ISSN 2472-1972

Functional Profiling of FSH and Estradiol in Ovarian Granulosa Cell Tumors

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Adult-type granulosa cell tumors (AGCTs) are sex-cord derived neoplasms with a propensity for late relapse. Hormonal modulators have been used empirically in the treatment of recurrent AGCT, albeit with limited success. To provide a more rigorous foundation for hormonal therapy in AGCT, we used a multimodal approach to characterize the expressions of key hormone biomarkers in 175 tumor specimens and 51 serum samples using RNA sequencing, immunohistochemistry, RNA *in situ* hybridization, quantitative PCR, and circulating biomarker analysis, and correlated these results with clinical data. We show that FSH receptor and estrogen receptor beta (ERβ) are highly expressed in the majority of AGCTs, whereas the expressions of estrogen receptor alpha (ERα) and G-protein coupled estrogen receptor 1 are less prominent. ERβ protein expression is further increased in recurrent tumors. Aromatase expression levels show high variability between tumors. None of the markers examined served as prognostic biomarkers for progression-free or overall survival. In functional experiments, we assessed the effects of FSH, estradiol (E2), and the aromatase inhibitor letrozole on AGCT cell viability using 2 *in vitro* models: KGN cells and primary cultures of AGCT cells. FSH increased cell viability in a subset of primary AGCT cells, whereas E2 had no effect on cell viability at physiological concentrations. Letrozole suppressed E2 production in AGCTs; however, it did not impact cell viability. We did not find preclinical evidence to support the clinical use of aromatase inhibitors in AGCT treatment, and thus randomized, prospective clinical studies are needed to clarify the role of hormonal treatments in AGCTs.

Key Words: granulosa cell tumor, estrogen receptor, aromatase, hormonal treatment

Adult-type granulosa cell tumors (AGCTs) are sex cord stromal tumors representing 3% to 5% of ovarian malignancies. A defining feature of AGCTs is a somatic mutation (c0.402C > G; p.C134W) in the *FOXL2* gene, which is thought to play a pivotal role in oncogenesis [1]. These tumors are characterized by slow growth and a generally favorable prognosis, with

Abbreviations: AGCT, adult-type granulosa cell tumor; E2, estradiol; ER, estrogen receptor; FSHR, FSH receptor; GPER, G-protein linked estrogen receptor; hGL, human granulosa-luteal; IHC, immunohistochemistry; qPCR, quantitative PCR; RNA-Seq, RNA sequencing; TMA, tumor tissue microarray

Received 10 October 2019
Accepted 11 March 2020
First Published Online 16 March 2020
Corrected and Typeset 13 April 2020

a 10-year survival of 84% [2]. Up to 30% of patients diagnosed with AGCT experience a late relapse. The mainstay of treatment for AGCT is surgical resection, but improved medical therapies are needed for advanced and relapsed disease. Current chemotherapeutic regimens show limited efficacy [3], and no prospectively validated targeted therapies exist for this unique tumor type.

AGCTs secrete estradiol (E2), inhibin B, and anti-Müllerian hormone, and tumor hormone production accounts for many of the signs and symptoms of the disease [4–6]. AGCTs are known to express certain hormone receptors [7–9], but the importance of hormonal signaling in AGCT progression remains uncertain. Hormonal therapies, such as GnRH-analogues and aromatase inhibitors, have been used empirically in AGCT with limited efficacy [10, 11]; however, the biological foundation for these treatments has not been clearly established.

Clinically, AGCTs are often diagnosed at perimenopause when gonadotropin levels rapidly increase, and FSH signaling has been proposed to be 1 of the main drivers of AGCT growth [12]. In normal granulosa cells, FSH promotes cell proliferation by cAMP-mediated signaling cascades, leading to increased aromatase (CYP19A1) expression and elevated serum E2 levels, essential for normal ovarian function [13, 14]. The gene expression profile of AGCTs has been shown to mimic that of FSH-stimulated granulosa cells [15], suggesting that this gonadotropin signaling pathway is active in these neoplasms. Regarding *CYP19A1*, FSH is known to increase its mRNA levels via specific transcription factors, and FOXL2 has been shown to act as a direct regulator of this gene [16]. Data concerning the expression and functionality of CYP19A1 in AGCTs are scarce, although the ability of AGCTs to secrete E2 implies its increased activity in tumor tissue.

Estrogens are known to exert strong mitogenic effects in breast and endometrial cancers [17, 18] and to inhibit apoptosis in normal granulosa cells [19], but their impact on AGCT proliferation is less clear. The effects of E2 are mediated through two distinct nuclear receptors estrogen receptor-α (ERα) and -β (ERβ), both expressed in AGCTs [20, 21]. Additionally, E2 can act through a membrane-bound G-protein linked estrogen receptor (GPER1) that has been observed in AGCTs [22]. The expression levels, prognostic implications, and functional roles of FSH, CYP19A1, and E2 in AGCTs have not been reported.

Here, using a multimodal approach that combines next-generation sequencing, quantitative real-time PCR (qPCR), RNA *in situ* hybridization, and immunohistochemistry (IHC), we profile the expression of the FSH receptor (*FSHR*), estrogen receptors (ERα, ERβ, *GPER1*), and CYP19A1 in a large cohort of *FOXL2* mutation-positive AGCTs with rich clinical and follow-up data. We augment this expression profiling with measurements of hormone levels in 51 preoperative serum samples. In functional analyses, we show that FSH increases *CYP19A1* expression and E2 production in an established AGCT cell line (KGN) and in primary cultures of AGCT cells. We demonstrate that stimulation with FSH increased cell viability in a subset of primary AGCT cells, whereas E2 had a similar effect only at high concentrations. Our results thus indicate a specific pattern of hormonal dependency in AGCTs and support the further clinical exploration of hormonal modulators in the treatment of AGCT.

1. Materials and Methods

A. Patients and Samples

Patient and sample data are shown in Tables 1 and 2. All of the AGCTs tested positive for the *FOXL2* (c0.402C > G; p.C134W) mutation, and histological diagnoses were verified by an expert pathologist (R.B.). We performed RNA sequencing of freshly frozen tissue from 6 primary and 4 recurrent tumors. We constructed a tumor tissue microarray (TMA) containing quadruple cores from 121 primary and 54 recurrent AGCTs from representative formalin-fixed, paraffin embedded samples (Table 1). Nine tumor samples were available both as freshly frozen tissue and formalin-fixed, paraffin embedded. For controls, normal

Table 1. Clinicopathologic Data of the (A) Patients and (B) Samples in the Tumor Tissue Microarray (TMA)

***A* Patients n = 138**	**n (% of Total)**
Age at diagnosis, years[a]	53 (26–81)
Tumor stage at diagnosis	
I	126 (91)
II	8 (6)
III	1 (1)
n/a	3 (2)
Follow-up time, years[a]	15.0 (0.7–42.3)
Recurrence	
Yes	51 (37)
No	87 (63)
Survival	
Alive	91 (66)
Dead of AGCT	24 (17)
Dead of other	23 (17)
***B* Tumor characteristics n = 175**	**n (% of total)**
Primary	121 (69)
Recurrent	54 (31)
Microscopic pattern	
Better-differentiated	72 (41)
Less-differentiated	98 (56)
n/a	5 (3)
Tumor size	
<10 cm	103 (59)
≥10 cm	70 (40)
n/a	2 (1)
Nuclear atypia	
High	50 (29)
Low	121 (69)
n/a	4 (2)
Mitotic index	
High	48 (27)
Low	123 (70)
n/a	4 (2)
MP status at sample retrieval	
Premenopausal	50 (29)
Postmenopausal	124 (71)
n/a	1 (0.5)

[a]Median (range)
MP = menopause; n/a = not available.

ovaries (n = 4) were obtained from women undergoing ovariectomy for benign indications. Serum samples from 47 AGCT patients were collected before surgery for either primary or recurrent AGCT (Table 2). Short-term primary tumor cell cultures were established from fresh AGCT samples from 6 patients (Table 3). Three samples of pooled human granulosa-luteal (hGL) cells were obtained from women undergoing *in vitro* fertilization treatment at the Department of Obstetrics and Gynecology, Helsinki University Hospital. Informed consent was obtained from patients who donated blood or fresh AGCT tumor samples for the study. The ethics committee of Helsinki University Central Hospital and the National Supervisory Authority for Welfare and Health in Finland approved this study.

