in 58% of the tumors with high Bcl-2 expression and in 67% of the tumors with high cyclin D2 expression.

Bcl-2 expression associates with parameters of well-differentiated cancer

When correlating the protein expression data with the clinicopathological parameters, we found that cyclin D2 expression did not associate with any of the parameters studied. Although the Bcl-2 expression in the GCT tissue correlated with that of GATA-4, no association between Bcl-2 and clinical stage or recurrence risk was observed. Instead, we found that high Bcl-2 expression correlated with a low rate of atypia ($P = 0.004$; Fig. 4C), low MI ($P = 0.005$; Fig. 4D), low Ki67 expression ($P = 0.002$; Fig. 4E), and tumor size less than or equal to 10 cm ($P = 0.013$; Fig. 4F). Previously, neither of these parameters associated with GATA-4. Interestingly, Bcl-2 expression also associated with the differentiated tumor subtype and AMH expression ($P = 0.049$ for both). Similarly to Bcl-2, we have previously found that AMH expression correlates inversely with tumor size (4). In conclu-

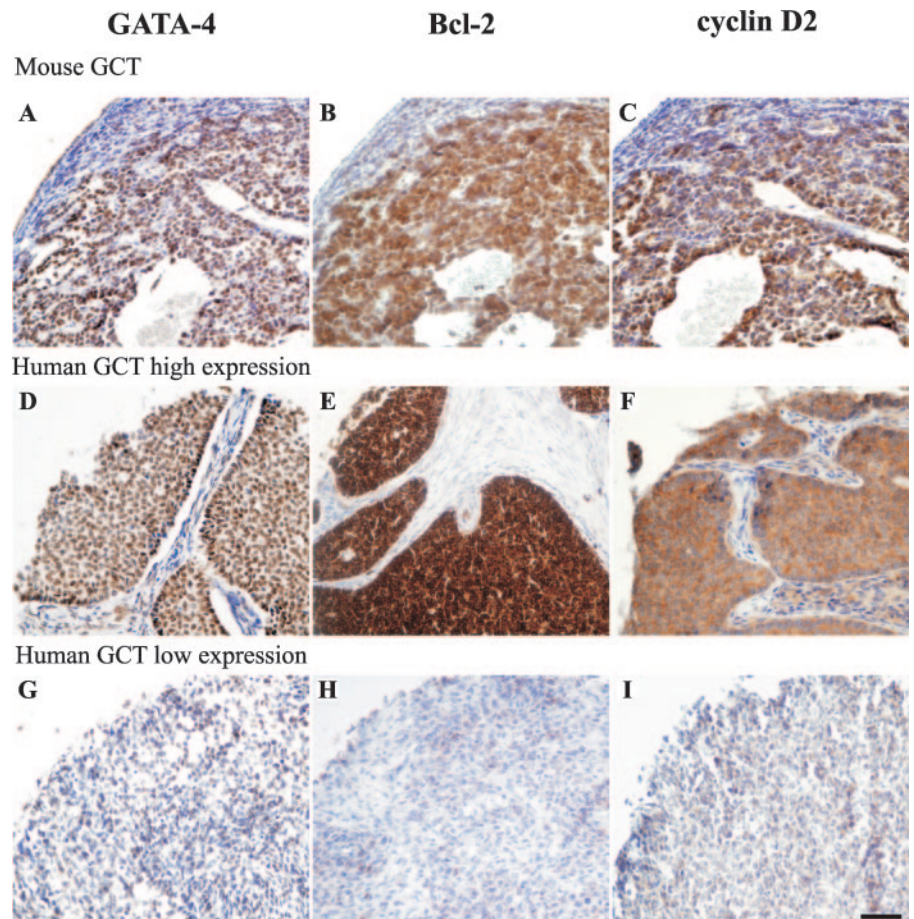


FIG. 3. Immunohistochemical analysis of GATA-4 (A, D, and G), Bcl-2 (B, E, and H), and cyclin D2 (C, F, and I) in mouse and human GCTs. All the studied proteins are highly expressed in GCT of 5-month-old transgenic mice (*upper row*). Examples of high and low expressions in human GCT are depicted in the *middle and the lowest row*, respectively. Brown indicates positive staining. Bars, 50 μ m.

