YAP regulates cell proliferation, migration, and steroidogenesis in adult granulosa cell tumors

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Abstract

The Hippo signaling pathway has been implicated as a conserved regulator of organ size in both *Drosophila* and mammals. Yes-associated protein (YAP), the central component of the Hippo signaling cascade, functions as an oncogene in several malignancies. Ovarian granulosa cell tumors (GCT) are characterized by enlargement of the ovary, excess production of estrogen, a high frequency of recurrence, and the potential for malignancy and metastasis. Whether the Hippo pathway plays a role in the pathogenesis of GCT is unknown. This study was conducted to examine the expression of YAP in human adult GCTs and to determine the role of YAP in the proliferation and steroidogenesis of GCT cells. Compared with age-matched normal human ovaries, GCT tissues exhibited higher levels of YAP expression. YAP protein was predominantly expressed in the nucleus of tumor cells, whereas the non-tumor ovarian stromal cells expressed very low levels of YAP. YAP was also expressed in cultured primary human granulosa cells and in KGN and COV434 GCT cell lines. siRNA-mediated knockdown of YAP in KGN cells resulted in a significant reduction in cell proliferation ($P < 0.001$). Conversely, overexpression of wild type YAP or a constitutively active YAP (YAP1) mutant resulted in a significant increase in KGN cell proliferation and migration. Moreover, YAP knockdown reduced FSH-induced aromatase (CYP19A1) protein expression and estrogen production in KGN cells. These results demonstrate that YAP plays an important role in the regulation of GCT cell proliferation, migration, and steroidogenesis. Targeting the Hippo/YAP pathway may provide a novel therapeutic approach for GCT.

Key Words

- Hippo/YAP pathway
- granulosa cell tumor
- cell proliferation
- steroidogenesis
- migration

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Introduction

The balance between cell growth and death plays a vital role in the maintenance of tissue homeostasis, organ size, and normal biological functions of the human body. Under physiological conditions, cell growth and apoptosis

are tightly controlled. Aged or damaged cells commit to programmed cell death, whereas adult stem cells may divide and differentiate to replace those dysfunctional cells to maintain tissue homeostasis and organ size.

Under pathological conditions, however, uncontrolled cell proliferation and decreased cell death and/or differentiation can lead to hyperplasia or even tumorigenesis. The mechanisms underlying the control of organ size are still largely unknown. Studies have shown that the Hippo signaling pathway (Salvador/Warts/Hippo pathway) plays a critical role in controlling organ size by regulating both cell proliferation and apoptosis in both *Drosophila* and mammals (Huang *et al.* 2005, Dong *et al.* 2007, Yu & Guan 2013).

Yes-associated protein 1 (YAP1 also known as YAP) is the major downstream effector of the Hippo pathway (Harvey *et al.* 2013). The expression and role of YAP in cancer are cell-type- and/or cellular-context-dependent (Strano *et al.* 2005, Harvey *et al.* 2013). Amplification of the YAP gene locus at 11q22 is found in hepatocellular carcinoma, breast cancer, oral squamous cell carcinomas, medulloblastomas, and esophageal squamous cell carcinomas (Snijders *et al.* 2005, Overholtzer *et al.* 2006, Zender *et al.* 2006, Fernandez *et al.* 2009, Muramatsu *et al.* 2011). In addition, overexpression and nuclear localization of YAP protein are found in colon, liver, lung, ovarian, and prostate cancers (Zender *et al.* 2006, Zhao *et al.* 2007, Steinhardt *et al.* 2008, Yu & Guan 2013). Moreover, overexpression of YAP leads to oncogenic transformation of an immortalized epithelial cell line MCF10A (Overholtzer *et al.* 2006). However, YAP enhances p73-dependent cell death during cisplatin-induced DNA damage (Strano *et al.* 2005, 2013), indicating that YAP may interact with other pathways to optimize a tumor suppressor response. In a subset of breast cancers, the expression of YAP protein was significantly decreased due to loss of heterozygosity, and short hairpin RNA (shRNA) knockdown of YAP increased migration and invasiveness, and enhanced tumor growth (Yuan *et al.* 2008). Therefore, the expression and function of YAP in specific cancers requires further investigation.

Granulosa cell tumors of ovary (GCTs), accounting for ~70% of malignant sex-cord stromal tumors and 5–8% of all ovarian malignancies, are poorly understood ovarian neoplasms (Jamieson & Fuller 2012). Patients with GCTs have a high overall survival rate, which is mainly attributed to diagnosis of GCT at an early stage. The prognosis is significantly poorer for patients with advanced tumors. The 10-year survival rates in stage III and IV are lower than 20% (Sehouli *et al.* 2004). Clinically, GCTs have a propensity for late recurrence (Crew *et al.* 2005, Villella *et al.* 2007). Approximately, 80% of patients with advanced stage or recurrent tumors succumb to their disease due to the limited treatment options for advanced and recurrent disease (Amsterdam & Selvaraj 1997, Jamieson & Fuller 2012). These tumors also have

malignant potential and metastatic ability. Indeed, metastases from GCTs have been reported in the lung, liver, brain, bone, diaphragm, abdominal wall, pancreas, and adrenal gland (Yamagami *et al.* 2012).