B. Serum Analyses

Preoperative samples were obtained from 47 AGCT patients (Table 2) within a month before surgery and prepared and stored at -80°C until the analysis. All the studied serum markers (FSH, E2, and inhibin B) were measured in the HUSLAB hospital clinical laboratory:

Table 2. Clinicopathologic Data of the (A) Patients and (B) Samples in the Serum Analysis

***A* Patients n = 47**	**n (%)**
Age at diagnosis, years[a]	56 (26–80)
Tumor stage at diagnosis	
I	47 (100)
Follow-up time, years[a]	5.3 (0.4–20.9)
Recurrence	
Yes	19 (40)
No	24 (51)
n/a	4 (9)
***B* Samples n = 51**	**n (%)**
Primary	31 (61)
Recurrent	20 (39)
Tumor size	
<10 cm	24 (47)
≥10 cm	15 (29)
n/a	12 (24)
MP status at sample retrieval	
Premenopausal	10 (20)
Postmenopausal	41 (80)

[a] Median (range)
MP = menopause; n/a = not available.

automated immunoassays for FSH [23] and E2 [24] and ELISA for inhibin B [25]. All the samples were classified as follows according to the patient's menopausal status at sample retrieval: "premenopausal" if the patient had 1 or 2 ovaries and menopause was not indicated in the medical records and "postmenopausal" if both ovaries had been removed, independent of age, or if the patient was postmenopausal according to her medical history.

C. RNA Sequencing

Freshly frozen AGCT tissue samples (n = 10) were lysed in RP1 lysis buffer and homogenized using a Precellys Lysing Kit and tissue homogenizer (Bertin Technologies, France). Total RNA was extracted from tumor samples according to the manufacturer's instructions and further purified with RNA purification kit (Nucleospin RNA/protein kit and RNA Clean up kit, Macherey-Nagel, Düren, Germany). RNA integrity was verified using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Poly (A)-containing mRNA molecules were purified from total RNA and fragmented into approximately 140- to 160-bp pieces using a standard fragmentation reagent. cDNA was generated using a first-strand using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis and cDNA purification using a standard purification kit. The synthesized cDNA was subjected to end repair by adding an "A," followed by the 3′ end adenylation. cDNA fragments were amplified using standard PCR and the products were purified. Libraries were quantified and qualified using Bioanalyzer 2100 (Agilent Technologies, CA, USA) and the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The libraries were paired-end sequenced (read length 100 bp) in a single lane using the Illumina HiSeq X ten system (Illumina, Inc, CA, USA). Library construction and sequencing were both performed in BGI Tech Solutions (Hong Kong).

The RNA-Sequencing (RNA-Seq) reads were processed and analyzed with SePIA [26] on the Anduril framework [27]. After trimming, reads shorter than 20 bp were discarded. Reads were quantified with Kallisto (v0.44.0) [28] using GENECODE (v25, GRCh38) [29] followed by tximport (v1.6.0) [30]. Limma (v3.38.3) [31] was used for batch correction and edgeR (v3.24.3) [32] for differential expression analysis. In differential expression analysis, false discovery rate < 0.25 was used as a threshold for statistical significance.

Table 3. Data on Primary Cultured AGCT Samples

Sample ID	Primary or Recurrent Tumor	Tumor Size (in cm)	Age of the Patient at Sample Retrieval and Menopause Status (Age at Primary Diagnosis)	Preoperative Serum Hormone Levels	Proportional Change in Cell Number After E2 Stimulation	Proportional Change in Cell Number After FSH Stimulation
1	Primary	11	53, postMP	E2 0.15 InhB 625 FSH 0.1	0.79	0.87
2	Recurrent	4	49, postMP (45)	E2 0.17 InhB 278 FSH 10.1	1.57	1.05
3	Primary	6,5	48, postMP	E2 0.55 InhB > 1000 FSH 41	1.40	1.28
4	Recurrent	5	68, postMP (59)	E2 0.17 InhB 61 FSH 42	1.23	1.43
5	Primary	4	49, postMP	E2 0.13 InhB 223 FSH 15.4	1.25	1.26
6	Recurrent	2,5	41, postMP (40)	E2 0.14 InhB 221 FSH 44.3	1.03	0.84

MP = menopause

D. Immunostaining

The AGCT TMA and normal ovary tissue sections were subjected to IHC as described [33]. Staining was performed using mouse monoclonal antibody for CYP19A1 [34], rabbit monoclonal antibody for ERα [35], and mouse monoclonal antibody for ERβ [36]. Samples were scored by 2 independent researchers (U-M.H. and N.A.) and disagreements were resolved by a joint review. Staining intensity was classified from 0 to 3 (0 = negative, 1 = weak, 2 = moderate, 3 = strong intensity). Images were generated using 3DHISTECH Panoramic 250 FLASH II digital slide scanner at Genome Biology Unit (Research Programs Unit, Faculty of Medicine, University of Helsinki, and Biocenter Finland). The scanning was done using 40× objective (Zeiss Plan-Apochromat 40x/NA 0.95) and applying the extended focus option using 7 focus layers.

E. RNA In Situ Hybridization

RNA *in situ* hybridization was performed on freshly cut 5-µm sections of the TMA using RNAscope 2.5 HD detection kit-BROWN (#322310, ACDBio, Milano, IT) for target mRNA detection. In short, tissue sections were baked for 1 hour at 60°C, then deparaffinized and treated with hydrogen peroxide for 10 minutes at room temperature. Target retrieval was performed for 15 minutes at 100°C, followed by protease plus treatment for 15 minutes at 40°C. The probes Hs-*FSHR* (#400501), Hs-*CYP19A1* (#430861), Hs-*GPER* (#553361), positive control probe Hs-*PPIB* (#313901), and negative control probe *DapB* (#310043) were hybridized for 2 hours at 40°C followed by signal amplification steps. The samples were incubated for 60 minutes with AMP 5–reagent. The sections were next treated with DAB for 10 minutes at room temperature followed by counterstaining with 50% hematoxylin. The sections were dipped in ammonium water and dehydrated in ethanol series and UltraClear before mounting. Two researchers (U-M.H. and N.A.) performed the scoring independently and disagreements were resolved by a joint review. Staining intensity was classified from 0 to 3 (0 = negative, 1 = weak, 2 = moderate, 3 = high intensity).

F. Hormone Stimulation in KGN Cell Line and Primary Cultured AGCT Cells

The *FOXL2* c0.402C > G mutation-positive KGN cell line [37], originating from a recurrent AGCT, was obtained from the Riken BioResource Center. KGN cells were passaged < 6 months following receipt or resuscitation of a frozen cell vial, and tested negative for mycoplasma infection. The cells were tested positive for the *FOXL2* (c0.402C > G; p.C134W) mutation. Short-term primary tumor cell cultures were established as described [38] from fresh primary (n = 3) or recurrent (n = 3) AGCT samples, all tested positive for the *FOXL2* (c0.402C > G; p.C134W) mutation. To increase the cell number, primary AGCT cells were first cultured for 3 to 4 days in DMEM/Ham's F-12 medium, supplemented with 10% fetal calf serum, penicillin/streptomycin, and L-glutamine in a humidified environment at 37°C and 5% CO. hGL cells were obtained from women undergoing *in vitro* fertilization. Each of the 3 hGL pools consisted of granulosa-luteal cells derived from 78 to 122 mature ovulatory follicles from 4 to 7 different patients. The hGL cells were isolated as previously described [39], followed by suspension in DMEM/F12 growth medium, supplemented with 2.5% Nu-serum I, ITS+^{TM} Premix (both from BD Biosciences, Bedford, MA, USA), penicillin/streptomycin, and L-glutamine (Gibco).