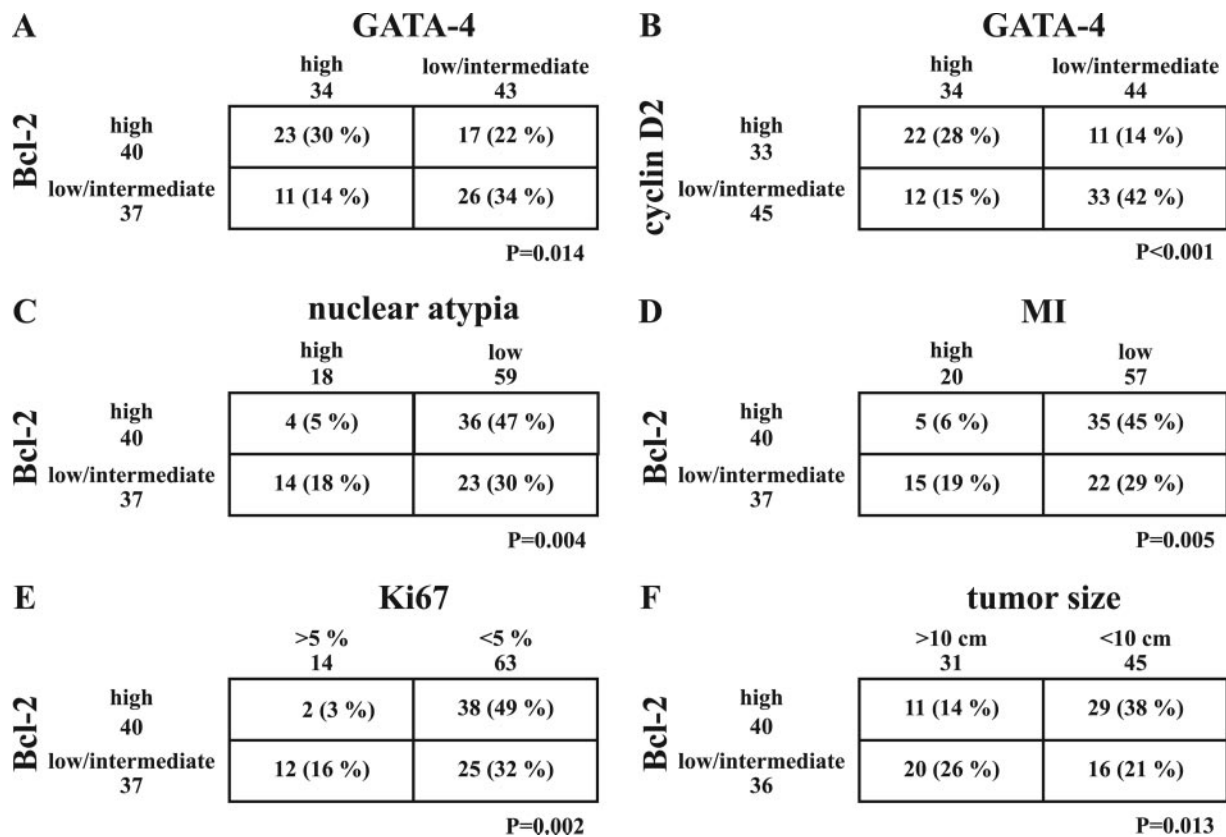


FIG. 4. Association of GATA-4, Bcl-2, and cyclin D2 expression with clinicopathological parameters in human GCT. A, GATA-4 and Bcl-2 expression associates in human GCTs (hGCTs) ($P = 0.014$; $n = 77$). B, GATA-4 and cyclin D2 expression associates in human GCTs ($P < 0.001$; $n = 78$). Bcl-2 expression inversely associates with nuclear atypia in human GCTs ($P = 0.004$; $n = 77$) (C), MI ($P = 0.005$; $n = 77$) (D), Ki67 ($P = 0.002$; $n = 77$) (E), and tumor size less than 10 cm ($P = 0.013$; $n = 76$) (F). A value of $P < 0.05$ was considered significant. Data are given as number and percentages of all the tumors in each subgroup.

sion, Bcl-2 expression associates with parameters that were previously connected to low-malignancy GCTs.

