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Angaben zur Veröffentlichung / Publication details:

Czogalla, Bastian, Alexandra Partenheimer, Udo Jeschke, Viktoria von Schönfeldt, Doris Mayr, Sven Mahner, Alexander Burges, et al. 2020. "β-arrestin 2 is a prognostic factor for survival of ovarian cancer patients upregulating cell proliferation." *Frontiers in Endocrinology* 11: 554733. <https://doi.org/10.3389/fendo.2020.554733>.



β -arrestin 2 Is a Prognostic Factor for Survival of Ovarian Cancer Patients Upregulating Cell Proliferation

Bastian Czogalla^{1†}, Alexandra Partenheimer^{1†}, Udo Jeschke^{1,2}, Viktoria von Schönfeldt¹, Doris Mayr³, Sven Mahner¹, Alexander Burges¹, Manuela Simoni^{4,5,6,7}, Beatrice Melli⁴, Riccardo Benevelli⁴, Sara Bertini⁴, Livio Casarini^{4,5†} and Fabian Trillsch^{1‡}

OPEN ACCESS

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*Correspondence:

Bastian Czogalla
bastian.czogalla@
med.uni-muenchen.de

[†]These authors share first authorship

[‡]These authors share
senior authorship

Specialty section:

This article was submitted to
Cancer Endocrinology,
a section of the journal
Frontiers in Endocrinology

Received: 22 April 2020

Accepted: 12 August 2020

Published: 18 September 2020

Citation:

Czogalla B, Partenheimer A,
Jeschke U, von Schönfeldt V, Mayr D,
Mahner S, Burges A, Simoni M,
Melli B, Benevelli R, Bertini S,
Casarini L and Trillsch F (2020)
 β -arrestin 2 Is a Prognostic Factor for
Survival of Ovarian Cancer Patients
Upregulating Cell Proliferation.
Front. Endocrinol. 11:554733.
doi: 10.3389/fendo.2020.554733

¹ Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Munich, Germany, ² Department of Obstetrics and Gynecology, University Hospital Augsburg, Augsburg, Germany, ³ Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany, ⁴ Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy, ⁵ Center for Genomic Research, University of Modena and Reggio Emilia, Modena, Italy, ⁶ Unit of Endocrinology, Department of Medical Specialties, Azienda Ospedaliero-Universitaria, Modena, Italy, ⁷ PRC, INRA, CNRS, IFCE, Université de Tours, Nouzilly, France

Establishing reliable prognostic factors as well as specific targets for new therapeutic approaches is an urgent requirement in advanced ovarian cancer. For several tumor entities, the ubiquitously spread scaffold protein β -arrestin 2, a multifunctional scaffold protein regulating signal transduction and internalization of activated G protein-coupled receptors (GPCRs), has been considered with rising interest for carcinogenesis. Therefore, we aimed to elucidate the prognostic impact of β -arrestin 2 and its functional role in ovarian cancer. β -arrestin 2 expression was analyzed in a subset of 156 samples of ovarian cancer patients by immunohistochemistry. Cytoplasmic expression levels were correlated with clinical as well as pathological characteristics and with prognosis. The biologic impact of β -arrestin 2 on cell proliferation and survival was evaluated, *in vitro*. Following transient transfection by increasing concentrations of plasmid encoding β -arrestin 2, different cell lines were evaluated in cell viability and death. β -arrestin 2 was detected in all histological ovarian cancer subtypes with highest intensity in clear cell histology. High β -arrestin 2 expression levels correlated with high-grade serous histology and the expression of the gonadotropin receptors FSHR and LHCGR, as well as the membrane estrogen receptor GPER and hCG β . Higher cytoplasmic β -arrestin 2 expression was associated with a significantly impaired prognosis (median 29.88 vs. 50.64 months; $P = 0.025$). Clinical data were confirmed in transfected HEK293 cells, human immortalized granulosa cell line (hGL5) and the ovarian cancer cell line A2780 *in vitro*, where the induction of β -arrestin 2 cDNA expression enhanced cell viability, while the depletion of the molecule by siRNA resulted in cell death. Reflecting the role of β -arrestin 2 in modulating GPCR-induced proliferative and anti-apoptotic signals, we propose β -arrestin 2 as an important prognostic factor and also as a promising target for new therapeutic approaches in advanced ovarian cancer.

Keywords: β -arrestin 2, ovarian cancer, immunohistochemistry, survival analysis, G protein-coupled receptor, *in vitro* analyses

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy and is considered the fifth leading cause of death among women (1), mainly due to late diagnosis in advanced tumor stage leading to overall poor prognosis. Lack of inadequate screening methods and rising resistances toward chemotherapy over the clinical course further contribute to the relatively low 5-years survival at around 45% (1, 2). Standard treatment for advanced EOC consists of primary cytoreductive surgery, followed by platinum-based combination chemotherapy followed by targeted therapies like the anti-angiogenic antibody bevacizumab or PARP inhibitors. To date, the most reliable prognostic factors include volume of post-operative residual disease, tumor stage diagnosed according to the International Federation of Gynecology and Obstetrics (FIGO) staging system, patient's age and histology (2–7). EOCs are classified into four histological subtypes: serous, mucinous, endometrioid, and clear-cell, which vary in terms of phenotype, etiology and molecular background (8). Taking the heterogeneity of ovarian cancer into account appears crucial for developing new prognostic and therapeutic strategies.

β -arrestin 2 is a multifunctional scaffold protein regulating signal transduction and internalization of activated G protein-coupled receptors (GPCRs) like follicle-stimulating hormone receptor (FSHR) and luteinizing hormone/choriogonadotropin receptor (LHCGR) by promoting clathrin-dependent endocytosis (9). The β -arrestin family is a known activator of signaling pathways involved in tumorigenesis (10–12) and proliferation of certain tumor entities (13–17), including ovarian cancer (18). Functional association between FSHR/LHCGR and β -arrestin in granulosa cells could be demonstrated after gonadotropin treatment *in vitro* (19, 20). In immortalized ovarian cell lines, β -arrestin 2 triggers the activation of FSH-induced mitogen-activated protein (MAP) kinase signaling cascades linked to anti-apoptotic and proliferative events (21).