Some factors and pathways have been shown to affect the development of GCT (reviewed in Jamieson & Fuller (2012)). Importantly, recent studies have shown that a somatic mutation in FOXL2 (C402G) is a potential driver in the pathogenesis of adult-type GCTs (Shah *et al.* 2009, Jamieson *et al.* 2010, Al-Agha *et al.* 2011, Jamieson *et al.* 2012, Rosario *et al.* 2012, Benayoun *et al.* 2013, Georges *et al.* 2013). However, the exact mechanisms underlying GCT progression, recurrence, and metastasis are largely unknown. Oftentimes, GCTs are hemorrhagic and manifest as painful abdominal masses, with an average diameter more than 10 cm (Young *et al.* 1984, Aboud 1997, Ko *et al.* 1999, Sehouli *et al.* 2004). Occasionally, the diameter of the GCT tumor can be over 30 cm (Stenwig *et al.* 1979, Ohel *et al.* 1983, Aboud 1997, Cronje *et al.* 1999). This volume is several hundredfold larger than the regular human ovary, which is around 5 cm³. The significant increase in the volume of the ovary indicates that the system controlling organ size in the patients with GCTs is disrupted. As GCTs are derived from ovarian granulosa cells, the proliferation of granulosa cells in the patient's ovary may be deregulated. However, the molecular pathology underlying this remarkable increase in tissue size is unknown.

In this study, we compare YAP expression in normal human ovarian tissues and GCT tissues using immunohistochemistry. We also examine the role of YAP in the regulation of GCT cell proliferation, migration, and steroidogenesis using the KGN human GCT cell line as a cellular model. Our results indicate that the Hippo/YAP signaling pathway plays a critical role in the regulation of GCT progression.

Materials and methods

Chemicals

Human FSH was from NHPP/NIDDK (Torrance, CA, USA). DMEM and other cell culture medium were from Invitrogen. FBS was from Atlanta Biologicals, Inc. (Lawrenceville, GA, USA). The Ribogreen RNA quantification kit and Alexa-conjugated secondary antibodies were from Life Technologies Corp.; YAP and phospho-YAP (ser127) antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The aromatase antibody was purchased from Serotec (Oxford, UK). Antibodies against

β -actin and β -tubulin were from Sigma–Aldrich. YAP small interfering RNA (siRNA) was from Dharmacon/Thermo Scientific (Pittsburgh, PA, USA). Secondary antibodies for western blotting chemiluminescence were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA); PCR reagents were from Invitrogen, Qiagen or Bio-Rad. All other molecular-grade chemicals were purchased from Sigma, Fisher (Pittsburgh, PA, USA), or United States Biochemical (Cleveland, OH, USA).

Cell lines and human GCT tissue slides

The KGN cell line, an adult GCT cell line expressing a mutated *FOXL2* (C402G) gene, was obtained from the Riken Biosource Center (Riken Cell Bank, Ibaraki, Japan). The COV434 cell line, a juvenile GCT cell line expressing WT *FOXL2* gene, was received from Dr C E van der Minne (University Hospital, Leiden, The Netherlands). The SKOV-3 ovarian cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The IGROV-1 ovarian cancer cell was received from Dr Bo R Reuda (Massachusetts General Hospital, MA, USA). Human ovarian granulosa cells isolated from two medium-sized follicles (5–10 mm in diameter) were obtained from a 33-year-old patient who received oophorectomy for causes other than an ovarian disorder. The collection of this tissue was permitted by a protocol approved by the University of Nebraska Medical Center Institutional Review Board. The cells were isolated manually with a needle and cultured in DMEM supplemented with 5% FBS. All cell lines used in this study were passaged fewer than ten times in our laboratories and were validated for their authenticity by short tandem repeat (STR) analysis. Formalin-fixed, paraffin-embedded normal human ovarian tissues ($n=10$) and human GCT ($n=12$) slides were obtained from the Department of Pathology, Tianjin Medical University Cancer Hospital and UNMC. The retrospective use of these human tissue slides was permitted by protocols approved by the UNMC Institutional Review Board and Tianjin Medical University Institutional Review Board.

The KGN GCT cells were derived from a patient with recurrent, metastasized GCT in the pelvic region (Nishi *et al.* 2001). These cells maintain many features of ovarian granulosa cells such as expression of the FSH receptor and induction of aromatase (CYP19A1) and production of estrogen in response to FSH (Nishi *et al.* 2001). To date, to our knowledge, the KGN cell line is the only appropriate cellular model for studying the growth and metastasis of the adult GCTs (Imai *et al.* 2008, Jamieson *et al.* 2010, Jamieson & Fuller 2012). In this study, we examined the

role of YAP in the proliferation and migration of KGN cells. The KGN cells used in these experiments were from passages 6 to 10. The cell line was validated by STR polymorphism analysis performed by both the Riken Biosource Center (Riken Cell Bank) and the Genetica DNA Laboratories (Burlington, NC, USA).