GATA-4 transactivates Bcl-2 and Ccnd2 promoters in COS-7 cells and KK-1 granulosa cells

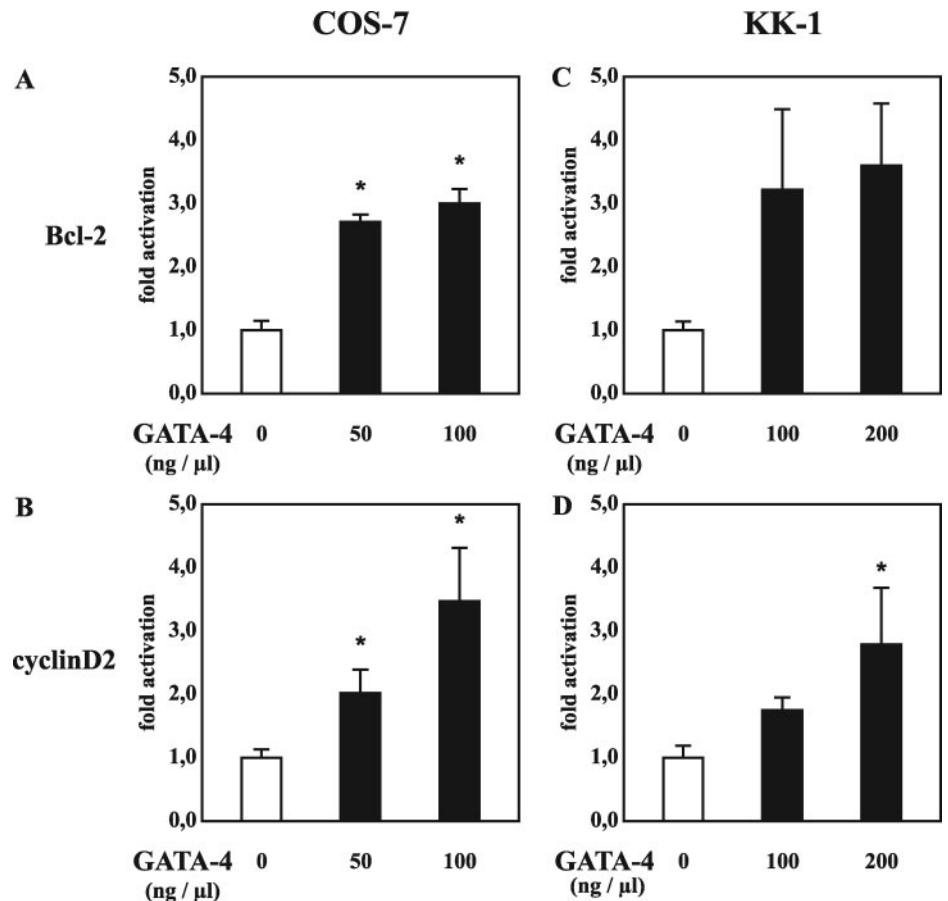
To examine the effect of GATA-4 on Bcl-2 and cyclin D2 gene transactivation *in vitro*, the -1281 -bp *bcl-2* gene promoter or the -680 -bp *Ccnd2* gene promoter was transfected into COS-7, KK-1, and KGN cell lines together with the GATA-4 expression plasmid. COS-7 cells did not endogenously express GATA-4 as found by Western blot and immunocytochemistry (data not shown), allowing us to explore directly the impact of GATA-4 on the activation of *Bcl2* and *Ccnd2* promoters in this cell line. In COS-7 cells, GATA-4 significantly dose dependently activated the *Bcl2* and the *Ccnd2* promoters (Fig. 5, A and B). Although the mouse granulosa tumor cell line KK-1 expresses GATA-4 endogenously, the overexpression of this transcription factor still transactivated Bcl-2 and cyclin D2 promoters up to 3.6- and 2.8-fold in these cells, respectively (Fig. 5, C and D; $P < 0.05$). Like KK-1, the KGN human granulosa tumor cells express GATA-4, as found by Western blot and immunocytochemistry (Fig. 6C) (data not shown). However, despite the use of multiple approaches, we were unable to transfect efficiently these cells with the promoter constructs and expression plasmids.