In this study, we aimed to evaluate the possible prognostic impact of β -arrestin 2 on clinical course of ovarian cancer. The role of β -arrestin 2 in modulating cell viability and survival was elucidated by functional analyses *in vitro*, suggesting new therapeutic approaches in ovarian cancer.

MATERIALS AND METHODS

Patients and Specimens

156 ovarian cancer samples of patients that underwent surgery between 1990 and 2002 at the Department of Obstetrics and Gynecology, Ludwig Maximilian University in Munich, Germany, were analyzed (Table 1). The clinical data were received from the patient's charts, whereas the follow-up data from the Munich Cancer Registry (MCR). Only patients with a clear diagnosis of ovarian cancer and were included in this study, while benign tumors, just as borderline tumors were excluded. These patients had no history of previous or secondary cancers. Also, none of the patients had neoadjuvant chemotherapy. After the samples had been formalin-fixed and paraffin-embedded (FFPE), they were evaluated by a specialized pathologist at the

TABLE 1 | Patient characteristics.

Parameters	N	Percentage (%)
HISTOLOGY		
Serous	110	70.5
Clear cell	12	7.7
Endometrioid	21	13.5
Mucinous	13	8.3
LYMPH NODE		
pNX	61	39.1
pN0	43	27.6
pN1	52	33.3
DISTANT METASTASIS		
pM0/X	150	96.2
pM1	6	3.8
GRADING SEROUS		
Low	24	23.0
High	80	77.0
ENDOMETRIOID		
G1	6	31.6
G2	5	26.3
G3	8	42.1
MUCINOUS		
G1	6	50.0
G2	6	50.0
G3	0	0
CLEAR CELL		
G3	9	100
FIGO		
I	35	23.1
II	10	6.6
III	103	68.2
IV	3	2.0
AGE		
≤60 years	83	53.2
>60 years	73	46.8

department of Pathology, Ludwig Maximilian University, who classified them into histological subtypes [serous ($n = 110$), endometrioid ($n = 21$), mucinous ($n = 13$), clear-cell ($n = 12$)] and rated the tumor grading. The serous ovarian cancer samples were divided into low and high grading. Endometrioid ovarian cancer was graded according to G1–G3. For the mucinous carcinoma, there is no WHO classification; however, the subtype is often classified into G1–G3. The clear cell cancer was always categorized as G3. Staging was performed using the FIGO (WHO) and TNM classification. I ($n = 35$), II ($n = 10$), III ($n = 103$), and IV ($n = 3$). Data on primary tumor extension was available in 155 cases: T1 ($n = 40$), T2 ($n = 18$), T3 ($n = 93$), T4 ($n = 4$), and on lymph node involvement in 95 cases: N0 ($n = 43$), N1 ($n = 52$). In nine cases available was the data on distant metastasis: M0 ($n = 3$), M1 ($n = 6$).

Ethical Approval

This study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 227-09 and 18-392). All tissue samples used for this study were obtained from leftover material from the archives of LMU Munich, Department Gynecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany, initially used for pathological diagnostics. The diagnostic procedures were completed before the current study was performed. During all experimental and statistical analysis, the observers were fully blinded to patient's data. All experiments were performed according to the standards of the Declaration of Helsinki (1975). As per declaration of our ethics committee no written informed consent of the participants or permission to publish is needed given the circumstances described above.

Immunohistochemistry

The formalin-fixed and paraffin-embedded ovarian cancer tissue samples were dewaxed in xylene for 20 min, before adding 100% ethanol to completely remove the Xylol. Unspecific color responses were avoided by blocking the endogenous peroxidase with 3% H_2O_2 in methanol (3 ml 30% H_2O_2 + 97 ml methanol), followed by rehydrating it in 70%, then 50% ethanol. Afterwards the slides were placed in a pressure cooker for 10 min, using sodium citrate buffer (pH = 6), made from 0.1 M citric acid and 0.1 M sodium citrate). After cooling down, this was followed by washing the samples first in distilled water, then in phosphate buffered saline (PBS) twice. After preprocessing, the slides were incubated in a blocking solution (ZytoChem Plus HRP Polymer System, Berlin, Germany, POLHRP-100) for 5 min to prevent an unspecific staining reaction. This was followed by a 16-h incubation overnight at 4°C with the primary antibodies: anti- β -arrestin 2, rabbit IgG, polyclonal, Abcam, ab151774 at a 1:1,500 dilution; anti-FSHR, rabbit IgG, polyclonal, Novus Biologicals, NLS2231 at a 1:100 dilution, anti-LHCGR, rabbit IgG, polyclonal, Acris, SP4594P at a 1:800 dilution; anti-GPER, rabbit IgG, polyclonal, Lifespan Biosciences, LS92467 at a 1:600 dilution and anti-hCG β , rabbit IgG, polyclonal, Dako, A-0231 at a 1:300 dilution—then washed in PBS twice. Next step was the application of reagent 2 (ZytoChem Plus HRP Polymer System, Berlin, Germany, POLHRP-100), consisting of a corresponding biotinylated secondary anti-rabbit IgG antibody and the associated avidin-biotin-peroxidase complex, for 20 min. For the visualization reaction 3,3 Diaminobenzidine (DAB) and a substrate buffer (Liquid DAB and Substrate Chromogen System, Dako, Munich, Germany, K3468) was used for 30 min, followed by distilled water, to stop the reaction. After counterstaining the slides with Mayer's acidic hematoxylin (Waldeck-Chroma, Münster, Germany, catalog-number 2E-038) for 2 min, they were dehydrated in an ascending series of alcohol (70, 96, 100%), brightened by adding xylol and then covered. Negative and positive controls were used to assess the specificity of the immunoreactions. Negative controls (colored in blue) were performed in kidney, placental and uterine tissue by replacement of the primary antibodies by species-specific (rabbit) isotype control antibodies (Dako, Glostrup, Denmark). For positive controls, placental and kidney tissues were used.