Immunohistochemistry analysis of YAP expression in ovarian tissues

The expression of YAP protein in paraffin-embedded human ovarian tissues was detected using a previously described peroxidase-based immunohistochemistry protocol (Wang *et al.* 2012a). Immunosignals were visualized with a 3,3'-diaminobenzidine (DAB) kit (Invitrogen). The sections were counterstained with Mayer's hematoxylin. In case of negative controls, the primary antibody was replaced by blocking buffer containing the same amount of IgG from non-immune rabbit serum. The sections were scanned with an iSCAN Coreo Slide Scanner (Ventana Medical Systems, Inc., Oro Valley, AZ, USA). The positivity (i.e., the number of positively stained cells relative to the total number of cells in the tissue section) and the intensity of the positive immunosignals were quantified with Aperio ImageScope software (Vista, CA, USA).

Localization of YAP protein in KGN cells by fluorescent immunocytochemistry

KGN cells were seeded onto glass coverslips and incubated in a growth medium (DMEM-F12 supplemented with 5% FCS) for 36 h before fixation in ice-cold 4% paraformaldehyde for 10 min. Staining was performed using methods described previously (Wang *et al.* 2012a). The fixed cells were incubated with YAP antibody (1:100) overnight at 4 °C in a humidified chamber. Antigens were visualized by applying Alexa-488-conjugated donkey anti-rabbit secondary antibodies. Actin filaments were stained with rhodamin-conjugated phalloidin and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The images were captured and analyzed with an LSM 710 confocal microscope (Thornwood, NY, USA). The exposure time of the camera was set for subtracting background fluorescence that was present in the sections incubated with the nonimmune IgG of the host species. YAP-specific fluorescence signals (immunosignal) were merged with the nuclear and actin signals to determine the sub-cellular site of protein expression.

YAP mRNA expression

YAP mRNA expression in GCT cell lines was detected with RT-PCR as described previously (Wang et al. 2012a). Total RNA was isolated from primary cultures of normal human granulosa cells, cultured GCT cell lines (KGN cells and COV434 cells), and epithelial ovarian cancer (EOC) cells (SKOV-3, CAOV-3, and IGROV-1 cells) using the RNeasy mini kit (Qiagen, Inc.) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 µg total RNA using iScript cDNA synthesis kit (Bio-Rad). The sequences of primers for YAP were forward, 5'-CAACTCCAACCAGCAGCAACA-3'; reverse: 5'-GCAGCCTCTCCTTCTCCATC TG-3'. The sequences of primers for GAPDH (used as a loading control) were forward: 5'-TGCACCACCAACTGCTTAGC-3'; reverse: 5'-GGCATG-GACTGTGGTCATGAG-3'. PCR products were loaded onto an agarose gel and separated by electrophoresis. The images were captured by a UVP gel documentation system (UVP, Upland, CA, USA). The PCR products were validated by sequence analysis.

Western blot analysis

Western blot was performed as described previously (Wang et al. 2012b). Briefly, normal cell lines or treated KGN cells were harvested on ice with ice-cold cell lysis buffer containing 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate and protease and phosphatase inhibitor cocktails. Proteins (30 µg) were loaded onto a 10% SDS-PAGE gel, separated by electrophoresis, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA and probed with appropriate primary and HRP-conjugated secondary antibodies. The immunosignal was detected with a Thermo Scientific SuperSignal West Femto Chemiluminescent Substrate Kit. The images were captured and analyzed with a UVP gel documentation system.

Establishment of KGN cell lines expressing WT and mutant YAP protein

KGN cells were cultured to 40% confluence and then transfected with retrovirus-based human YAP expression constructs. The characteristics and use of these vectors have been reported previously (Dong et al. 2007). Two days following transfection, cells were selected with G-418 (400 µg/ml) for 7 days. Three stable cell lines were

established: i) the KGN-MXIV control cell line was transfected with the control vector MXIV and expresses endogenous YAP; ii) the KGN-YAP cell line overexpresses wild-type YAP protein; and iii) the KGN-YAP^{S127A} cell line expresses a constitutively activated YAP mutant. Mutation of YAP protein (serine to alanine at residue 127) prevents YAP phosphorylation, leading to its nuclear localization and constitutive activation.

Cell proliferation assay

To determine the effect of YAP on GCT cell proliferation, KGN cells were plated in 60 mm cell culture dishes and incubated in a growth medium supplemented with 5% FBS until 60% confluent. The cells were then transfected with siGLO (a cy5-labeled nontargeting siRNA as control) or YAP siRNA for 6 h using METAFECTENE (Biontexas-USA, San Diego, CA, USA) according to the manufacturer's instructions. The cells were harvested 72 h after siRNA transfection for determination of protein levels or cell numbers. YAP protein expression was determined by western blot analysis and the cell numbers were quantified with an Invitrogen Countess automated cell counter.

The effect of YAP on GCT cell proliferation was also determined in KGN cell lines that overexpressed the WT or mutant YAP proteins. Each cell line was cultured in the growth medium containing 10% FBS for up to 3 days. Cell number and cell size for each cell line were determined daily with an Invitrogen Countess Automated cell counter.

Cell viability analysis

Experiments were performed to determine whether alterations in YAP expression were associated with changes in cell viability. KGN cells transfected with scramble siRNA (siGLO) or YAP siRNA were cultured for 96 h before performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a Vybrant MTT Assay Kit (Life Technologies) according to the manufacturer's instructions. In another experiment, KGN-MXIV cells (control cell line expressing endogenous YAP), KGN-YAP cells (KGN cells overexpressing WT YAP), and KGN-YAP^{S127A} cells (KGN cells expressing mutant YAP (S127A) as described in the text) were cultured for 48 h before cell viability was measured with the Vybrant MTT Assay Kit according to the manufacturer's instructions.