DN GATA-4 construct down-regulates whereas overexpression of GATA-4 up-regulates Bcl-2 in human KGN granulosa cells

To investigate further the role of GATA-4 in Bcl-2 and cyclin D2 regulation, KGN cells were transfected with adenovirus expressing GATA-4-engrailed fusion protein having DN GATA-4 activity (DnG4) (31). Transfecting KGN cells with DnG4 had no effect on cyclin D2 mRNA or protein levels, or on cell proliferation studied by 5-bromo-2-deoxyuridine assay (Fig. 6B) (data not shown). Instead, DnG4 was able to significantly down-regulate Bcl-2 mRNA compared with cells transfected with control adenovirus (Fig. 6A; $P < 0.05$). Notably, the GATA-4 DN approach also caused a clear down-regulation of Bcl-2 protein levels as analyzed by Western blot (Fig. 6A). However, disrupting GATA-4 function had no effect on KGN cell apoptosis as studied by caspase activity assay (data not shown).

Finally, we tested the effect of GATA-4 overexpression on Bcl-2 and cyclin D2 expression by transfecting KGN cells with an adenoviral GATA-4 construct. Up-regulation of GATA-4 protein was confirmed by Western blot (Fig. 6C). Bcl-2 mRNA was significantly up-regulated compared with cells transfected with control adenovirus, whereas Bcl-2 protein remained unchanged (Fig. 6C) (data not shown; $P < 0.05$). In contrast, no effect on cyclin D2 mRNA or protein levels was detected (Fig. 6D) (data not shown).

FIG. 5. GATA-4 transactivates Bcl-2 and Ccnd2 promoters in COS-7 and KK-1 cells. A and B, The COS-7 cells were transfected either with Bcl-2 (A) or cyclin D2 (B) promoter-luciferase reporter constructs. Cells were cotransfected with empty control vector or increasing amount (50, 100 ng) of GATA-4 expression plasmid. C and D, The KK-1 cells were transfected either with Bcl-2 (C) or cyclin D2 (D) promoter-luciferase reporter construct. Cells were cotransfected with empty control vector or increasing amount (100, 200 ng) of GATA-4 expression plasmid. For all experiments, luciferase activity is normalized to β -galactosidase activity and presented relative to control as the mean \pm SEM of three independent experiments performed in triplicate. *, $P < 0.05$.



Discussion

Based on the similarities in gene expression patterns and morphology, GCTs are thought to emerge from normal granulosa cells of the ovary (7). Both failure of apoptosis and excessive proliferation probably participate in the events leading to tumor formation. These processes are regulated by several transcription factors that are expressed in normal and in neoplastic granulosa cells (1). Among these, a member of the GATA transcription factors family, GATA-4, is highly expressed in normal ovarian follicles and GCTs (4, 8, 10). Our previous studies on 80 GCTs with clinical follow-up have shown that high GATA-4 expression correlates with a higher aggressiveness of tumors, suggesting that this transcription factor has a role in granulosa cell tumorigenesis and may serve as a prognostic marker (4).

Earlier studies have revealed that GATA-4 is able to transactivate antiapoptotic Bcl-2 and cell cycle regulator cyclin D2 in other cell types (17, 25). In this study we assessed the expression of GATA-4, Bcl-2, and cyclin D2 in normal proliferating granulosa cells and GCTs, and addressed the role of GATA-4 in the regulation of Bcl-2 and cyclin D2 in granulosa cells *in vitro*. GATA-4, Bcl-2, and cyclin D2 are expressed in human granulosa cells in primary and antral follicles (this study and Ref. 24). We now found that in human GCTs, the expression profile of Bcl-2 and cyclin D2 associates with that of GATA-4, suggesting a linkage between expression levels of these three genes also in granulosa cells.