Staining Evaluation

All EOC specimens were examined with a Leitz photomicroscope (Wetzlar, Germany) and specific β -arrestin 2 immunohistochemically staining reaction was observed in the cytoplasm of the cells. The intensity and distribution pattern of β -arrestin 2 staining was rated using the semi-quantitative immunoreactive score (IR score, Remmele's score). To obtain the IR score result, the optional staining intensity (0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and the percentage of positive stained cells (0 = no staining, 1 = <10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells and 4 = >81%) were multiplied. Cut-off points for the IR scores were selected for the β -arrestin 2 staining considering the distribution pattern of IR scores in the collective. β -arrestin 2 staining was regarded as low with IRS 0–3 and as high with IRS \geq 4. Renal biopsies from patients with different *ARRB2* expression levels served as positive and negative control staining (22) (Figures 1E,F).

Cell Lines and Transfection Protocol

The immortalized human granulosa (hGL5) cell line of ovarian origin (23), the human embryonic kidney (HEK293) and the ovarian carcinoma (A2780) cell lines were used for *in vitro* experiments. Cells were cultured and handled as previously described (21, 24–26). hGL5 cells culture medium was DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/mL penicillin and 50 μ g/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 2% ITS + Premix Universal Culture Supplement (#354352; Corning Incorporated, Corning, NY, USA). The HEK293 cell line was cultured in DMEM additioned of 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin and 50 μ g/mL streptomycin. The A2780 cell line served as a model of ovarian cancer and was cultured in RPMI1640 medium (Sigma-Aldrich) additioned of 10% FBS, 1% Hepes (Sigma-Aldrich), 2 mM L-glutamine, 100 IU/mL penicillin and 50 μ g/mL streptomycin. Cell lines were maintained in an incubator at 37°C and 5% CO_2 .

The transfection protocol was described previously (25–28). Briefly, 3.5×10^4 cells were transiently transfected in a 96-well plate using increasing amount (range: 0–200 ng/well) of β -arrestin 2-encoding pcDNA3.1 plasmid (29) or commercial siRNA probes against β -arrestin 2 (#14387; Life-Technologies, Carlsbad, CA, USA) (21), using an electroporator (Gene Pulser MXcell; Bio-Rad Laboratories Inc., Hercules, CA, USA) in accordance with established protocol and settings (21, 30). Control probes without specific target mRNAs were loaded for achieving equal amount of siRNA among wells.

Western Blot Analysis and Antibodies

Overexpression and knock-down of β -arrestin 2 and pro-caspase 3 were evaluated by Western blotting, as previously described (21, 24). Briefly, transfected cells were seeded in 24-well plates (1×10^5 cells/well) and cultured 48 h before to be lysed using 4°C RIPA buffer. β -arrestin 2 and pro-caspase 3 were evaluated by 12% SDS-page and Western blotting, using specific antibodies (#3857, Cell Signaling Technology Inc., Danvers, MA, USA; #MA1-91637, Thermo-Fisher Scientific,

respectively). β -actin signals served as loading controls and were detected using a horseradish peroxidase (HRP)-conjugated specific antibody (#A3854; Sigma-Aldrich). Signals were revealed by ECL chemiluminescent compound (GE HealthCare), after incubation of the membranes with a secondary anti-rabbit HRP-conjugated antibody (#NA9340V; GE HealthCare), except for β -actin. Western blotting signals were acquired by the Quantity One analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Analysis of Cell Viability and Death

hGLC, HEK293, and A2780 cells were seeded in 96-well plates (1×10^4 cells/well) before viability assessment the by MTT colorimetric assay (31), using a previously validated protocol (21, 30). The absorbance was detected by a Victor3 plate reader (Perkin Elmer Inc., Waltham, MA, USA). A measure of death cells was obtained by incubating samples 30 min with propidium iodide (32). Cells were washed twice by PBS before excitation and light emission detection by a CLARIOstar microplate reader (BMG LabTech, Ortenberg, Germany), following the manufacturer's protocol. Data were represented as box and whiskers plots in a graph, as a measure of cell viability, and control of protein amount per well was performed by Bradford assay.

Statistical Analysis

IBM SPSS Statistics, version 25.0 (IBM Corporation, Armonk, NY, USA) was used for collecting and analyzing all the data for statistical significance. Overall-survival was compared with Kaplan-Meier curves and log-rank testing was used to detect differences in patients' overall survival times. The Spearman correlation coefficient (cc) was used for detecting correlations between immunohistochemical staining outcomes. For multivariate analyses, the cox regression was performed. Results obtained from cell lines were analyzed by D'Agostino and Pearson normality test before applying the Kruskal-Wallis test together with Dunn's correction for multiple comparisons, using the GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). For all analysis a P -value of <0.05 was considered to be statistically significant.

RESULTS

β -arrestin 2 Expression and the Correlation to Clinical and Pathological Characteristics

The clinicopathologic characteristics of the analyzed ovarian cancer patients are listed in **Table 1**. 104 (67%) out of 156 ovarian cancer specimens showed positive cytoplasmic β -arrestin 2 expression. The median immunoreactive score (IRS) of cytoplasm staining was 2 (with a range of 0–12). Clear cell histology exhibited the strongest β -arrestin 2 staining (Kruskal-Wallis test; $p = 0.01$) when compared with different histological subtypes (**Figures 1A–D**).

Representative staining of benign ovarian and fallopian tube tissue show positive results in epithelial cells, granulosa cells and theca cells. The ovarian and fallopian tube stroma did not show a β -arrestin 2 staining (**Figures 2A,B**).

We analyzed the correlation between β -arrestin 2 and clinicopathological data, such as histology, grading, and FIGO classification (**Table 2**). A positive correlation was observed between high β -arrestin 2 expression and high-grade serous histology (Spearman's correlation analysis; $p = 0.041$; $cc = 0.169$). Moreover, no other correlations were observed.