Cell-cycle and apoptosis analysis was performed by flow cytometry. KGN cells were transfected with scramble

siRNA (siGLO) or YAP siRNA for 6 h and then cultured for 96 h before analysis. Briefly, cells were trypsinized, fixed, and permeabilized with 70% ethanol overnight at -20°C . The cells were then labeled with propidium iodide for 30 min at 37°C and flow cytometry was used to determine the cell-cycle distribution of the KGN cells.

Apoptosis was also analyzed using cell surface expression of Annexin V. KGN cells were transfected with scramble siRNA (siGLO) or YAP siRNA for 6 h and then cultured for 96 h before determining the extent of apoptosis using the Annexin V-FITC Apoptosis Assay Kit as described by the manufacturer (BioVision, Inc., Milpitas, CA, USA). The cells were trypsinized and incubated for 5 min at room temperature with Annexin V-FITC and propidium iodide before analysis by flow cytometry.

Cell migration assay

A chemotaxis assay was used to determine the effect of YAP on KGN cell migration. KGN cells (4×10^5) in 250 μl of serum-free DMEM were placed in a Transwell insert (8 μm pore size, Corning-Costar, Lowell, MA, USA). The inserts were then placed in the wells of a 24-well-plate containing 750 μl of DMEM-FBS (5%) and incubated at 37°C for 6 h. After incubation, the cells on the top of the membrane were removed with a cotton swab. The cells which had migrated to the underside of the membrane were fixed and stained with 0.04% crystal violet in methanol for 30 min. The cells were then photographed (100 $\times$ magnification) and ten pictures per group were quantified under a microscope.

The wound-healing assay was also used to determine whether YAP regulates KGN cell motility. KGN cells were cultured in six-well cell plates until confluent. Wounds were made by scratching the cell layer with a 100 μl pipette tip. After washing away the cell debris, pictures were taken for each 'wound' with an Olympus inverted microscope equipped with a DP71 digital camera (Olympus America, Inc., Center Valley, PA, USA). The cells were incubated in serum-free medium for 20 h and then another picture was taken for each 'wound'.

17 β -estradiol ELISA

KGN cells were cultured in 24-well plates with androstenedione (5 μM) and treated as described in the figure legends. The conditioned media were collected for 17 β -estradiol determination using an estradiol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated at least three times unless otherwise noted. Data were analyzed for significance by one-way ANOVA with Tukey's post-test. A value of $P < 0.05$ was considered to be significant. Statistical analysis was conducted using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Expression of YAP protein in GCTs

Adult GCTs are commonly identified in women during the perimenopausal period (Jamieson & Fuller 2012). The expression of YAP in the normal human ovary and GCTs was detected by immunohistochemistry. In control perimenopausal ovarian sections, the main cellular component and the stroma, exhibited very little or no staining for YAP (Fig. 1A, C, and E). In control ovarian sections, few small follicles or luteinized structures were observed. When follicles were present, YAP immunosignals were detected in granulosa cells and were distributed in both nucleus and cytoplasm (too few follicles, data not shown). When luteinized cells were present, YAP was predominantly localized to the cytoplasm (Fig. 1C and E). Compared with the control ovarian tissues, YAP immunosignals were significantly increased ($P < 0.001$) in the GCT tumor tissues and the protein was predominantly located in the nucleus of tumor cells (Fig. 1B, D, and F). Nontumor stromal cells had low levels of YAP expression. Immunostaining controls revealed no non-specific staining in GCT tissues (Fig. 1G). The intensities of YAP immunostaining signals were quantified with Aperio imagescope software (Aperio). The data showed that both YAP signal intensity and YAP positivity (percentage of the YAP positive cells relative to the total number of cells per section) in the ovarian GCT tissues were significantly higher than the signals in the normal ovarian tissues (Fig. 1H, $P < 0.001$).

Expression of YAP in ovarian GCT tumor cell lines

As mentioned earlier, the KGN cell is a suitable cellular model for the GCT research. Fluorescent immunocytochemistry showed that YAP is expressed in KGN cells (Fig. 2A). Most of the immunosignal was localized to the nucleus. Interestingly, the YAP immunosignal was also localized to punctate regions in the periphery of the cells that co-localized with β -actin (Fig. 2A). It is very possible that YAP may interact with the focal adhesion proteins.

Figure 1

YAP is expressed in human granulosa cell tumors (GCTs). Immunohistochemistry was used to examine the expression of YAP in normal ovarian tissues (A, C, and E) and human GCT tissues (B, D, and F). YAP staining is indicated in brown. The counter stain is hematoxylin, shown in blue.

(A) Whole-tissue section scan of a representative normal ovary stained with YAP antibody; (B) scanned image of partial ovarian GCT tissue stained with YAP. Amplified representative images of normal ovarian tissues (C) and GCT tissues (D) stained with YAP antibody to show cell-type-specific YAP expression. Representative high-resolution images were also presented to show the subcellular localization of YAP in the normal ovarian tissues (E)

Antibody controls using the same amount of IgG to replace the primary YAP antibody were not stained (Fig. 2A, lower panel).