Both KGN and primary cultured AGCT cells were grown for 10 or 3 days before hormone stimulation, respectively, in phenol red free DMEM:F12 medium supplemented with 10% charcoal stripped fetal calf serum, L-glutamine, and antibiotics to deplete the cells from external hormones. Next, 10 000 KGN and primary AGCT cells per well were plated on 96-well plates in hormone-depleted medium for the cell viability assay. For RNA extraction and medium collection, 600 000 cells per well were plated on 6-well plates. After the cells had attached, they were treated with 100 nM of FSH (#HOR-253, Immuno Diagnostics, Hämeenlinna, Finland), 1000 nM of E2 (#E2758, Sigma-Aldrich, St Louis, MO, USA), 2 μM testosterone (#86500, Sigma-Aldrich), or 5 μM dose of letrozole (#L6545, Sigma-Aldrich). Cell viability was measured after 96 hours using the WST-1 assay (Sigma-Aldrich).

G. qPCR

Total RNA was extracted using the NucleoSpin RNA/Protein kit (Macherey-Nagel, Düren, DE). Reverse transcription was performed using the Reverse Transcriptase Core Kit, and qPCR was carried out using the MESA GREEN qPCR MasterMix Plus for SYBR Assay (both from Eurogentec, Seraing, BE). *ACTB* was used as reference gene for mRNA expression.

H. E2 Measurement

Medium was collected from KGN and AGCT primary cells at 96-hour time point and analyzed for E2 concentration using a mass spectrophotometer at HUSLAB. Calibrators containing 25 to 1000 pmol/L of estradiol (Cerilliant) were prepared in 50% methanol. Forty microliters of sample extracts and calibrators were analyzed on a liquid chromatography tandem mass spectometry system equipped with an AB Sciex 5500 triple quadrupole mass spectrometer. Data were acquired and processed with the Analyst Software (Ver 1.6.2; AB Sciex).

I. Statistical Analyses

The immunohistochemical data and categorical variables were analyzed with contingency tabling (2 × 2) and chi-square or Fisher exact tests. For cell culture data, 1-way ANOVA followed by Mann-Whitney *U*-test, with-control Dunnett or Student *t*-test was used. The comparison between primary and recurrent tumors was performed by matched-pair *t*-test analysis. Serum hormone levels differed from normal distribution even after performing logarithmic transformation and thus Spearman´s rho was reported. Survival curves of different groups were illustrated by Kaplan-Meier plots and compared with the log-rank test.

Two-sided *P* value less than 0.05 was considered statistically significant. All data were analyzed using JMP pro13.

2. Results

A. Primary and Recurrent AGCTs Show Distinct Transcriptional Profiles

To characterize the expression levels of the hormonal pathway genes, we first performed RNA sequencing of 6 primary and 4 recurrent AGCTs. Altogether, 1091 genes were differentially expressed between primary and recurrent tumors [40]. Six of these genes were involved in estrogen signaling, including 3 adenylate cyclase genes contributing to formation of cAMP in response to G-protein signaling, and CREB3L1, encoding for cAMP-responsive element, showing higher expression in primary tumors compared with recurrent tumors (Fig. 1A). Among the genes with high expression in both primary and recurrent tumors were heat shock protein 90B1 and members of the PI3K/Akt pathway. No significantly differentially expressed genes were found between relapsed and nonrelapsed primary tumors after multiple testing correction [40]. However, when analyzing the hormonal pathway genes (KEGG estrogen biosynthesis hsa04915) using unsupervised hierarchical clustering, the primary and recurrent tumors clustered separately, indicating distinct transcriptional profiles. Of the key hormonal regulators, *FSHR* was robustly expressed in all studied samples (Fig. 1A-B). Interestingly, *CYP19A1* expression was highly variable (range -0.07 to 9.85 Log2tpm) among the tumors. Of the estrogen receptors, *ESR2* was the most highly expressed (range 5.75–7.15 Log2tpm), whereas *GPER1* expression levels were generally low at the mRNA level (range 0.20–2.84 Log2tpm).

B. AGCT Cells Express Highly Variable Levels of CYP19A1 Gene

To compare the gene expression levels of key hormone receptors in AGCTs to those in primary granulosa cells, we measured mRNA levels of *FSHR, CYP19A1, ESR1, ESR2*, and *GPER1* in hGL cells, an AGCT cell line KGN, and primary AGCT cells by qPCR analysis (Fig. 1C-G). *FSHR* expression levels were higher in hGL cells compared with expression in the KGN cell line and the expression levels of *ESR2* were higher in both AGCT and hGL cells compared with KGN cells (Fig. 1C, F). By contrast, the mRNA expression levels for *ESR1* were notably higher in hGL cells compared with KGN or primary AGCT cells (Fig. 1E). Interestingly, *CYP19A1* expression was minimal in hGL and KGN cells, whereas the expression in AGCT cells varied widely among the individual samples (Fig. 1D), in line with the result of the RNA-Seq analysis. The expression levels of GPER1 in AGCT cells were similar to hGL and KGN cells (Fig. 1G).

C. FSHR Is Widely Expressed in AGCTs

Because gonadotropins are speculated to play a role in AGCT progression, we next characterized the expression of *FSHR* in a larger cohort using RNA *in situ* hybridization on a TMA of 175 AGCTs. *FSHR* mRNA was detected in 90% of AGCTs (Fig. 2A-D), and the signal was strong or moderate in 60% of the tumors (Fig. 2A-D, Table 4). *FSHR* expression was lower in tumors with high mitotic activity ($P = 0.01$), but expression did not correlate with other clinical parameters such as tumor size, stage, or risk of recurrence.

D. Serum Inhibin B Levels Correlate Inversely With FSH Levels in AGCT Patients

Next, we analyzed FSH, E2, and inhibin B levels in 51 preoperative serum samples. FSH levels were generally low in AGCT patients, with median levels of 1.55 IU/l (range 0.05–15.3 IU/L) and 6.6 IU/L (range 0.1–60.3) in premenopausal and postmenopausal patients, respectively. For E2 the median level was 0.125 nmol/L (range 0.01–0.48) in premenopausal patients and 0.15 nmol/L (range 0.04–1.05) in postmenopausal patients. Serum FSH levels