β -arrestin 2 Correlates With Gonadotropin Receptors Expression

We then analyzed the correlation between β -arrestin 2 expression and other parameters previously examined (33–37) in the same ovarian cancer cohort (**Table 3**). A positive correlation was detected to the expression of the gonadotropin receptors FSHR ($p = 0.029$; $cc = 0.183$) and LHCGR ($p = 0.001$; $cc = 0.284$), the G protein-coupled membrane estrogen receptor GPER ($p = 0.0009$; $cc = 0.215$) as well as with the choriogonadotropin beta subunit (hCG β) ($p < 0.001$; $cc = 0.36$). Representative stainings of the correlated parameters are shown in **Supplementary Figure 1**.

High β -arrestin 2 Expression Is Associated With Impaired Overall Survival

Age of the patient cohort was 58.7 ± 31.4 years (median \pm standard deviation, SD; $n = 156$) with a range of 31–88 years. The overall survival of the EOC patients was 34.4 ± 57.8 months (median \pm SD; $n = 156$). High β -arrestin 2 expression (IRS 4–12) is associated with shorter overall survival. As depicted on the Kaplan-Meier curve, higher cytoplasmic β -arrestin 2 expression correlates with a significantly impaired prognosis (**Figure 3**; 50.64 vs. 29.88 months, median; $p = 0.025$).

Clinicopathological Parameters as Independent Prognostic Factors

A multivariate cox-regression analysis was performed to detect which parameters were independent prognostic factors for overall survival in the present cohort. In this analysis, patients' age ($p = 0.014$) and FIGO stage ($p < 0.001$) were independent factors for overall survival. High β -arrestin 2 ($p = 0.152$), however, was not confirmed as an independent factor of prognostic independence (**Table 4**).

Effects of β -arrestin 2 Overexpression and Depletion on Cell Viability

Since β -arrestin 2 expression levels were found to correlate with overall survival of ovarian cancer patients, the impact of the molecule on cell proliferation and survival was evaluated, *in vitro*. Cell viability and death are represented by box and whiskers plots (**Figure 4**).

Western blot analysis demonstrated that β -arrestin 2 expression levels increase together with the amount of cDNA encoding the molecule, in the HEK293, hGL5 and A2780 cell lines (**Figure 4A**). We found increased viability of all cell models overexpressing β -arrestin 2, compared to mock-transfected cells used as controls (**Figures 4B–D**; Kruskal-Wallis test; $p < 0.01$; $n = 8$). Moreover, highest levels of cell viability corresponded to the highest amount of plasmid transfected. These results are corroborated by data from propidium iodide-stained HEK293

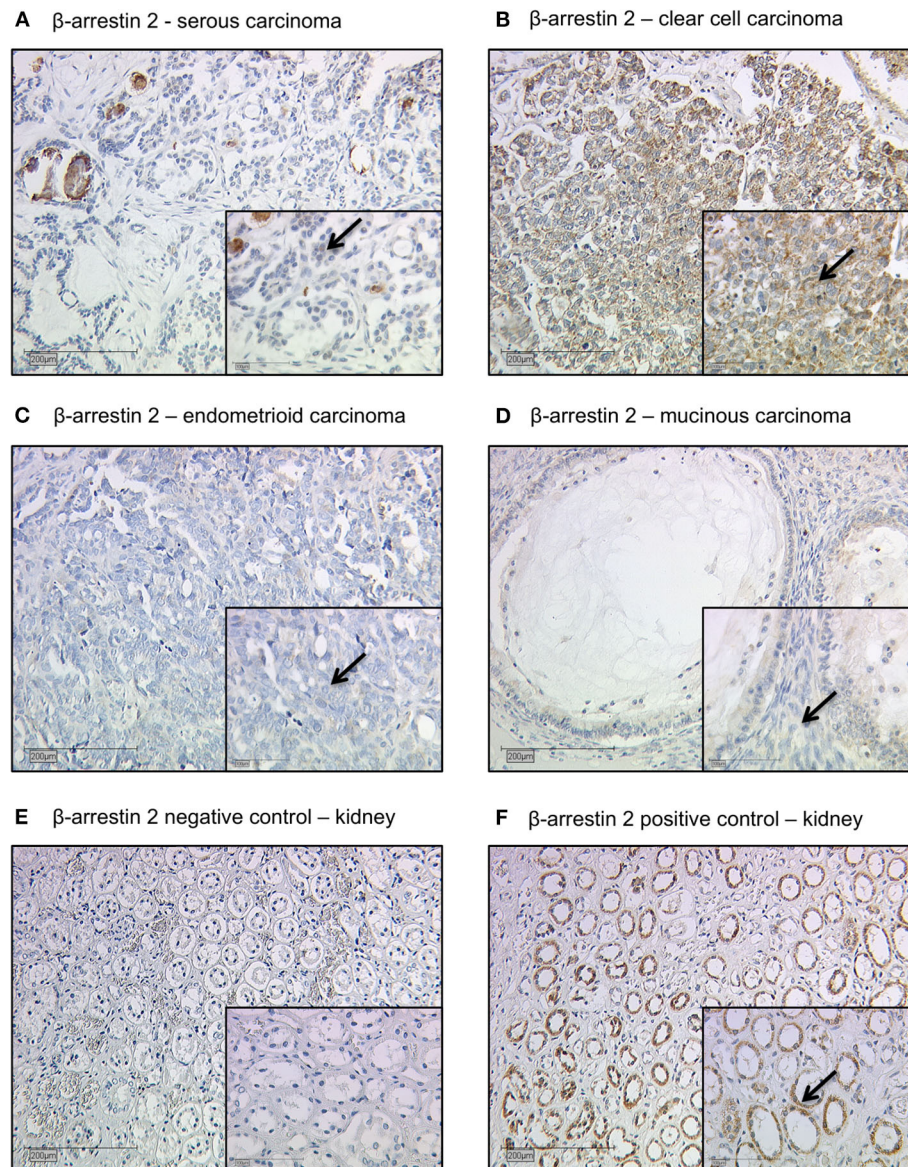


FIGURE 1 | Detection of β -arrestin 2 with immunohistochemistry. **(A)** Cytoplasmic β -arrestin 2 staining in ovarian cancer with serous, **(B)** clear cell, **(C)** endometrioid, and **(D)** mucinous histology. **(E)** β -arrestin 2 negative control **(F)** and positive control in human kidney tissue. Clear cell histology exhibited the strongest cytoplasmic β -arrestin 2 expression (Kruskal-Wallis test; $p = 0.01$) when compared with different histological subtypes. Arrows demonstrate representatively β -arrestin 2 stained cells. Images representative of three independent experiments.