RT-PCR and western blot analysis showed that YAP mRNA and YAP protein were expressed in primary cultures of human granulosa cells, KGN and COV434 GCT cell lines (Fig. 2B and C). YAP RNA and YAP protein were also expressed in three ovarian epithelial cancer cell lines (Fig. 2B and C). IGROV-1 cells had lower expression of YAP compared with SKOV-3 and CAOV-3 cells. It is well known that YAP protein activity is highly regulated by phosphorylation (Dong *et al.* 2007). Western blot analysis revealed that phospho (S127)-YAP was present in each of the cell lines. Our results also showed that compared with the KGN cells YAP was less phosphorylated in COV434 cells (Fig. 2B and C), a juvenile GCT cell line (Jamieson *et al.* 2010, Jamieson & Fuller 2012).

Knockdown of YAP expression in KGN cells inhibits cell proliferation

To explore the role of YAP in GCT cell proliferation, we transiently transfected KGN cells with two YAP siRNAs to knockdown YAP expression. Cy5-labeled scrambled siRNA

and GCT tissues (F). Tissue sections probed with the same amount of nonimmunized rabbit IgG were used as antibody control (G).

(H) Quantitative results of DAB intensity were also presented to show the difference of positivity (percentage of the number of DAB positive cells relative to number of total cells in each tissue section) and relative intensity of YAP immunosignal between normal human ovarian tissues (CTRL) and tumor tissues (GCT). Each bar represents the mean $\pm$ S.E.M. Significantly different ($*P < 0.001$) from the control (CTRL) groups. Scale bars = 2 mm in A and B; scale bars = 100 μ m in C and D; scale bars = 50 μ m in E and F; SC, stromal cells; CL, corpus luteum cells; TC, tumor cells.

(siGLO) was used as a control. Results showed that the transfection efficiency was very high in the KGN cells, with more than 95% of cells being siGLO positive (Fig. 3A). Western blot analysis revealed that both YAP siRNA1 and siRNA 2 significantly ($P < 0.001$) reduced YAP protein in KGN cells (Fig. 3B). As shown in Fig. 3C, the YAP-knockdown KGN cells were only 50–60% confluent compared with siGLO-treated cells, which were nearly 100% confluent (Fig. 3C). Quantification of cell numbers showed that compared with the normal KGN cells or the KGN cells treated with siGLO, the cell numbers in the YAP-knockdown KGN cells was significantly reduced ($\sim 55\%$; $P < 0.001$; Fig. 3D). Knockdown of YAP not only suppressed cell proliferation but also affected cell morphology. As shown in Fig. 3C, YAP-knockdown KGN cells were more spindle-shaped compared with control KGN cells, indicating that YAP may regulate the actin cytoskeleton of the KGN cells. Additional experiments were performed to determine whether the decrease in cell numbers was due to a reduction in cell viability. Cell viability/apoptosis assays using the MTT assay (Supplementary Figure S1, see section on supplementary data given at the end of this article), cell-cycle analysis (Supplementary Figure S2), and apoptosis analysis using Annexin V (Supplementary Figure S3), revealed that

Figure 2

YAP is expressed in human ovarian GCT cell lines and epithelial ovarian cancer cell lines. (A) Localization of YAP protein in KGN cells using fluorescent immunocytochemistry. (A1) Total YAP (green); (A2) YAP protein (green) merged with actin filaments (red, stained with Rhodamine-phalloidin). The nucleus was stained with DAPI (blue). (A3) KGN cells stained with same amount of rabbit IgG (antibody control) and Rhodamine-phalloidin.

Scale bars = 25 μ m. (B) RT-PCR detection of YAP mRNA expression in cultured human granulosa cells (human-GC), GCT cell lines (KGN and COV434) and ovarian cancer cell lines (IGROV-1, SKOV-3, and CAOV-3). (C) Western blot detection of YAP protein expression in the same cell lines. Phospho-YAP(S127): Serine127 phosphorylated YAP protein. GAPDH and tubulin were used as mRNA and protein loading controls respectively.

knockdown of YAP did not reduce the viability of KGN cells.

Overexpression of YAP in KGN cells promotes cell proliferation and migration

To further explore the role of YAP in GCT cell proliferation, we established cell lines which express the WT YAP protein (KGN-YAP) or the constitutively active YAP protein (KGN-YAP^{S127A}). The empty vector (MXIV) was also transfected into KGN cells and served as a control cell line (KGN-MX). Results showed that compared with the control cell line (KGN-MX), both KGN-YAP and the KGN-YAP^{S127A} cells expressed more YAP protein (Fig. 4A). Morphologically, KGN-YAP^{S127A} cells were smaller and much more elongated compared with control cells (Fig. 4B). Compared with the control group, cell numbers almost tripled in KGN-YAP^{S127A} cells and almost doubled in KGN-YAP cells within 24 h of plating (Fig. 4C). After 3 days of culture, the number of KGN-YAP^{S127A} cells increased more than tenfold, the number of KGN-YAP cells increased more than sixfold, while the number of

control cells (KGN-MX) increased only 2.5-fold (Fig. 4C). We also found that in comparison to the KGN-MX control cells, the diameter of KGN-YAP and KGN-YAP^{S127A} cells was reduced by ~20% ($P < 0.05$) (Supplementary Figure S4, see section on supplementary data given at the end of this article).