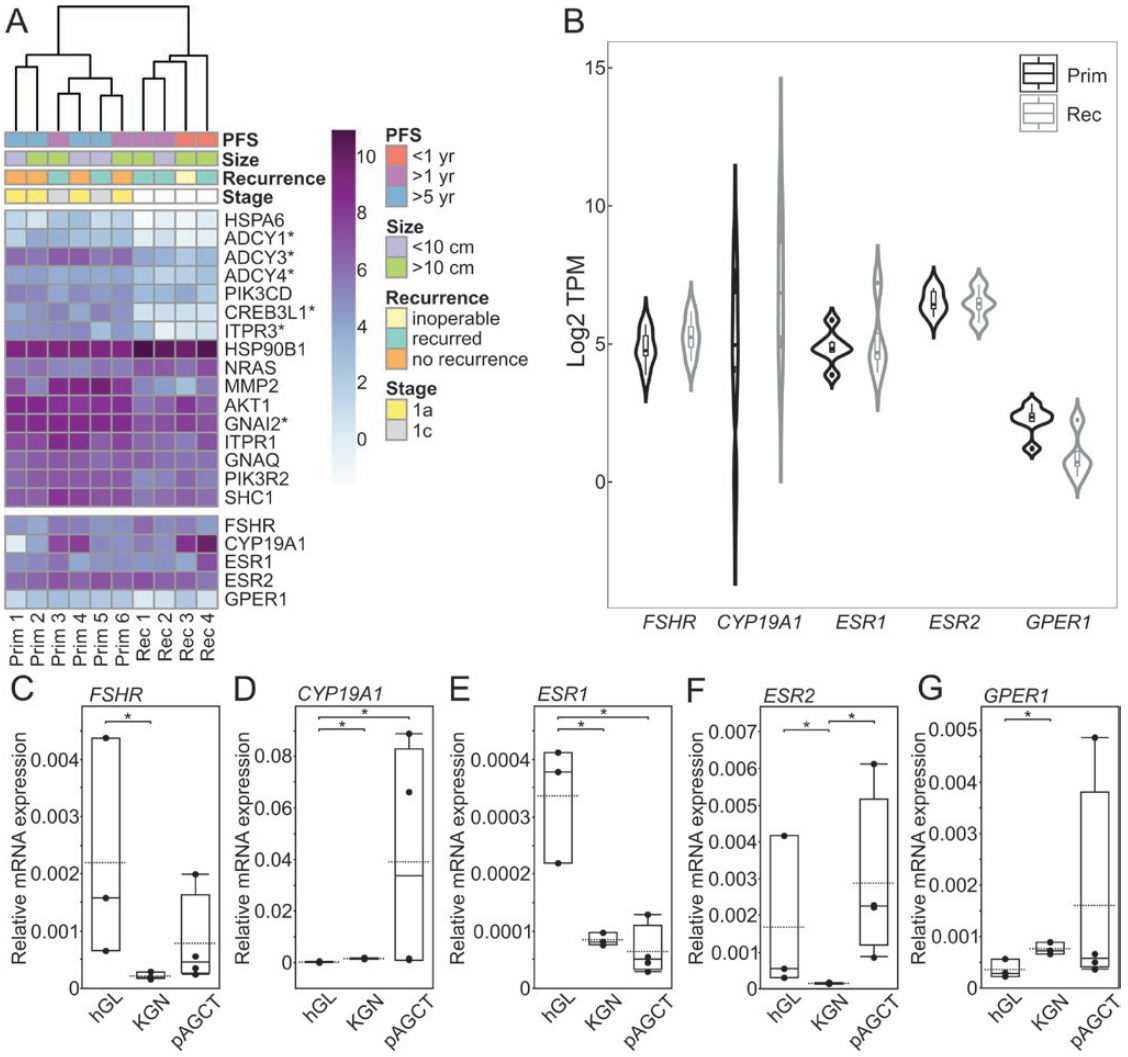

Figure 1. Transcriptional profiling of 6 primary and 5 recurrent AGCTs using next-generation mRNA sequencing. (A) Heatmap showing the expression levels of differentially expressed estrogen signaling genes between primary and recurrent samples (upper). Genes with statistically significant differential expression ($P < 0.05$, false discovery rate < 0.25) are marked with an asterisk. Lower part of the heatmap presents scaled expression levels of key hormonal signaling genes in primary and recurrent AGCTs. (B) Violin plots showing the expression levels of key hormone receptors and *CYP19A1* enzyme in the primary and recurrent samples. (C-G) qPCR analysis showing the expression levels of key hormone receptors *FSHR, ESR1, ESR2, GPER1*, and *CYP19A1* enzyme in human granulosa lutel (hGL) cells, KGN cell line and AGCT primary cells. All studied tumors were tested positive for *FOXL2* mutation and derived from postmenopausal patients. The data are shown as boxplots, where dots represent individual samples, the box represents the interquartile range, and the whiskers represent the first and fourth quartile. In comparisons between the groups, the asterisks indicate significant statistical difference ($P < 0.05$).

were significantly lower in patients with large tumors (>10 cm in diameter) when compared with patients with smaller tumors (<10 cm in diameter) ($P = 0.001$). FSH and E2 levels did not correlate with each other; however, FSH and inhibin B levels showed a strong inverse correlation (Spearman rho -0.75, $P < 0.0001$), consistent with the suppressive effect of circulating inhibin B on FSH secretion from the pituitary. In accordance with previous findings [41], inhibin B levels were higher in patients with larger tumors ($P = 0.01$). Next, we assessed the preoperative serum FSH levels from synchronous samples of 36 AGCT patients whose tumors were represented in the TMA. We observed significantly increased

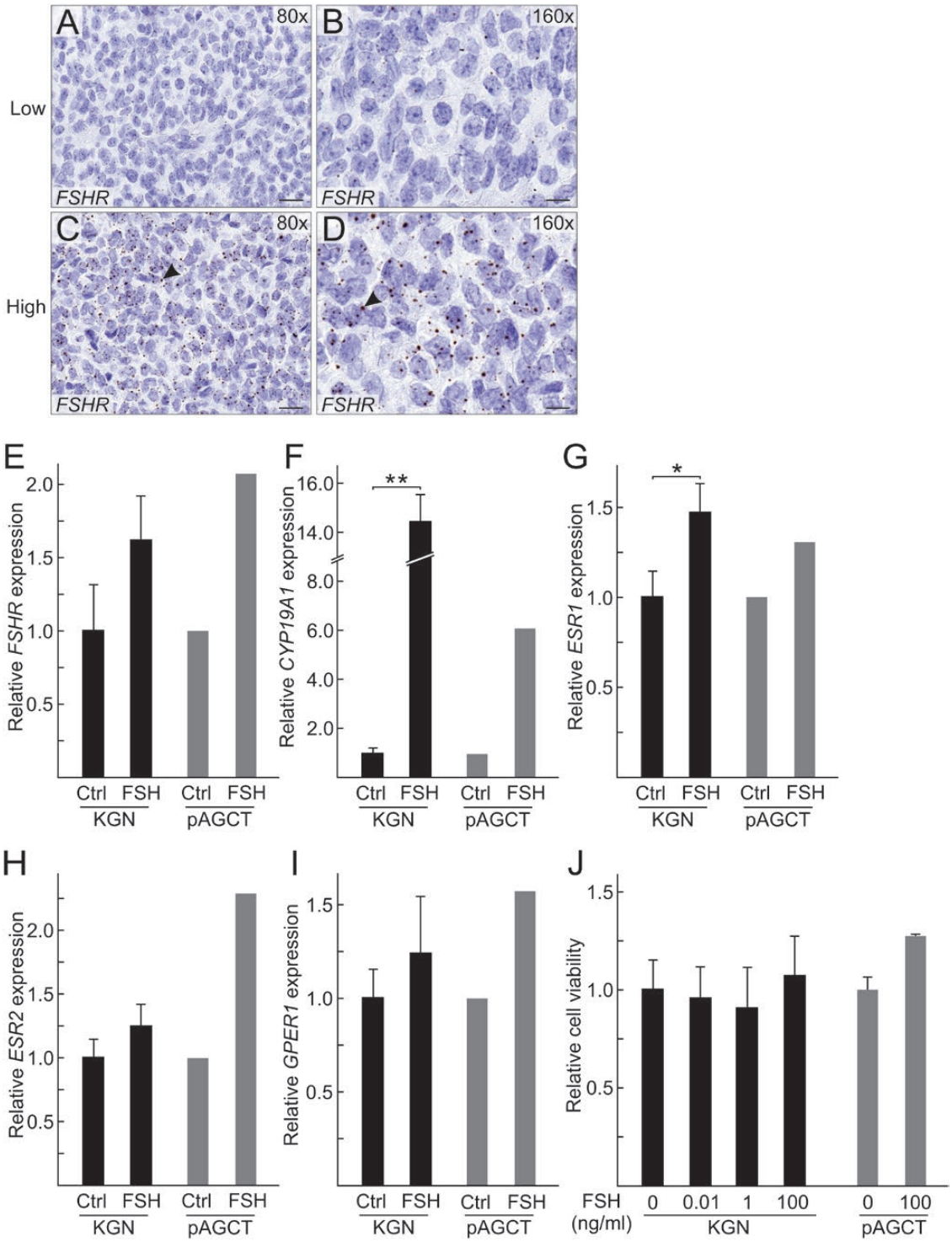

Figure 2. FSHR mRNA expression and the effect of FSH stimulation in AGCTs. RNA *in situ* hybridization was used to quantify *FSHR* expression in the AGCT TMA. Representative images of (A,B) low and (C,D) high staining patterns. Original magnifications 80 × (scale bar: 20 μm) and 160 × (scale bar: 10 μm). Expression of (E) *FSHR*, (F) C*YP19A1*, (G) *ESR1*, (H) *ESR2*, and (I) *GPER1* was quantified in KGN cell line and cultured primary AGCT cells by qPCR after stimulation with FSH (0 or 100 ng/mL) for 96 hours. Black columns: KGN cell line; gray columns: primary AGCT cells. (J) KGN data represents the average of 3 independent experiments. Results are shown as mean ± SEM and statistical significance ($P < 0.05$) was assessed by 1-way ANOVA followed by Dunnett test.