and A2780 cells overexpressing β -arrestin 2, indicating that the least cell death occurs with the highest amounts of β -arrestin 2-encoding plasmid transfected (**Figures 4E–G**; Kruskal-Wallis test; $p < 0.01$; $n = 8$). However, data from transfected hGL5 cells did not reveal any association between β -arrestin 2 expression and cell death (**Figure 4F**; Kruskal-Wallis test; $p \geq 0.05$; $n = 8$).

Since a positive correlation between β -arrestin 2 expression and cell viability was found (**Figure 4**), control experiments were performed by evaluating the cell viability and death of cells maintained under β -arrestin 2 depletion, *in vitro*. Therefore,

HEK293, hGL5, and A2780 cells were treated by increasing concentrations of siRNA probes silencing the endogenous molecule, then MTT assay and propidium iodide staining were performed (**Figure 5**).

The viability of all the cell lines is significantly reduced upon β -arrestin 2 depletion (**Figures 5A–C**; Kruskal-Wallis test; $p < 0.05$; $n = 8$). These data are reflected by increased propidium iodide signals occurring together with increasing amounts of siRNAs, in HEK293 cells (**Figure 5D**; Kruskal-Wallis test; $p < 0.05$; $n = 8$), while similar signals were found between siRNA-

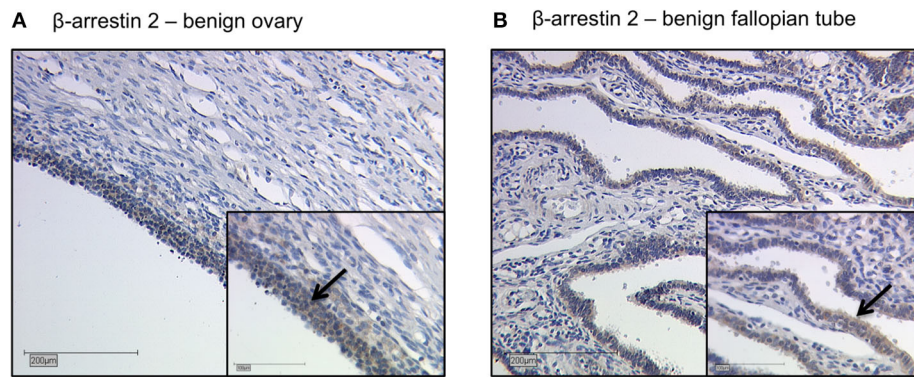


FIGURE 2 | Detection of β -arrestin 2 with immunohistochemistry in benign ovary and fallopian tube. Detection of β -arrestin 2 with immunohistochemistry in benign ovary (A) and fallopian tube (B). Positive β -arrestin 2 staining was observed in epithelial cells, granulosa cells and theca cells. The ovarian and fallopian tube stroma did not show a β -arrestin 2 staining. Arrows demonstrate representatively β -arrestin 2 stained cells. Images representative of three independent experiments.

TABLE 2 | Correlation analysis between β -arrestin 2 expression and clinicopathological data.

Variable	P	Correlation coefficient
Histology	0.075	−0.083
FIGO stage	0.673	0.086
Age	0.206	0.105
GRADING		
Clear cell, endometrioid, and mucinous (G1–G3)	0.520	−0.088
Serous—low grading	0.052	−0.160
Serous—high grading	0.041*	0.169

Clinicopathologic data and β -arrestin 2 expression were correlated to each other using Spearman's correlation analysis. Significant correlations are indicated by asterisks (* $p < 0.05$). p , two-tailed significance.

and mock-treated hGL5 and A2780 cells (Figures 5E,F; Kruskal-Wallis test; $p \geq 0.05$; $n = 8$). Western blotting analysis confirmed the siRNA-dependence of β -arrestin 2 silencing, which is linked to pro-caspase 3 cleavage in all the cell lines (Figure 5G).

DISCUSSION

Our study revealed for the first time that β -arrestin 2 expression significantly correlates with impaired overall survival of ovarian cancer patients and these results are consistent with the proliferative role of β -arrestin 2 demonstrated *in vitro*. β -arrestins are known to act as scaffold proteins controlling multiple cellular functions, such as MAP kinase signaling, GPCR trafficking and transcriptional modulations (38, 39). Several studies demonstrated that β -arrestins are involved in carcinogenesis of the ovary (11, 18) and other types of cancer (10, 12–17). The relevance of the GPCR/ β -arrestin pathway in ovarian cancer cells is demonstrated by the finding that endothelin-1 receptor/ β -arrestin complex activates beta-catenin signaling pathways with impact on carcinogenesis and metastatic progression (11). Nevertheless, conflicting results have been

TABLE 3 | Correlation analysis of β -arrestin 2.