Transwell migration assays showed that cells in KGN-YAP group migrated faster than cells in KGN-MXIV group, while cells in the KGN-YAP^{S127A} group migrated faster than cells in the KGN-YAP group (Fig. 5A and B). This result was further confirmed by the wound-healing assay (Fig. 5C). The morphology of the migrated cells (Fig. 5A) demonstrated that the KGN-YAP^{S127A} cells were smaller than the corresponding control KGN-MX cells, a finding consistent with cell diameter measurements shown in Supplementary Figure S4.

YAP regulates steroidogenesis in GCT cells

An important feature of the GCT is its ability to produce excessive estrogen. As expected, FSH treatment increased the expression of aromatase protein (Fig. 6A) and

Figure 3

YAP is required for KGN cell proliferation. (A) Fluorescence-labeled scramble siRNA (siGLO) indicates siRNA transfection efficiency in KGN cells. (B) Western blot shows the downregulation of YAP protein after YAP siRNA treatments. β -tubulin was used as a protein loading control. The lower panel shows the quantitative results for the western blot. Bars represent means $\pm$ s.e.m. ($n=3$). (C) Morphology of siGLO-transfected KGN cells and

YAP siRNA-transfected cells. Scale bars = 100 μ m. (D) KGN cells were transfected with YAP siRNAs for 6 h and then cultured for another 72 h. Cell numbers in each group were determined with an INVITROGEN Countess cell counter. Each bar represents the mean $\pm$ s.e.m. ($n=5$). Bars with the same letter are not significantly ($P>0.05$) different from each other.

significantly stimulated the production of 17 β -estradiol in the KGN cells ($P<0.001$, Fig. 6B). Treatment of KGN cells with YAP siRNA to knockdown YAP protein reduced FSH-induced aromatase expression (Fig. 6A) and significantly suppressed production of 17 β -estradiol ($P<0.001$, Fig. 6B). FSH treatment had no effect on β -tubulin expression in the KGN cells (Fig. 6A). Treatment of KGN cells with scramble siRNA had no effect on YAP and β -tubulin protein expression nor did it affect the basal or FSH-stimulated estrogen production (Fig. 6A and B).

Discussion

More than one and half centuries have passed since the first report of GCTs in women by (Rokitansky 1859,

Malmström *et al.* 1994). The etiology and mechanisms underlying the progression and metastasis of GCT are largely unknown. GCTs are derived from granulosa cells, which are highly regulated by reproductive hormones and growth factors in an endocrine, paracrine, and autocrine manner (Craig *et al.* 2007, Smith & Xu 2008). Therefore, hormones, growth factors, and signaling pathways involved in the regulation of normal ovarian granulosa cell functions may also affect the initiation and/or progression of GCT. Indeed, several hormones and growth factors have been implicated in the development of GCT. Inhibin- α has been suggested to be a critical GCT tumor suppressor in rodents because the targeted deletion of the inhibin- α subunit in mice resulted in ovarian granulosa-stromal tumors with very high

Figure 4

Overexpression of YAP stimulates KGN cell proliferation. (A) Empty plasmid-transfected KGN cells (KGN-MX), WT YAP overexpression cells (KGN-YAP), and mutant YAP-transfected KGN cells (KGN-YAP^{S127A}) were plated in six-well plates and the levels of YAP and phosphorylated YAP in these established stable cell lines were examined by western blot. β -tubulin was used as a protein loading control. (B) Upper panel: morphological difference between mutant YAP-transfected KGN cells (KGN-YAP^{S127A}) and Empty plasmid-transfected KGN cells (KGN-MX). Scale bars = 100 μ m. Lower panel: actin filaments stained with Rhodamine-phalloidin to demonstrate the morphological change between the two established cell lines. Nuclei were stained with DAPI. Scale bars = 25 μ m. (C) Effect of YAP overexpression on KGN cell proliferation. WT KGN cells (KGN-MX), KGN-YAP cells, and KGN-YAP^{S127A} cells were incubated for 24, 48, and 72 h, respectively, and the cell number was counted with an INVITROGEN Countess automatic cell counter. Each bar represents the mean $\pm$ S.E.M., $n=5$. Bars with the same letter are not significantly ($P>0.05$) different from each other.