Table 4. Marker Distributions in AGCT Tissue Microarray

A	**Immunohistochemical Staining**							
Marker	**Negative n (%)**		**Weak n (%)**		**Intermediate n (%)**		**High n (%)**	
	Primary	**Recurrent**	**Primary**	**Recurrent**	**Primary**	**Recurrent**	**Primary**	**Recurrent**
ERα (n = 165)	82 (71)	31 (62)	11 (10)	8 (16)	22 (19)	11 (22)	0	0
ERβ (n = 152)	7 (6)	2 (5)	34 (31)	6 (14)	29 (27)	11 (25)	38 (35)	25 (57)
Cyp19A1 (n = 156)	60 (54)	21 (48)	36 (32)	13 (30)	12 (11)	5 (11)	4 (4)	5 (11)
B	**RNA in situ hybridization**							
Marker	**Negative n (%)**		**Weak n (%)**		**Intermediate n(%)**		**High n (%)**	
	Primary	**Recurrent**	**Primary**	**Recurrent**	**Primary**	**Recurrent**	**Primary**	**Recurrent**
FSHR (n = 165)	11 (9)	6 (13)	31 (26)	18 (38)	60 (51)	21 (44)	15 (13)	3 (6)
Cyp19A1 (n = 167)	104 (89)	38 (76)	8 (7)	5 (10)	5 (4)	7 (14)	0	0
GPER (n = 161)	106 (95)	33 (66)	5 (5)	17 (34)	0	0	0	0

tumor ERα expression in patients with low serum inhibin B levels ($P = 0.04$), suggesting diminished inhibin B–FSH feedback in high ERα expressing AGCTs. We found no significant correlations between serum FSH or E2 levels and other estrogen receptors.

E. FSH Increases CYP19A1 Expression and E2 Production in AGCT Cells In Vitro

To study the functional role of FSH in AGCTs, KGN and primary AGCT cells were stimulated with FSH for 96 hours and then gene expression levels and E2 production were measured by qPCR and mass spectrometry, respectively. In KGN cells, *FSHR* expression was increased by FSH stimulation (Fig. 2E). Also, *CYP19A1* mRNA levels (Fig. 2F) and the amount of E2 in cell culture supernatants (Fig. 3A) were significantly increased by FSH. Additionally, FSH stimulated the expression of *ESR1*, the gene encoding ERα, whereas no statistically significant change in ERβ coding *ESR2* or *GPER1* levels was seen in KGN cells (Fig. 2G-I). FSH stimulation did not have an effect on viability in this cell line (Fig. 2J).

In primary patient-derived AGCT cells, consistent with our findings in KGN cells, FSH stimulation significantly increased the expression of *CYP19A1* (Fig. 2F) and secretion of E2 into the culture medium (Fig. 3B). Interestingly, the level of FSH or E2 in patient´s preoperative serum sample did not correlate with the amount of E2 measured in the culture media (Table 3). In contrast to the KGN cell line, FSH stimulation increased the mRNA expression of all the estrogen receptors (*ESR1*, *ESR2,* and *GPER1)* in all 6 of the patient-derived primary AGCT cells (Fig. 2G-I). Moreover, FSH stimulation increased cell viability in 3 of 6 AGCT primary cell cultures by 26% to 43% (Fig. 2J, Table 3). Importantly, these results indicate active hormonal signaling in AGCT cells.

F. CYP19A1 Is Expressed in a Subset of AGCTs

Aromatase inhibitors have been used in the treatment of relapsed AGCT [3]. These drugs inhibit the synthesis of estrogens from androgen precursors by binding to aromatase. To reveal the expression of CYP19A1 in a larger sample set, we performed RNA *in situ* hybridization and IHC on the TMA. *CYP19A1* mRNA was detected in 15% of the tumor specimens

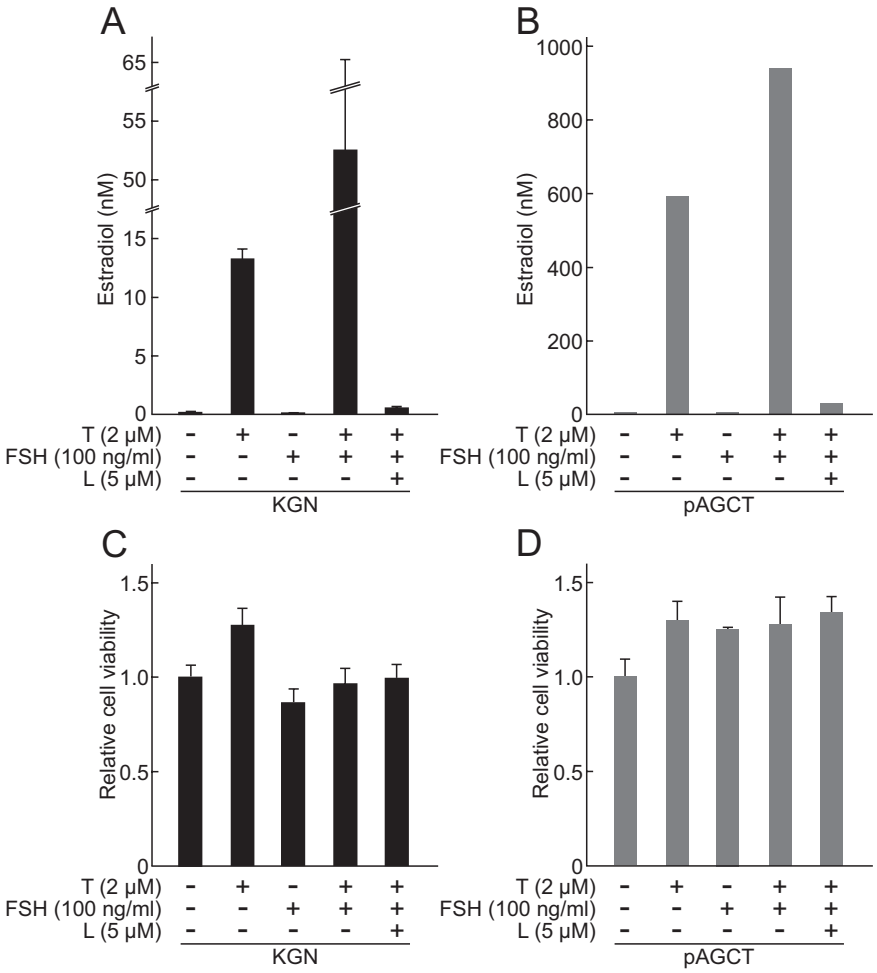

Figure 3. Letrozole blocks E2 production but does not affect cell viability in primary AGCT cells. KGN cells and 6 primary AGCT cultures were stimulated with FSH and letrozole (L) for 96 hours. Testosterone (T) was provided as a substrate. (A-B) E2 concentrations in cell culture supernatants were measured by mass spectrometry. (C-D) After stimulation cell viability was assessed by WST-1 assay. *P* values for (C) and (D) are shown in Table 5.

by RNA *in situ* hybridization (Fig. 4A-D). CYP19A1 immunoreactivity was detected in 48% of the tumors, and 17% showed moderate or strong staining (Fig. 4E-H, Table 4). *CYP19A1* mRNA expression correlated significantly with the moderate/high CYP19A1 protein expression ($P < 0.0001$). CYP19A1 protein expression also correlated positively with ERα expression ($P = 0.009$). There were no correlations between CYP19A1 expression and clinical parameters such as tumor size, stage, or recurrence rate. Also, CYP19A1 expression in the tumor tissue did not correlate with circulating E2, FSH, or inhibin B levels.