Staining	β -arrestin 2	FSHR	LHCGR	GPGR	hCG β
β-ARRESTIN 2					
cc	1.000	0.183	0.284	0.215	0.360
p	–	0.029	0.001	0.009	0.000
n	148	143	146	148	137
FSHR					
cc	0.183	1.000	0.164	0.214	0.082
p	0.029	–	0.046	0.009	0.340
n	143	151	149	148	137
LHCGR					
cc	0.284	0.164	1.000	0.170	0.200
p	0.001	0.046	–	0.037	0.018
n	146	149	154	151	140
GPGR					
cc	0.215	0.214	0.170	1.000	0.114
p	0.009	0.009	0.037	–	0.179
n	148	148	151	153	141
hCGβ					
cc	0.360	0.082	0.200	0.114	1.000
p	0.000	0.340	0.018	0.179	–
n	137	137	140	141	142

cc, correlation coefficient; p , two-tailed significance; n , number of patients.

reported with a positive impact of angiotensin-mediated β -arrestin expression in a clinical-genomic dataset from 820 ovarian cancer patients (40). Indicative results may be provided by the analysis of gene expression data stored in public cancer repositories. For instance, The Human Protein Atlas database (<https://www.proteinatlas.org>; last accession: 03 March 2020) revealed that β -arrestin 2-encoding gene (*ARRB2*) expression is an unfavorable prognostic marker of prostate cancer, although the association is not replicated for ovarian cancer. These data corroborate, at least in part, our finding although they should be considered cautiously, since gene expression levels

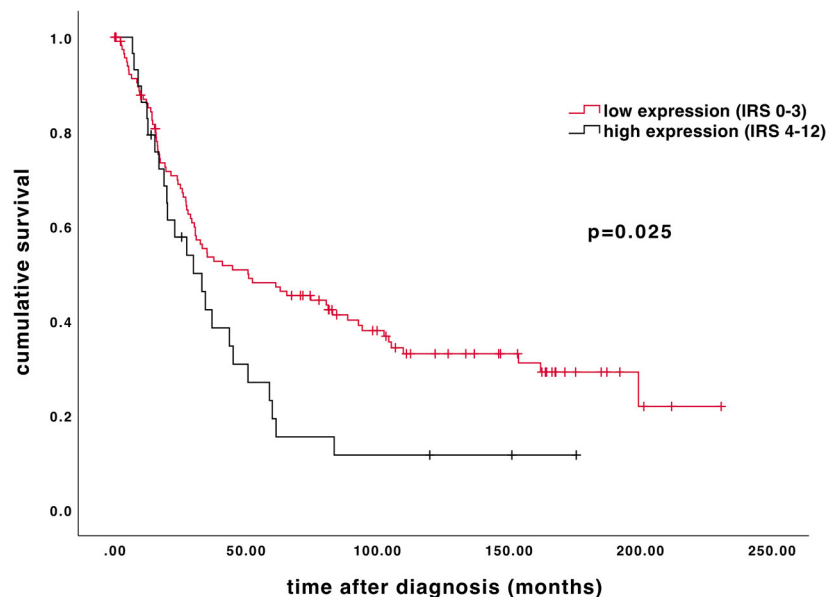


FIGURE 3 | Kaplan–Meier estimate of β -arrestin 2. β -arrestin 2 expression (IRS 4–12) was associated with impaired overall survival (50.64 vs. 29.88 months, median; $p = 0.025$). Censoring events have been marked in the graphs.

TABLE 4 | Multivariate analysis of the analyzed ovarian cancer patients ($n = 156$).

Covariate	Coefficient (b_i)	[HR Exp (b_i)]	95% CI		P-value
			Lower	Upper	
Patient's Age (≤ 60 vs. >60)	0.625	1.869	1.133	3.084	0.014
FIGO (I, II vs. III, IV)	0.813	2.255	1.499	3.393	<0.001
GRADING					
Clear cell, endometrioid, and mucinous (G1–G3)	0.142	1.153	0.749	1.774	0.517
Serous—low grading	−0.695	0.499	0.160	1.562	0.232
Serous—high grading	0.654	1.924	0.765	4.838	0.164
High β -arrestin 2	−0.097	0.907	0.795	1.036	0.152

are not necessarily corresponding to protein levels and the database does not allow to analyze all the specific histological tumor subtypes.

The link between GPCR/ β -arrestins complexes and cancer is an established concept (41) and seems to be result of sustained intracellular signaling occurring upon dysregulation of intracellular β -arrestin protein levels (42). These molecules were associated with drug resistance of breast (43) and lung cancer cells (44), cancer cell migration and invasion (45), tumor progression (46) and metastasis (47), as well as a number of other tumors (48). Thus, β -arrestins were proposed as a target for therapeutic strategies (49). Our data *in vitro* confirm the proliferative role of β -arrestin 2 also in an ovarian cancer cell line, as well as in hGL5 and HEK293 cells. There are converging evidences that β -arrestin 2 depletion leads to decreasing cell viability and/or activating a caspase 3-mediated cell death, at least in HEK293 cells and confirmed in the

ovarian cancer cell line A2780. Interestingly, in hGL5 cells, increased β -arrestin 2 expression is linked with upregulation of cell viability, while the depletion of this molecule by siRNA did not result in cell death. This is likely due to the integration of exogenous *E6* and *E7* oncogenes in the hGL5 cell line genome (23), which served for immortalizing them and, likely, results in increased resistance to caspase 3 activation. On the other hand, these oncogenes encode proteins inhibiting tumor protein 53 (p53) and retinoblastoma (Rb) molecules and interfering with apoptosis and cell cycle blockade. hGL5 have characteristics reflecting epithelial tumor cells, such as fibroblast-like spindle shape and relatively high proliferation rate, a feature depending on β -arrestins functioning (21), as well as in other ovarian cancer cell lines (40). However, these results underline that β -arrestins are modulators of proliferative signals exerted through a molecular signaling module with GPCRs and the extracellular-regulated MAP kinases 1 and