penetrance (Matzuk *et al.* 1992). However, human GCTs are generally associated with high levels of circulating inhibins (Watson *et al.* 1997). Therefore, the function of inhibin in the development of GCT in humans requires further evaluation. Transgenic mice with constitutively activated WNT/ β -catennin signaling in their granulosa cells developed GCT (Boerboom *et al.* 2005), indicating that the WNT/ β -catennin signaling pathway may be involved in the rodent GCT initiation and progression. However, it remains unclear if the molecular mechanisms of tumorigenesis in this mouse model are related to those involved in GCT development in women. A more recent whole-transcriptome sequencing study with human samples has demonstrated that a somatic mutation in FOXL2 (C402G; Cys134Trp) was present in almost all morphologically identified adult-type GCTs and indicated that mutant FOXL2 is a potential driver in the pathogenesis of adult-type GCTs (Shah *et al.* 2009, Jamieson *et al.* 2010, Al-Agha *et al.* 2011, Jamieson & Fuller 2012, Rosario *et al.* 2012, Benayoun *et al.* 2013, Georges *et al.* 2013). More recently, Rosario *et al.* found that patients with homozygous FOXL2 mutations had a significantly higher relapse rate ($P=0.04$). However, they did not find a significant correlation between FOXL2 mutation status or FOXL2 expression and any other clinical variables. The author's indicated that due to the limitation of the sample size, the prognostic significance of this gene mutation still needs to be confirmed (Rosario *et al.* 2013). Therefore, although nearly all adult GCTs express the mutant FOXL2 gene, the mechanisms underlying the progression, recurrence, and metastasis of ovarian GCT remain unknown. In this study, our data clearly show that YAP, a central component of the Hippo pathway, is highly expressed in human GCT tissues and that overexpression of YAP protein significantly stimulates the proliferation and migration of the KGN GCT cell line, demonstrating that the Hippo pathway may be involved in the progression of GCT.

YAP is the major downstream effector of the Hippo pathway. Activation of the Hippo pathway suppresses YAP activity by phosphorylating YAP and subsequently retaining it in the cytoplasm. In this study, we found that YAP immunostaining was significantly higher in human GCT tumor tissues compared with normal ovarian tissues. Moreover, YAP was mainly localized in the nucleus of tumor cells. In comparison, little YAP protein was found in age-matched normal ovarian tissues. When luteinized cells were present in age-matched normal ovarian tissues, YAP was mainly localized to the cytoplasm. Consistent with our results, elevated YAP expression and nuclear localization have been observed in multiple types of

Figure 5

YAP regulation of KGN cell migration. (A) KGN-MXIV cells, KGN-YAP cells, and KGN-YAP^{S127A} cells were loaded to a transwell migration assay chamber and the migration assay was performed as described in the Materials and methods section. Top panel, representative image of the migrated KGN-MXIV cells. Lower panel, representative image of the migrated KGN-YAP^{S127A} cells. Scale bars, 100 μ m. (B) Migrated cells in each

group were counted and the fold change (relative to the KGN-MXIV control group) of the migrated cells was determined. Each bar represents the mean $\pm$ s.e.m. ($n=12$). Bars with the same letter are not significantly ($P>0.05$) different from each other. (C) Evaluation of the cell migration ability with the wound-healing assay. Representative images from triplicate experiments were presented. Scale bars, 100 μ m.

human cancers, including liver cancers, colon cancers, EOCs, lung cancers, and prostate cancers (Zender *et al.* 2006, Dong *et al.* 2007, Zhao *et al.* 2007, Steinhardt *et al.* 2008). In hepatocellular carcinoma, YAP was determined to be an independent prognostic marker for overall survival and disease-free survival (Xu *et al.* 2009). In EOC, very recent data indicated that subcellular levels of YAP showed an exceptionally strong association with poor patient survival (Hall *et al.* 2010). The authors suggested that high levels of nuclear YAP, or low levels of cytoplasmic phosphorylated YAP, were associated with poor survival in patients with EOC. Patients with both high-nuclear YAP and low-phosphorylated YAP had about 50% lower 5-year survival, and this combination serves as an independent prognostic marker for survival (Hall *et al.* 2010). In another study, levels of active nuclear YAP were reported to be expressed at high levels in around 14% of a cohort of 284 human ovarian cancer samples. Segregation by histotype showed that the correlation between nuclear YAP and poor survival is predominantly associated with clear cell tumors, independent of stage (Zhang *et al.* 2011). As patient survival data are not available for the samples used in this study, we cannot correlate YAP expression data with survival of GCT patients. However, the high level of expression and nuclear location of YAP protein in samples from GCT patients indicate that YAP may play important role in regulation of GCT progression.

Control of ovarian cell proliferation is critical for the normal function of the ovary. Uncontrolled granulosa cell proliferation and decreased cell death and/or

differentiation can lead to hyperplasia of the granulosa layer and the formation of GCTs. Our results indicate that dysregulation of the Hippo/YAP signaling pathway may contribute to uncontrolled granulosa cell proliferation and development of GCTs which expands the size of the ovary. We used the KGN cell line as a model to determine the role of YAP in GCT cell proliferation. Consistent with the results from the patient samples, immunohistochemical and molecular studies showed that KGN cells express YAP. Knockdown of YAP protein in KGN cells with YAP-targeting siRNA significantly suppressed cell proliferation without reducing cell viability, indicating that YAP is critical for the proliferation of KGN cells. Furthermore, overexpression of YAP or its constitutively active mutant (YAP^{S127A}) in KGN cells stimulated KGN cell proliferation and migration, indicating that the Hippo/YAP pathway may play a very important role in the regulation of GCT cell growth and potentially GCT cell metastasis. Taken together, our results indicate that YAP protein is required for the proliferation of KGN cells and the Hippo/YAP pathway might be one of the major pathways that are involved in the regulation of GCT progression. To our knowledge, this is the first direct evidence showing the function of the Hippo/YAP pathway in the progression of GCTs.