Evidence for the functional association of GATA-4 and Bcl-2 was obtained from *in vitro* studies. Namely, GATA-4 transactivated the Bcl-2 promoter and up-regulated Bcl-2 expression, whereas inhibiting GATA-4 function down-regulated Bcl-2 levels in cultured granulosa tumor cells. This is in accordance with the binding of GST-GATA-4 fusion protein to a consensus GATA site of the *Bcl-2* gene promoter (25); this finding, using recombinant GATA-4 protein, is not cell type dependent. In many tissues, Bcl-2 is known to function as an antiapoptotic factor (32). However, down-regulation of Bcl-2 by inhibiting GATA-4 function did not induce apoptosis in granulosa tumor cells. This finding is in line with that by Aries *et al.* (33), who found that disturbing GATA-4 function by DN means in cardiomyocytes significantly increases apoptosis only in the presence of doxorubicin, an anticancer drug promoting apoptosis. On the other hand, the loss of antiapoptotic Bcl-2 may be compensated for by the concurrent loss of proapoptotic members of the Bcl-2 family.

An association between GATA-4 and cyclin D2 has been reported in pulmonary artery smooth muscle cells, where GATA-4 is able to activate cyclin D2 (17). In our GCT material, we found a correlation between GATA-4 and cyclin D2 expression suggesting an association between these factors also in granulosa cells. This link is further supported by experiments with granulosa tumor cells in which we provide new evidence that GATA-4 is able to transactivate the cyclin D2 promoter. However, the inhibition of GATA-4 function *in vitro* did not attenuate the basal expression of cyclin D2 in