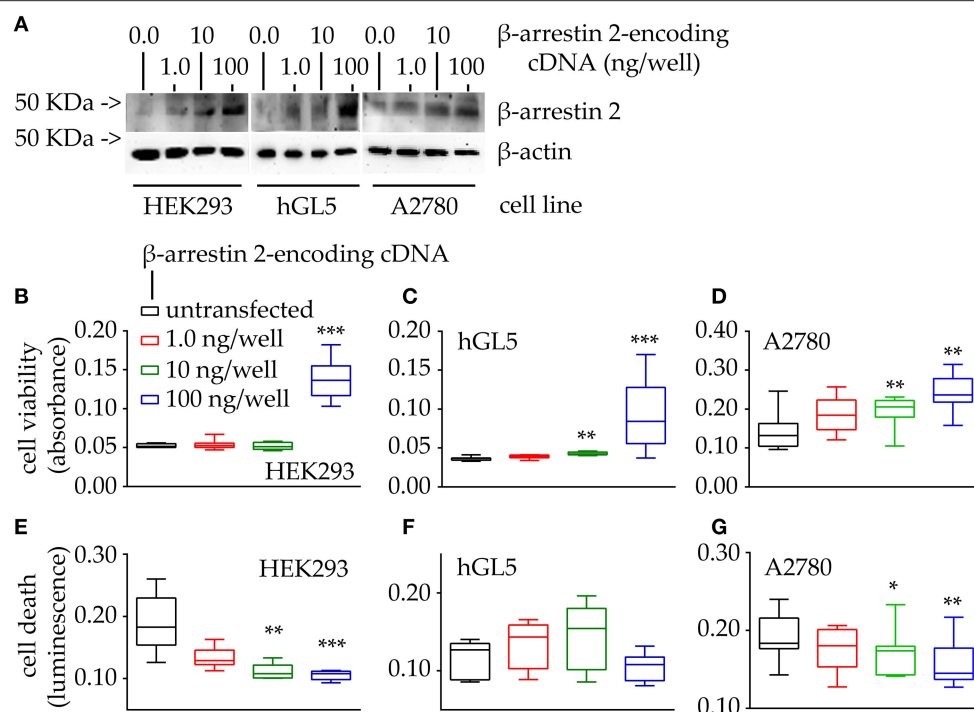


FIGURE 4 | Evaluation of cell viability and death under 48-h β -arrestin 2 overexpression. HEK293, hGL5, and A2780 cells were transfected by increasing amount of plasmid encoding β -arrestin 2 and cell viability and death were assessed by MTT and propidium iodide, respectively. Total protein content from 0.5×10^4 70% confluent log phase cells were loaded. **(A)** Representative image of β -arrestin 2 detected in cell lysates by Western blotting using a specific rabbit antibody using β -actin as a normalizer. **(B)** Box and Whiskers plot of MTT data, collected from transfected HEK293 cells overexpressing increasing levels of β -arrestin 2, representing cell viability. **(C)** hGL5 cell viability evaluated by MTT. **(D)** β -arrestin 2-dependent cell viability of the A2780 ovarian cancer cell line. **(E)** Data from the β -arrestin 2-overexpressing HEK293, representing cell death, in propidium iodide-stained samples. **(F)** β -arrestin 2-overexpressing hGL5 cell death. **(G)** β -arrestin 2-overexpressing A2780 ovarian cancer cell death. Brightness/contrast of Western blotting pictures were adjusted uniformly in all panels. Asterisks indicate significantly different data distribution than mock-transfected (untransfected) cells (Kruskal-Wallis test and Dunn's correction for multiple comparisons; * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$; $n = 8$).

2 (ERK1/2), in a number of cells (50), including ovarian cells (21). Gonadotropin receptors are expressed in ovarian cells and targeted by β -arrestin 2 (51). It is well-known that gonadotropin receptor expression is dysregulated in ovarian cancer (34, 36, 52) as well adrenal tumor (53), although the specific influence of these receptor on carcinogenesis is still not determined (54). One reason for this, may be the bi-directional regulation of pro- and anti-apoptotic signals which depend on FSHR and LHCGR expression levels (55). Interestingly, the depletion of β -arrestin 2 may be linked to increased tumor cell growth and angiogenesis, at least in certain cases, such as lung cancer (56). In this case, the absence of β -arrestin 2 would lead to uncontrolled activation of proliferative signals induced by other GPCRs, e.g., the interleukin 8 receptor beta (CXCR2). Taken together, we could speculate that β -arrestins may positively or negatively impact proliferative events, modulating the activity of various GPCRs specifically expressed in the tumor cells.

In correlation analyses, a link between β -arrestin 2 and the G-protein coupled estrogen receptor (GPER) was identified. This receptor is known for rapidly mediating non-genomic

signals induced by estrogens and was found in both healthy and neoplastic human tissue (57, 58). Indeed, previous studies revealed that GPER is related to better overall survival in ovarian cancer patients (34, 59, 60). Since GPER belongs to the GPCRs family, we may suppose that β -arrestin 2 might interact with this membrane estrogen receptor and influence its physiological function. However, this issue must be further investigated because GPER recycling and intracellular trafficking seems to be regulated by mechanisms not involving β -arrestins (61).

Interestingly, we also found an association between β -arrestin 2 and one of the two LHCGR ligands, hCG β . This hormone was shown to be associated with an increased 5-years survival in LHCGR positive/FSHR negative ovarian cancer cases (33). Although this study had limitations related to low specificity of the anti-gonadotropin receptor antibodies employed (62), it suggests that hCG β and its receptor might be linked to survival in these patients. These data would be in line with the relatively high potency of hCG β in inducing LHCGR-mediated intracellular cAMP increase (27), as an effect linked to the steroidogenic potential of the hormone and to pro-apoptotic effects (24). On the other hand, these data would