G. Aromatase Inhibition With Letrozole Suppresses E2 Production in AGCT Cells

Next, we studied the functional effects of CYP19A1 inhibition in KGN and primary AGCT cells. For this purpose, cells were treated with a 5-μM dose of the aromatase inhibitor letrozole and a 2-μM dose of testosterone as a substrate for CYP19A1. Testosterone significantly stimulated both E2 production and cell viability in both KGN and primary AGCT cells, confirming the presence of functional CYP19A1. FSH consistently enhanced the production of E2 in the presence of testosterone (Fig. 3A-B). Interestingly, letrozole treatment completely suppressed E2 production in the cell cultures (with and without FSH stimulation), but had no effect on cell viability in either KGN or primary AGCT cells (Fig. 3C-D, *P* values shown in Table 5).

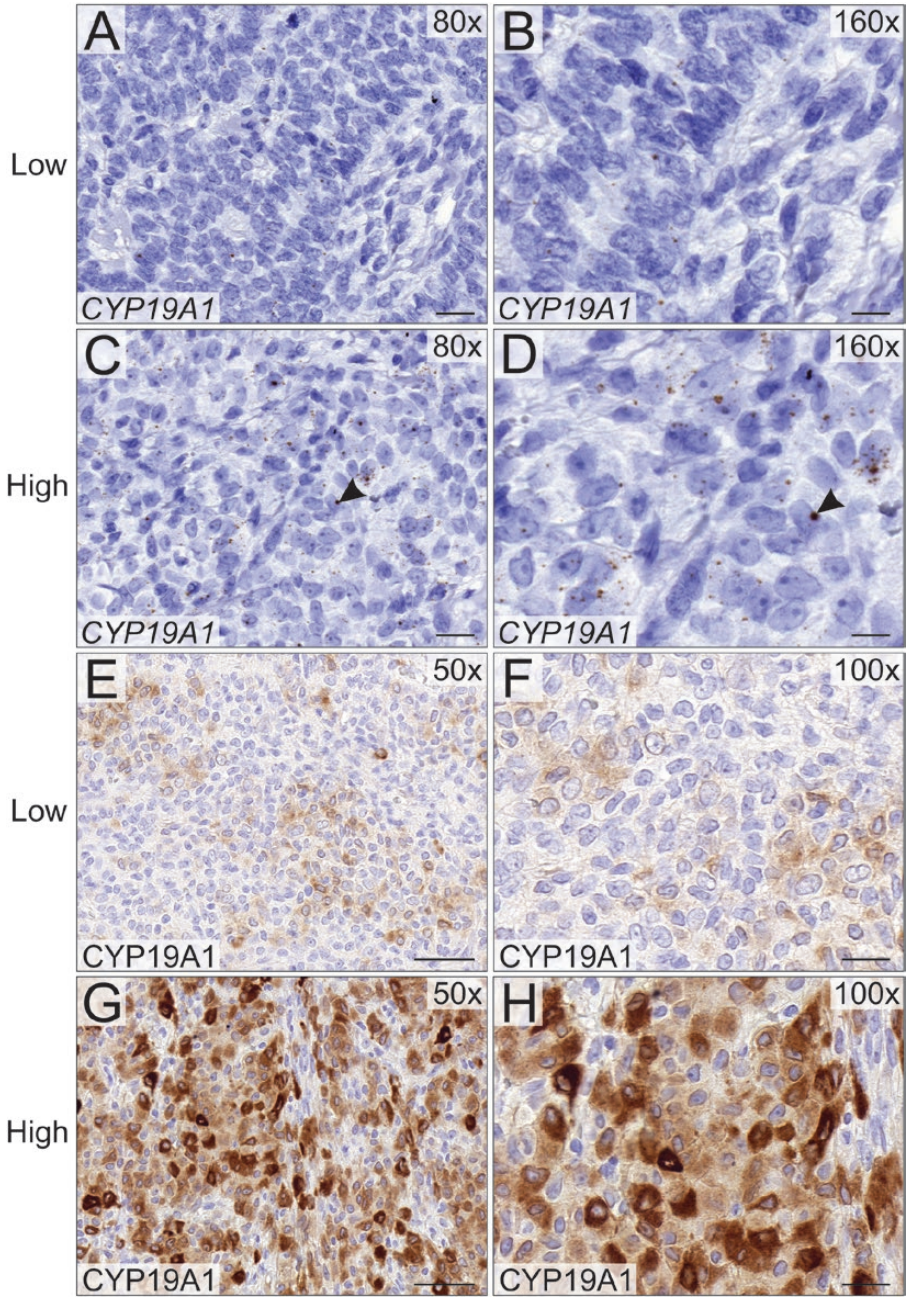

Figure 4. CYP19A1 is expressed in a subset of AGCTs. *CYP19A1* expression was assessed in the TMA by (A-D) RNA *in situ* hybridization and (E-H) IHC. Representative images of (A-B, E-F) low and (C-D, G-H) high staining of AGCTs. Magnifications: (A-D) 80× (scale bar: 20 μm) and (E-H) 160× (scale bar: 10 μm) and 50× (scale bar: 50 μm) and 100× (scale bar: 20 μm). Arrows indicate positively stained cells.

H. ERβ Is the Main Estrogen Receptor in AGCTs and Its Expression Is Higher in Recurrent Tumors

The expressions for estrogen receptors were further studied in the AGCT TMA using IHC or RNA *in situ* hybridization. ERα immunoreactivity was observed in 33% of the tumors, and ERβ protein was detected in 94% (Fig. 5A-H, Table 4). In the ERα-positive tumors, the staining pattern was nuclear in 59% and cytoplasmic in 48% of the tumors. ERβ immunoreactivity was exclusively nuclear and classified as moderate or strong in 67% of

Table 5. *P* **Values of the Hormone Stimulations (in Fig. 3) Using Student** *t*-**Test**

A				
KGN	**Control**	**Testosterone**	**FSH**	**Testosterone + FSH**
Testosterone	0.0001	-	-	-
FSH	<0.0001	<0.0001	-	-
Testosterone + FSH	0.506	<0.0001	0.061	-
Testosterone + FSH + Letrozole	0.897	<0.0001	0.021	0.591
B				
pAGCT	**Control**	**Testosterone**	**FSH**	**Testosterone + FSH**
Testosterone	<0.0001	-	-	-
FSH	0.0002	0.473	-	-
Testosterone + FSH	<0.0001	0.771	0.667	-
Testosterone + FSH + Letrozole	<0.0001	0.447	0.146	0.297

the tumors. *GPER1* mRNA expression was detected only in 14% of the tumors by RNA *in situ* hybridization (Fig. 5I-L). Our results are in line with the data from mRNA sequencing and thus confirm that ERβ is the predominant estrogen receptor in AGCT.

ERβ expression levels were significantly higher in recurrent AGCTs when compared to primary tumors ($P = 0.001$). Furthermore, paired analysis of primary and recurrent samples from same patients demonstrated a significantly stronger ERβ immunoreactivity in the recurrent AGCTs ($P = 0.0013$) compared with the primary tumor (Fig. 6A). A similar pattern between primary and recurrent samples was noted for *GPER1* mRNA expression, although the levels of expression were low (Fig. 6B). There were no significant differences in the expression levels of ERα between primary and recurrent samples. We also found no correlation between any of the estrogen receptor expression patterns and tumor size, stage at primary diagnosis, recurrence rate, or menopause status at the time of sample retrieval. None of the estrogen receptors had prognostic significance in terms of disease-free or overall survival.