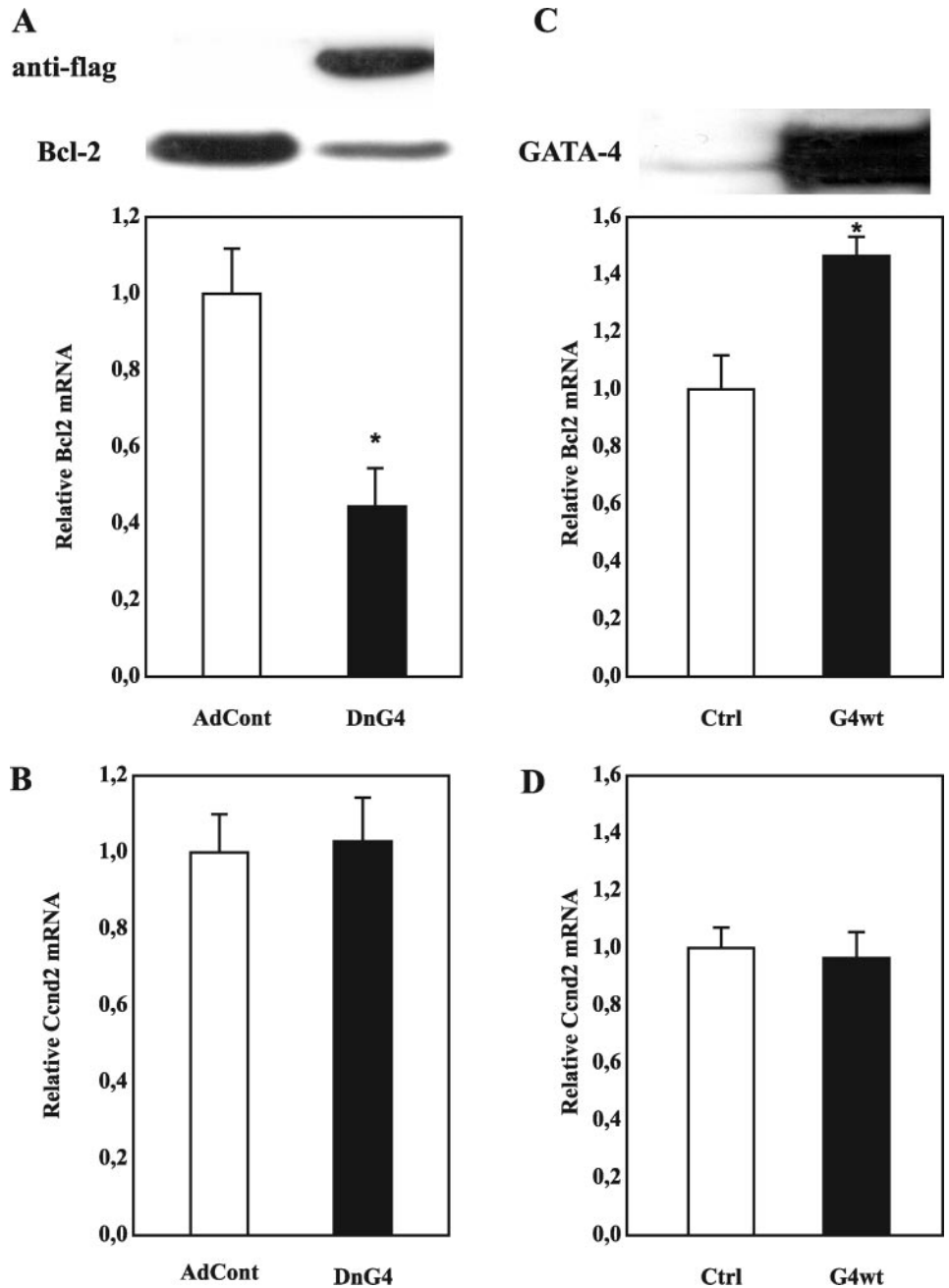


FIG. 6. Effect of DN and WT GATA-4 on Bcl-2 and cyclin D2 mRNA and protein. A and B, The KGN cells were transfected with adenovirus expressing DN GATA-4 or control adenovirus, and 48 h later, both mRNA and protein were collected. C and D, The KGN cells were transfected with GATA-4 overexpressing adenovirus or control adenovirus, and mRNA and protein were collected 96 h later. For all experiments the relative mRNA levels were determined using quantitative real-time RT-PCR. The level of expression was normalized to GAPDH and is presented relative to control (defined as 1.0) as the mean \pm SEM of three independent experiments, each performed in triplicate. *, $P < 0,05$. Protein levels of GATA-4, Bcl-2, and FLAG-tagged GATA-4 were determined using Western blot analysis. AdCont, Control adenovirus for DnG4 (adenovirus without fusion protein that disturbs GATA-4 function).

these cells. The inability of GATA-4 alone to regulate cyclin D2 may be due to other granulosa cell-specific transcription factors inhibiting the effects of GATA-4. Expression of cyclin D2 in granulosa cells is known to be tightly regulated by FOXO1 as well as activin-Smad3 signaling (18). Up-regulation of cyclin D2 requires detachment of the FOXO1 transcription factor from its promoter. The possible effects of FOXO1 and the previously reported GATA-4/Smad3 cooperation (12) were not assessed in our study, and this or other cofactors may well explain why the inhibition of GATA-4 only, had no effect on the basal expression of cyclin D2. The interplay between GATA-4 and cooperating factors, *e.g.* Smad proteins, as well as the effects of relevant signaling

cascades, *e.g.* activin and FSH pathways, on GATA mediated transcription in granulosa cells remains to be elucidated.

The results for cyclin D2 expression in our large GCT series are in line with previous reports implicating a role for this factor in the pathogenesis of GCTs (1, 14, 16, 34). Although we now find that cyclin D2 mRNA levels are usually high in GCTs, and that only a few human GCTs exhibited low-cyclin D2 protein expression, we were unable to find a correlation between cyclin D2 and clinicopathological parameters. Importantly, we find that Bcl-2 is expressed in human granulosa cell pathobiology. High Bcl-2 expression associated with parameters typical for well-differentiated can-

cer or favorable clinical behavior. The connection between GATA-4 and Bcl-2 remains somewhat ambiguous as to the indicators of adverse clinical behavior, *i.e.* the association of GATA-4 with clinical stage or recurrence rate (4). Nevertheless, the present and previous *in vitro* results indicate that GATA-4 participates in the regulation of ovarian Bcl-2 gene expression.