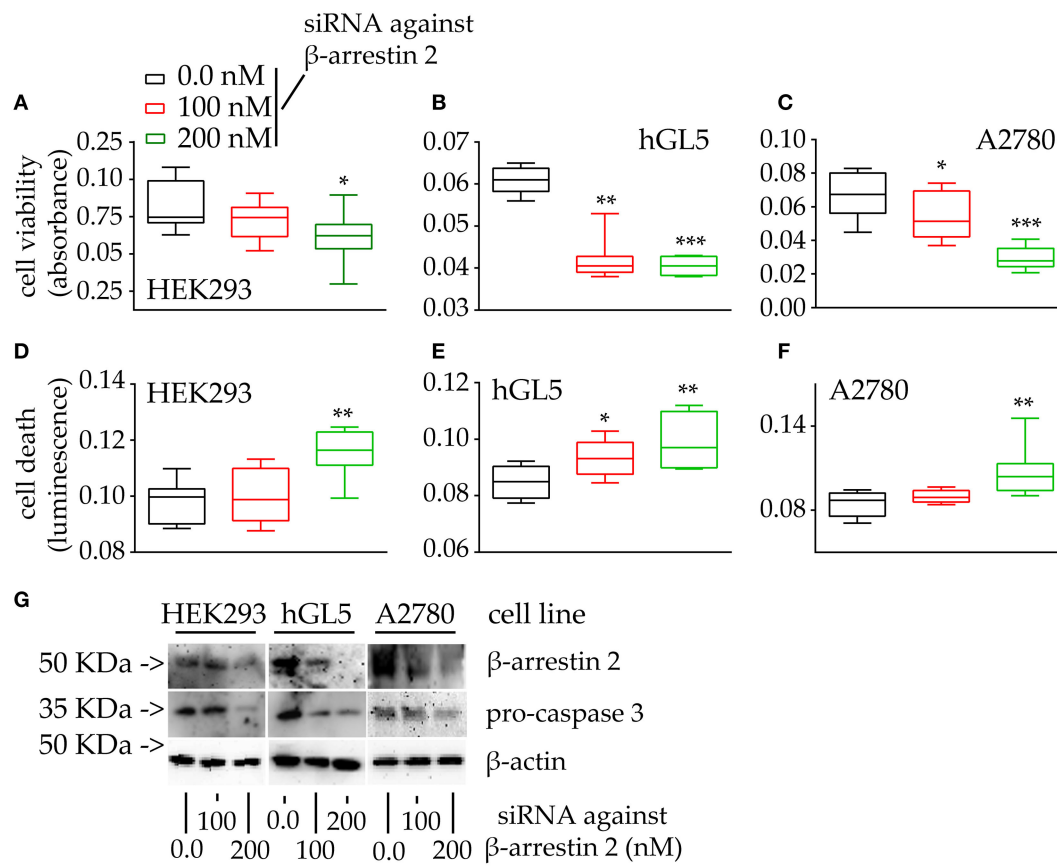


FIGURE 5 | Viability and death of 48-h β -arrestin 2-silenced HEK293, hGL5, and A2780 cells. Samples were transfected by increasing amount of siRNA probes targeting the β -arrestin 2 mRNA and 48-h cell viability and death were assessed by MTT and propidium iodide. Total protein content from 3×10^4 70% confluent log phase cells were loaded. **(A)** Box and Whiskers plot representing the HEK293 cell viability under β -arrestin 2 depletion. **(B)** β -arrestin 2-silenced hGL5 cell viability. **(C)** β -arrestin 2-silenced A2780 ovarian cancer cell viability. **(D)** Data from the β -arrestin 2-silenced HEK293, representing cell death evaluated using propidium iodide staining. **(E)** hGL5 cell death evaluated by propidium iodide. **(F)** Cell death in β -arrestin 2-silenced A2780 evaluated by propidium iodide. **(G)** Representative Western blotting images demonstrating β -arrestin 2 depletion and pro-caspase 3 cleavage in HEK293, hGL5, and A2780 cell lysates. β -actin was the normalizer. Brightness/contrast of Western blotting pictures were adjusted uniformly in all panels. Significantly different data distribution than mock-transfected (untransfected) cells were indicated by asterisks. Kruskal-Wallis test and Dunn's correction for multiple comparisons; * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$; $n = 8$).

be in contrast with the fact that hCG β has been used as a marker of certain tumors, such as the choriocarcinoma (63), which reflects the involvement of β -arrestin 2 in the hCG β -mediated intracellular signaling (29) and suggests that the connection between the two molecules in cancer merits further investigations.

β -arrestin 2 is associated with a significantly impaired overall survival of ovarian cancer patients. This finding is supported by *in vitro* data demonstrating that β -arrestin 2 upregulates the viability of an ovarian cancer cell line, and transfected HEK293 and hGL5 cells. Reflecting the role of β -arrestins as scaffold proteins linked to the GPCRs activity, correlations between the β -arrestin 2 and FSHR and LHCGR expression was identified ovarian cancer tissue. Taken together, we indicate that β -arrestin 2 may be a promising new target in ovarian cancer so that clinical implications should be further addressed in future research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BC, UJ, MS, LC, and FT: conceptualization. DM, SM, and AB: validation. BC, AP, UJ, and LC: formal analysis. AP, BM, RB,

and SB: investigation. BC, AP, LC, and FT: writing-original draft preparation. UJ, VS, DM, SM, AB, MS, BM, RB, and SB: writing—review and editing. AP, BC, and LC: visualization. UJ, SM, and MS: supervision. All authors have read and agreed to the published version of the manuscript.

FUNDING

MS was a Le Studium Research Fellow, Loire Valley Institute for Advanced Studies, Orléans & Tours, France, INRA—Centre Val de Loire, 37380 Nouzilly, France, receiving funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 665790. This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

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ACKNOWLEDGMENTS

The authors were grateful to Mrs. Christina Kuhn for excellent technical assistance and Professor Gaetano Marverti (University of Modena and Reggio Emilia, Modena, Italy) for providing the A2780 cell line. Authors were grateful to the Italian Ministry of University and Research for supporting the Department of Biomedical, Metabolic, and Neural Sciences (University of Modena and Reggio Emilia, Italy) in the context of the Departments of Excellence Programme.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2020.554733/full#supplementary-material>

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Conflict of Interest: Research support, advisory board, honoraria, and travel expenses from AstraZeneca, Clovis, Medac, MSD, Novartis, PharmaMar, Roche, Sensor Kinesis, Tesaro, Teva have been received by SM and from AstraZeneca, Medac, PharmaMar, Roche, Tesaro/GSK by FT. All above mentioned companies did not influence the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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