I. E2 Increases AGCT Cell Viability Only at High Concentrations

Because FSH stimulation increased aromatase expression, E2 production, and AGCT cell viability, we next explored whether E2 alone affects AGCT cell viability. We found that exogenous E2 (0.1-1000 nM) did not affect the viability of KGN cells (Fig. 5P). Also, in primary AGCT cells E2 did not increase the cell number at 0.1 to 100 nM concentrations. However, the supraphysiological dose (1000 nM) increased the cell number on average by 36% in 4 of the 6 studied samples (range 23%–57%) (Fig. 5P, Table 3). This 1000-nM dose reflects the E2 concentrations that were seen in the AGCT cell culture supernatants after stimulation with FSH, indicating that high local E2 concentrations may exist in AGCTs. In 1 recurrent tumor, high E2 had no effect and in 1 primary tumor E2 decreased cell number by 21%. Interestingly, the tumors that did not respond to high E2 also did not respond to FSH stimulation, suggesting heterogeneous hormonal dependencies in individual AGCTs. No correlation between the level of FSH or E2 in patient´s preoperative serum sample and response to E2 stimulation could be seen (Table 3). We also found that E2 had no effect on the expression levels of estrogen receptors *ESR1*, *ESR2*, and *GPER1* in KGN cells (Fig. 5M-O). However, in primary AGCT cells, E2 treatment increased *ESR2* expression in all of the cultures indicating a positive feedback mechanism of E2 and *ESR2* expression in AGCTs (Fig. 5N).

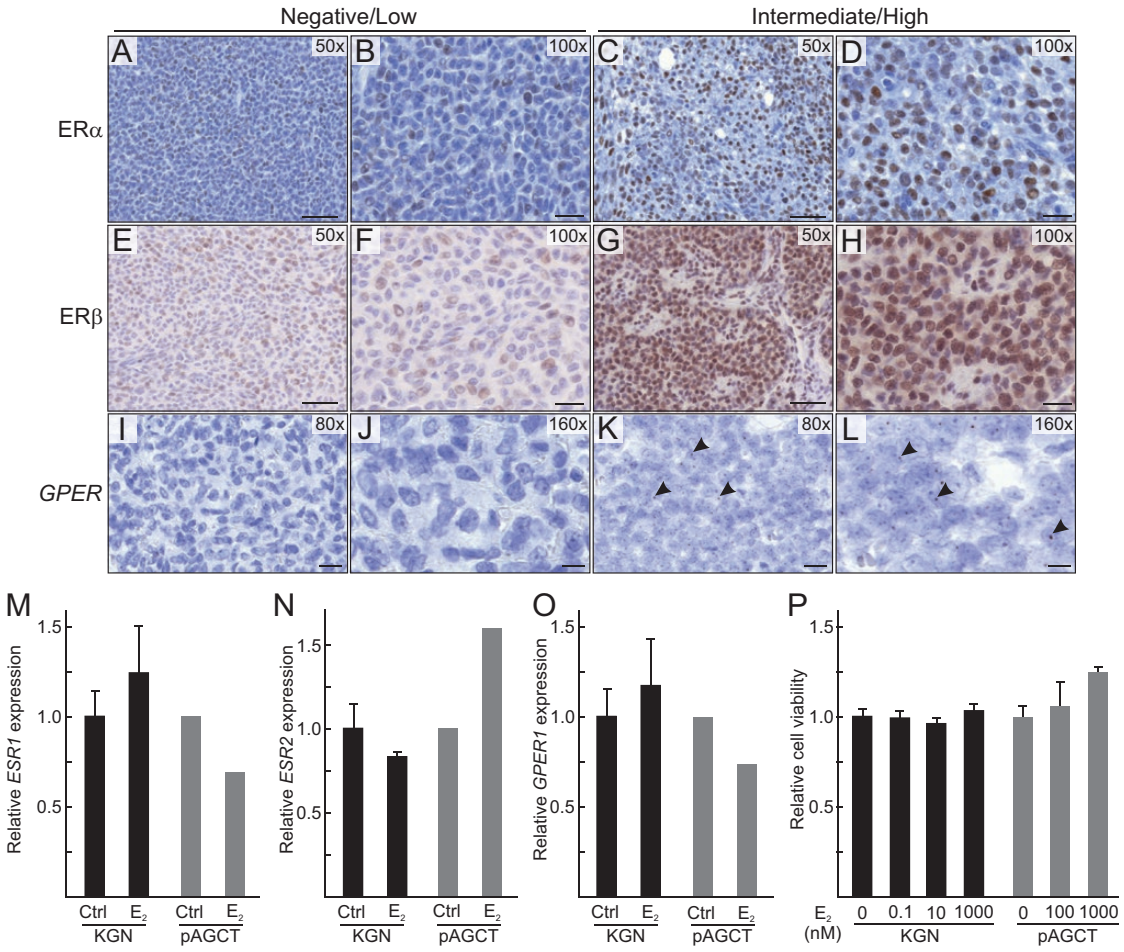

Figure 5. Expression of estrogen receptors and effects of E2 stimulation in AGCTs (A-H). Expression of ERα and ERβ was assessed by IHC. Representative images of low and high staining patterns, magnifications 50× (scale bar: 50 μm) and 100× (scale bar: 20 μm). (I-L) Expression of *GPER1* mRNA was determined by RNA *in situ* hybridization. Representative images of low and high staining patterns, magnifications 80× (scale bars: 20 μm) and 160× (scale bars: 10 μm). (M-O) Expression of *ESR1, ESR2,* and *GPER1* was assessed by qPCR after stimulation with E2 (0-1000 nM) for 96 hours. (P) Cell viability was measured after stimulation with E2 (0-1000 nM) for 96 hours. Black columns: KGN cell line; gray columns: primary AGCT cells.

3. Discussion

Hormonal activity is one of the hallmarks of AGCTs. Indeed, these tumors are often diagnosed in perimenopause because of signs or symptoms of hormone production, such as vaginal bleeding caused by estrogen-induced endometrial hyperplasia. As the peak incidence of AGCTs is in perimenopause or early postmenopause, it has been suggested that alterations in circulating hormone levels, such as the rise in serum gonadotropin levels, contributes to tumor formation. Additional knowledge on the possible hormonal modulation of these unique tumors is needed given that anti-hormone therapies are currently used in AGCT patient care.

Here, we first characterized the expression of sex hormone receptors at transcriptional level using a large set of diagnostically validated AGCTs with an extensive clinical data and follow-up time. In the transcriptional profiling, we did not find any significant differences in the expression of hormone receptors between primary and recurrent tumors. Even though our sample size in this experiment was limited, it supports earlier findings, as a previous

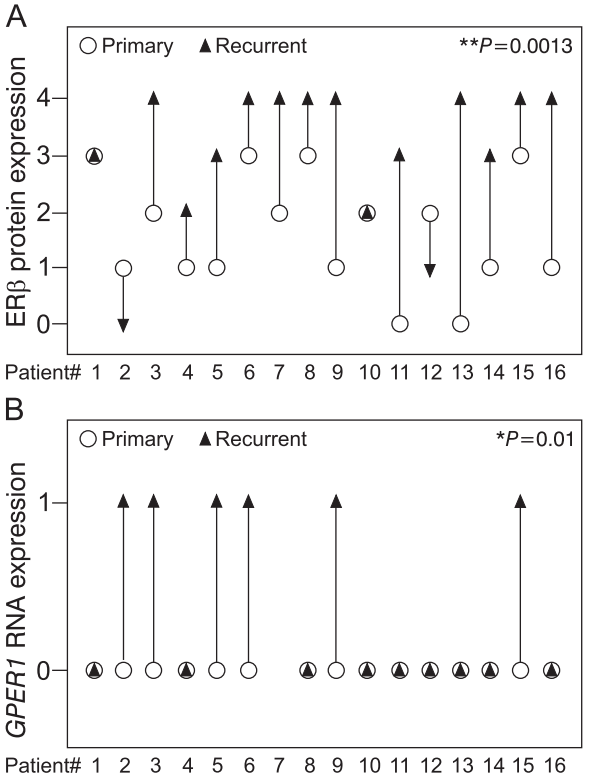

Figure 6. ERβ and *GPER1* expression levels were increased in recurrent AGCTs compared with primary tumors. Paired analysis of the expression of estrogen receptors (A) ERβ and (B) *GPER1* in 16 patients with matched primary and recurrent tumor samples in the TMA.

transcriptomic analysis reported mainly similar expression patterns between stage I and advanced AGCTs [42].