Our present findings shed further light on the pathophysiological cascades important in GCTs. We herein demonstrate that Bcl-2 and cyclin D2 colocalize with GATA-4 in normal and neoplastic human granulosa cells. Although GATA-4 was able to transactivate *Ccnd2*, the inhibition of this transcription factor in the granulosa cells did not affect the expression of cyclin D2 in these cells. Conversely, GATA-4 was functionally linked to Bcl-2 and is likely to be an important regulator of this antiapoptotic factor both in normal granulosa cells as well as in GCTs.

Acknowledgments

We thank Drs. Yoshihiro Nishi and Toshihiko Yanase for providing the KGN cell line, and Drs. Jeffery Molkentin, David Wilson, Mary Hunziker-Dunn, and Qiangrong Liang for providing the plasmid and adenoviral constructs. We also thank Ms. Taru Jokinen for excellent technical assistance.

Received February 1, 2008. Accepted July 15, 2008.

Address all correspondence and requests for reprints to: Markku Heikinheimo, M.D., Ph.D., Children's Hospital, University of Helsinki, P.O. Box 22 (Stenbäckinkatu 11), 00014 Helsinki, Finland. E-mail: markku.heikinheimo@helsinki.fi.

This work was supported by the Finnish Pediatric Research Foundation (to A.K. and M.H.), Finnish Cancer Organizations (to R.B. and M.H.), Helsinki University Central Hospital Research Funds (to L.U.-K., R.B., M.H., and M.A.), Sigrid Juselius Foundation (to M.H.), Jalmari and Rauha Ahokas Foundation (to M.A.), and Emil Aaltonen Foundation (to M.A.).

Disclosure Statement: The authors have nothing to disclose.