The mechanism by which YAP regulates GCT cell proliferation is unclear. Granulosa cells are highly regulated by gonadotropins, steroid hormones, and growth factors. Abnormal activities in the pathways activated by any of these factors may induce transformation of follicular granulosa cells and may promote GCT

Figure 6

(A) YAP is required for FSH-stimulated estradiol secretion. Normal KGN cells (CTRL), siGLO-transfected KGN cells, and YAP siRNA-transfected KGN cells were incubated with or without 100 ng/ml of FSH for 48 h. The protein levels of YAP and aromatase was determined by western blotting. β -tubulin was used as a protein loading control. (B) 17 β -estradiol in the medium was measured by ELISA. Each bar represents the mean $\pm$ S.E.M., $n=5$. Bars with the same letter are not significantly ($P>0.05$) different from each other. CTRL, normal KGN cell as control; E_2 , 17 β -estradiol.

tumor growth, recurrence, or metastasis. As a major effector of the Hippo pathway, YAP was reported to interact with many important factors associated with GCT development. For example, YAP has been reported to regulate the Wnt/ β -catenin pathway in colorectal cancer. β -catenin has been reported to be a potential initiator of GCT (Boerboom *et al.* 2005, Richards *et al.* 2012). YAP was also reported to interact with the TGF β signaling pathway (Fujii *et al.* 2012), which is a critical regulator of granulosa cell proliferation (Skinner *et al.* 1987). We have recently found that TGF α , via EGFR signaling pathways, plays an important role in the proliferation of GCT cells (Wang *et al.* 2012a). Interestingly, YAP was reported to interact with EGFR to regulate cell proliferation

(Komuro *et al.* 2003, Zhang *et al.* 2009, Huang *et al.* 2013). Future studies on the interaction between the Hippo pathway and these critical regulators of ovarian function may reveal novel mechanisms by which YAP promotes the initiation and/or progression of GCT.

An important feature of GCT tumor is its ability to produce large amounts of estrogen (Schumer & Cannistra 2003, Jamieson & Fuller 2012). It is believed that the excess estrogen in GCT is mainly attributable to the high level of expression of aromatase. However, the high circulating levels of estrogen should suppress FSH secretion by estrogen feedback leading to the decreased expression of aromatase and reduced production of estrogen. Obviously, the feedback mechanism fails to regulate aromatase expression and estrogen production in GCT patients. The regulatory mechanisms controlling estrogen secretion in GCT patients is unclear. In this study, we found that FSH induced the expression of aromatase and stimulated the production of estrogen in KGN cells. Our studies also showed that knockdown of YAP blocked FSH-induced expression of aromatase and compromised FSH-stimulated production of estrogen. This novel finding indicates that the Hippo/YAP pathway is involved in the regulation of steroidogenesis in human GCT tissues.

Excessive estrogen produced by GCT has been shown to stimulate other estrogen target organs in GCT patients. For example, the sustained high level of estrogen has been reported to stimulate endometrium, resulting in the development of endometrial hyperplasia and endometrial adenocarcinoma in GCT patients (Schumer & Cannistra 2003). Whether estrogen produced by GCT also promotes GCT progression or metastasis is unknown. Since estrogen is produced by the GCT cells, the tumor cells themselves are immersed in an environment with sustained high level of estrogen. Data from rodent models indicates that estradiol alone has little effect on rodent granulosa cells, but is required for maximum FSH stimulation of aromatase (*CYP19*) expression (Fitzpatrick & Richards 1991) and estradiol synthesis (Adashi & Hsueh 1982, Zhuang *et al.* 1982). Therefore, it is very possible that increased estrogen in GCT patients may synergize with FSH to regulate human GCT cell proliferation and steroidogenesis. This hypothesis is supported by the findings that drugs with an anti-estrogenic effect, such as gonadotropin-releasing hormone agonists and aromatase inhibitors are effective in treatment of some types of GCTs (Freeman & Modesitt 2006, Korach *et al.* 2009, Alhilli *et al.* 2012). Importantly, the recent identification of G-protein-coupled estrogen receptor GPR30 (or GPER) in the ovary may provide a reasonable explanation of how estrogen may regulate GCT

cell proliferation (Carmeci *et al.* 1997, Wang *et al.* 2007, 2008). Our recent data showed that knockdown of GPR30 significantly compromised the proliferation of KGN cells (Wang *et al.* 2012b), indicating that the membrane estrogen receptor may mediate the action of estrogen in the GCT cells. The potential interaction between the Hippo pathway and estrogen receptor-mediated signaling pathways during GCT initiation and progression is an absorbing mystery that remains to be elucidated.

During revision of this paper Kawamura *et al.* (2013) reported that fragmentation of murine ovaries disrupted ovarian Hippo signaling, leading to increased expression of downstream growth factors, promotion of follicle growth, and the generation of mature oocytes.

In conclusion, our data show that YAP is highly expressed in human GCT tissues and is a critical regulator of proliferation, migration, and steroidogenesis in the KGN human GCT cell line. We propose that the Hippo/YAP signaling pathway may be involved in the regulation of GCT growth and progression. YAP may be a potential novel biomarker for GCT diagnosis and a promising target for the development of drugs for GCT therapy.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0339>.

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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