Genes with differential expression between primary and recurrent tumors included 3 adenylate cyclases, of which *ADCY4* expression has earlier been correlated with better survival in breast cancer patients and *ADCY3* has been recognized as a potential biomarker in gastric cancer [43, 44]. A lower expression level for gene encoding for transcription factor *CREB3L1* in recurrent tumors was intriguing as decreased CREB3L1 expression has earlier been linked to reduced progression free survival in breast cancer [45]. The high expression levels of *HSP90B1* in both primary and recurrent tumors were noteworthy as HSP90 chaperone proteins have been shown to regulate hormone receptors and have a crucial role in normal granulosa cell function [46, 47]. According to an earlier report, increased HSP90B1 expression levels have been positively associated with ovarian granulosa cell proliferation and survival [48]. The herein detected high expression levels of *HSP90B1* and members of the PI3K/Akt pathway suggest their role as potential targets for therapy. This is further supported by the results from our previous work in which we performed unbiased high-throughput drug screening on AGCT samples demonstrating that both HSP90 and PI3K-inhibitors reduced the cell viability of AGCT cells [49]. Regardless, that these drugs were not selective toward AGCT cells in comparison with normal granulosa cells in the screen, our present finding supports the therapeutic potential of HSP90 and PI3K-inhibitors in AGCT patients.

Consistent with our findings at the transcriptional level, we confirmed a strong uniform expression of *FSHR* in a large set of AGCTs by using RNA *in situ* hybridization, a finding that has not been shown earlier in more than a few individual AGCTs because of the lack of specific IHC-compatible antibodies. Importantly, we observed an increase in cell number in 3/6 of the tested primary AGCT samples following FSH stimulation. This FSH effect in

primary AGCT cultures is most likely mediated by P13K/Akt and MAPK pathways [50]. Cell viability was not affected in the KGN cell line by FSH, which may be due to the low FSHR expression status in these cells. Our data on the low serum FSH levels is in line with earlier findings in AGCT patients [51, 52], and the inverse correlation between serum FSH and inhibin B levels in this study supports the notion that tumor-derived inhibin B regulates pituitary FSH secretion.

The role of FSH in inducing CYP19A1 activity in normal granulosa cells is well established [53]. Regardless of the FSH stimulation priming of the hGL cells, the average *CYP19A1* mRNA expression levels tended to be higher in AGCTs. Further, we found a significant increase in expression of *CYP19A1* gene after FSH stimulation in both KGN and primary AGCT cells, supporting the role of FSH in E2 production in tumor cells. An earlier study using primary rat granulosa cells showed that FSH regulates *CYP19A1* via GATA4 [54], a transcription factor known to be overexpressed in AGCTs [55]. Another study reported the direct regulation of aromatase by FOXL2 and proposed that the C134W mutation increases this stimulatory effect [16]. Moreover, induction of aromatase activity has been observed as a consequence of the interaction of FOXL2C134W with SMAD3, a transcription factor widely expressed in AGCTs [56, 57]. We found that *CYP19A1* gene expression was highly variable among studied AGCT samples both at the transcriptomic and protein level. Moreover, the protein expression was positive in 48% of the studied tumors, which is a lower proportion than previously reported [58, 59]. This finding might explain the observed variation in the responsiveness to anti-estrogen therapies [3, 10]. Furthermore, CYP19A1 expression may be dependent on constitutive FSH stimulation, which is suppressed in AGCT patients through the downregulation of FSH secretion from the pituitary by tumor-derived inhibin B. Another factor secreted by AGCTs, anti-Müllerian hormone, has been shown to attenuate FSH-mediated stimulation of CYP19A1 expression [13]. Thus, the observed variability between individual AGCTs may be due to the complex regulatory networks of *CYP19A1* expression [60].

The role of E2 in AGCT growth and survival has been controversial. Estrogen promotes tumor growth in various cancers [61], and the safety of estrogen replacement therapy of AGCT patients remains questionable [62]. In a previous study with a long follow-up time, postmenopausal estrogen replacement therapy did not negatively impact the prognosis of AGCT patients [63]. Furthermore, earlier studies on KGN cells did not support a growth-stimulating effect of E2 [21, 22, 64]; indeed, it has been shown that E2 may decrease KGN cell migration [22]. In line with these reports, we did not find any effect on KGN cell viability in response to E2 stimulation. Further, in the primary AGCT cell cultures, no effects on cell viability were seen at physiological concentrations. However, an increase in cell number was detected in 4/6 of the primary AGCT cell cultures tested when using an extremely high estradiol dose. Our results thus confirm the earlier findings of negligible responsiveness of AGCTs cells to estradiol and provide further evidence on that estrogen replacement therapy can be considered safe in AGCT patients with no evidence of disease.

Consistent with earlier studies [7, 20], we found that ERβ was the principal estrogen receptor expressed in AGCTs. ERα expression was markedly lower than ERβ, but its correlation with circulating FSH and tissue CYP19A1 expression and that FSH stimulation increased ERα expression suggest that ERα may have a functional role in a subset of AGCTs. In other hormone-related malignancies, such as breast and prostate cancer, ERα is considered tumorigenic, whereas ERβ mostly functions as a tumor suppressor by blocking the proliferation and inducing apoptosis [61, 65, 66]. ERβ effects are also dependent on the ERβ-subtype [67, 68]. Increased ERβ expression in recurrent tumors suggests the significance of ERβ in this unique slow growing tumor type, but the specific effect of ERβ in tumor progression awaits further evidence. It is of note that *GPER1* expression was shown to be minimal both according to the RNA sequencing and RNA *in situ* methods, contradicting earlier findings reporting its expression in AGCT [22]. We observed robust RNA expression of *FSHR* and *ESR1* in hGL cells consistent with the FSH-priming associated with the IVF protocol. Consistent with previous observations, we confirmed strong expression

of *CYP19A1, ESR2*, and *FSHR* in AGCT cells. Because of the high variability of expression levels, especially in AGCT cells, increased numbers of biological replicates would be needed to make further clinical conclusions.

Several case studies report variable responses to divergent hormonal treatments in AGCTs, and among these therapies aromatase inhibitors have been the most promising [3]. We did not, however, observe any changes in cell viability after letrozole treatment, even though the used doses were sufficient to suppress E2 production in AGCT cells. This unresponsiveness to letrozole was also noted in our earlier study when the cell viability of 7 primary AGCT samples were studied by high-throughput drug screening [49]. In our current patient series, a total of 10 patients were treated with letrozole, generally as a last therapeutic option when other medical treatments had proven ineffective. Only 1 of these patients had stable disease, whereas 9/10 had disease progression during letrozole treatment. Our findings on negligible efficacy of aromatase inhibitors in AGCT *in vitro* are thus in keeping with the clinical practice. An increased cell viability in response to testosterone stimulation raises questions of the role of testosterone in AGCTs. According to earlier reports, testosterone inhibits apoptosis in normal granulosa cells contributing to follicular growth and proliferation [69, 70]. A recent study reported moderate staining for androgen receptor in AGCT cells, also implicating further studies on the role of androgens in this unique tumor type [21]. However, our results highlight a previously unexplored finding warranting further studies on the direct effects of androgens on AGCTs by, for instance, studying the effects of dihydrotestosterone on the proliferation and survival of AGCT cells.

The present work confirms the wide expression of sex hormone receptors, and active hormonal signaling in AGCTs, encouraging further studies on hormonal modulation in AGCT treatment. Even though we did not find *in vitro* evidence to support the use of aromatase inhibitors in AGCT treatment, considering the small sample size and variable effects of FSH and estradiol in primary AGCT cells, we cannot exclude that some patients may gain benefit of these compounds. Future studies should examine whether inhibition of both estrogen and testosterone receptors would be more efficient in the treatment of AGCTs. Finally, prospective, randomized clinical trials, accompanied by careful biomarker analyses, are needed to determine the efficacy of hormonal therapies in AGCTs.

Acknowledgments

The authors thank Rainer Lehtonen for help in manuscript preparation.

Financial Support: This study was supported by grants from the Academy of Finland, Sigrid Jusélius Foundation, Orion Research Foundation, Finnish Cancer Foundation, Helsinki University Hospital Research Funds, University of Helsinki Graduate school, Sladjana M. Crosley Foundation for GCT Research, and DoD Award W81XWH-16-1-0188.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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