References

- Fuller PJ, Chu S 2004 Signalling pathways in the molecular pathogenesis of ovarian granulosa cell tumours. *Trends Endocrinol Metab* 15:122–128
- Schumer ST, Cannistra SA 2003 Granulosa cell tumor of the ovary. *J Clin Oncol* 21:1180–1189
- East N, Alobaid A, Goffin F, Ouallouche K, Gauthier P 2005 Granulosa cell tumour: a recurrence 40 years after initial diagnosis. *J Obstet Gynaecol Can* 27:363–364
- Anttonen M, Unkila-Kallio L, Leminen A, Butzow R, Heikinheimo M 2005 High GATA-4 expression associates with aggressive behavior, whereas low anti-Müllerian hormone expression associates with growth potential of ovarian granulosa cell tumors. *J Clin Endocrinol Metab* 90:6529–6535
- Robertson DM, Pruyers E, Jobling T 2007 Inhibin as a diagnostic marker for ovarian cancer. *Cancer Lett* 249:14–17
- La Marca A, Volpe A 2007 The anti-Müllerian hormone and ovarian cancer. *Hum Reprod Update* 13:265–273
- Fuller PJ, Chu S, Fikret S, Burger HG 2002 Molecular pathogenesis of granulosa cell tumours. *Mol Cell Endocrinol* 191:89–96
- Laitinen MP, Anttonen M, Ketola I, Wilson DB, Ritvos O, Butzow R, Heikinheimo M 2000 Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. *J Clin Endocrinol Metab* 85:3476–3483
- Anttonen M, Ketola I, Parviainen H, Pusa AK, Heikinheimo M 2003 FOG-2 and GATA-4 are coexpressed in the mouse ovary and can modulate müllerian-inhibiting substance expression. *Biol Reprod* 68:1333–1340
- Heikinheimo M, Ermolaeva M, Bielinska M, Rahman NA, Narita N, Huhtaniemi IT, Tapanainen JS, Wilson DB 1997 Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* 138:3505–3514
- Tremblay JJ, Viger RS 2003 Transcription factor GATA-4 is activated by phosphorylation of serine 261 via the cAMP/protein kinase signaling pathway in gonadal cells. *J Biol Chem* 278:22128–22135
- Anttonen M, Parviainen H, Kyrönlahti A, Bielinska M, Wilson DB, Ritvos O, Heikinheimo M 2006 GATA-4 is a granulosa cell factor employed in inhibin- α activation by the TGF- β pathway. *J Mol Endocrinol* 36:557–568
- Kwintkiewicz J, Cai Z, Stocco C 2007 Follicle-stimulating hormone-induced activation of Gata4 contributes in the up-regulation of Cyp19 expression in rat granulosa cells. *Mol Endocrinol* 21:933–947
- Chu S, Rushdi S, Zumpe ET, Mamers P, Healy DL, Jobling T, Burger HG, Fuller PJ 2002 FSH-regulated gene expression profiles in ovarian tumours and normal ovaries. *Mol Hum Reprod* 8:426–433
- Sherr CJ 1995 D-type cyclins. *Trends Biochem Sci* 20:187–190
- Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ, Weinberg RA 1996 Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 384:470–474
- Suzuki YJ, Day RM, Tan CC, Sandven TH, Liang Q, Molkentin JD, Fanburg BL 2003 Activation of GATA-4 by serotonin in pulmonary artery smooth muscle cells. *J Biol Chem* 278:17525–17531
- Park Y, Maizels ET, Feiger ZJ, Alam H, Peters CA, Woodruff TK, Unterman TG, Lee EJ, Jameson JL, Hunzicker-Dunn M 2005 Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. *J Biol Chem* 280:9135–9148
- Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ 1991 Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 129:2799–2801
- Pru JK, Tilly JL 2001 Programmed cell death in the ovary: insights and future prospects using genetic technologies. *Mol Endocrinol* 15:845–853
- Vaskivuo TE, Tapanainen JS 2003 Apoptosis in the human ovary. *Reprod Biomed Online* 6:24–35
- Hsu SY, Lai RJ, Finegold M, Hsueh AJ 1996 Targeted overexpression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. *Endocrinology* 137:4837–4843
- Ratts VS, Flaws JA, Kolp R, Sorenson CM, Tilly JL 1995 Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology* 136:3665–3668
- Vaskivuo TE, Anttonen M, Herva R, Billig H, Dorland M, te Velde ER, Stenback F, Heikinheimo M, Tapanainen JS 2001 Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins, and transcription factor GATA-4. *J Clin Endocrinol Metab* 86:3421–3429
- Kobayashi S, Lackey T, Huang Y, Bisping E, Pu WT, Boxer LM, Liang Q 2006 Transcription factor gata4 regulates cardiac BCL2 gene expression in vitro and in vivo. *FASEB J* 20:800–802
- Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP 1998 Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4:844–847
- Kananen K, Markkula M, Rainio E, Su JG, Hsueh AJ, Huhtaniemi IT 1995 Gonadal tumorigenesis in transgenic mice bearing the mouse inhibin α -subunit promoter/simian virus T-antigen fusion gene: characterization of ovarian tumors and establishment of gonadotropin-responsive granulosa cell lines. *Mol Endocrinol* 9:616–627
- Nishi Y, Yanase T, Mu Y, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K, Takayanagi R, Kashimura Y, Haji M, Nawata H 2001 Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. *Endocrinology* 142:437–445
- Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB 1993 Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol* 13:2235–2246
- Charron F, Tsimiklis G, Arcand M, Robitaille L, Liang Q, Molkentin JD, Meloche S, Nemer M 2001 Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev* 15:2702–2719
- Liang Q, De Windt LJ, Witt SA, Kimball TR, Markham BE, Molkentin JD 2001 The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. *J Biol Chem* 276:30245–30253
- Borner C 2003 The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. *Mol Immunol* 39:615–647
- Aries A, Paradis P, Lefebvre C, Schwartz RJ, Nemer M 2004 Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. *Proc Natl Acad Sci USA* 101:6975–6980
- Burns KH, Agno JE, Sicinski P, Matzuk MM 2003 Cyclin D2 and p27 are tissue-specific regulators of tumorigenesis in inhibin α knockout mice. *Mol Endocrinol* 17:2053–2069