Published in final edited form as:

Mol Cancer Res. 2019 June ; 17(6): 1378–1390. doi:10.1158/1541-7786.MCR-18-0989.

Multifunctional APJ Pathway Promotes Ovarian Cancer Progression and Metastasis

Deepika Neelakantan^{1,*}, Samrita Dogra^{1,*}, Bharat Devapatla¹, Pharavee Jaiprasart¹, Marie Claire Mukashyaka¹, Ralf Janknecht^{2,6}, Shailendra Kumar Dhar Dwivedi³, Resham Bhattacharya^{3,6}, Sanam Husain⁴, Kai Ding⁵, and Sukyung Woo^{1,6}

¹Department of Pharmaceutical Sciences, College of Pharmacy, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

²Department of Cell Biology, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

³Department of Obstetrics and Gynecology, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

⁴Department of Pathology, College of Medicine, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

⁵Department of Biostatistics and Epidemiology, College of Public Health, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA,

⁶Peggy and Charles Stephenson Cancer Center, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Abstract

High mortality rates in ovarian cancer (OvCa) are due to late stage diagnosis when extensive metastases are present, coupled with the eventual development of resistance to standard chemotherapy. There is thus an urgent need to identify targetable pathways to curtail this deadly disease. In this study we show that apelin receptor APJ is a viable target that promotes tumor progression of high-grade serous OvCa (HGSOC). APJ is specifically overexpressed in tumor tissue, and is elevated in metastatic tissues compared to primary tumors. Importantly, increased

Corresponding Author: Sukyung Woo, PhD, Department of Pharmaceutical Sciences, College of Pharmacy, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave., Oklahoma City, OK 73117-1200, Tel: 405-271-6593 ext. 47471; Fax: 405-271-7505, sukyung-woo@ouhsc.edu.

*These authors contributed equally to this work

AUTHOR CONTRIBUTIONS

Conception and design: D. Neelakantan, S. Dogra, S. Woo

Development of methodology: D. Neelakantan, S. Dogra, R. Janknecht, MC. Mukashyaka

Acquisition of data (provided animals, acquired and managed patients, provided facilities,): D. Neelakantan, S. Dogra, B. Devapatla, P. Jaiprasart, SKD Dwivedi, R. Bhattacharya, S. Woo

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Neelakantan, S. Dogra, R. Janknecht, S. Husain, K Ding, S. Woo

Writing, review, and/or revision of the manuscript: D. Neelakantan, S. Dogra, MC. Mukashyaka, R. Bhattacharya, R. Janknecht, S. Woo

Study supervision: S. Woo

COMPETING INTERESTS:

The authors have declared that no conflict of interest exists.

APJ expression significantly correlates with decreased median overall survival by 14.7 months in HGSOC patients. Using various OvCa model systems, we demonstrate that APJ expression in cancer cells is both necessary and sufficient to increase pro-metastatic phenotypes *in vitro*, including proliferation, cell adhesion to various molecules of the extracellular matrix, anoikis resistance, migration, and invasion; and these phenotypes are efficiently inhibited by APJ inhibitor ML221. Overexpression of APJ also increases metastasis of OvCa cells *in vivo*. Mechanistically, the pro-metastatic STAT3 pathway is activated downstream of APJ, and in addition to the ERK and AKT pathways, contributes to its aggressive phenotypes. Our findings suggest that the APJ pathway is a novel and viable target, with potential to curb OvCa progression and metastasis.

INTRODUCTION

Patients with ovarian cancer (OvCa) are typically diagnosed at an advanced stage of the disease with widespread presence of metastases, and thus have an overall 5-year survival rate of around 30% (1). High-grade serous ovarian carcinoma (HGSOC) is the most commonly diagnosed OvCa subtype, and while the initial response rate of HGSOC patients to platinum and taxane-based drugs is around 70%, they often present with recurrences that are chemoresistant (1). Hence, there is an urgent need to identify novel and targetable pathways in this deadly disease.

The apelin receptor APJ, is a G-protein coupled receptor involved in regulating physiological processes such as angiogenesis, cardiovascular development, fluid homeostasis, and reprogramming of energy metabolism, when it is bound by its endogenous ligand apelin. (2,3). While specific regulators of these processes downstream of APJ remain largely unknown, multiple players including ERK, PLC β -PKC, and AKT cascades have been implicated (2). The role of this pathway in cancer is now slowly being recognized. APJ/apelin expression is elevated in many malignant tissues (4), indicating that its mis-expression/activation may be crucial in tumorigenesis. Studies have also shown that increased expression and/or activation of this axis in the cancer cells as well as in the stroma, contributes to cancer progression mainly by increasing tumor-related angiogenesis (5–7), and proliferation (8–10). However, the extent of APJ expression by cancer cells and its pathophysiological roles in human cancers remain largely unknown.

Herein, we demonstrate that APJ promotes HGSOC tumor progression and metastasis. Using various model systems, we show that increased APJ expression is both required and is sufficient to enhance numerous pro-metastatic phenotypes of OvCa cells *in vitro*, and increases metastasis *in vivo*. The APJ pathway is clinically relevant, as increased APJ expression in tumor tissues significantly correlates with worsened overall survival in HGSOC patients. Taken together, our data demonstrate that the APJ pathway plays tumor-promotional roles in OvCa and hence, presents the potential for a novel therapeutic strategy.

METHODS AND MATERIALS

Reagents and Cell Culture

Human OvCa cell lines OVCAR-5 and OVCAR-8 were purchased from the DCTD Tumor Repository, National Cancer Institute at Frederick, Maryland. Immortalized normal fallopian tube epithelial cells (FTE188) were a generous gift from Dr. Jinsong Liu (MD Anderson Cancer Center). Human normal ovarian surface epithelial cells (HOSE), OVCAR-4, and TykNu were a kind gift from Dr. Danny Dhanasekaran (OUHSC). SKOV-3 and OVCAR-3 were a generous gift from Dr. Youngjae You (OUHSC). The cell lines were profiled via short tandem repeat profiling to confirm their identity before receipt. The cell lines were cultured in RPMI (OVCAR-3, OVCAR-4, and OVCAR-5); ATCC-EMEM (TykNu), McCoy's (SKOV-3), and 1:1 MCDB-105 and M199 media (HOSE and FTE188). Media was supplemented with 10–20% fetal bovine serum (FBS). Cells and media were periodically tested for mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza), and if found positive, older freezes of mycoplasma-free cells were used. Experiments were performed on cells within 15 passages post thaw. APJ-overexpressing cells were transduced as per established protocols with pLenti-CMV-APLNR or empty vector (EV) and selected with 1 µg/ml (OVCAR-3) or 1.5 µg/ml (OVCAR-5) puromycin. Two different shRNA constructs (shAPJ1: 5' GCGCTCAGCTGATATCTTCAT-3' and shAPJ-2: 5'-GGCTTCTAGAAGGGAAGAAAT-3') were inserted into RNAi-Ready pSIREN-RetroQ vector at the BamHI and EcoRI restriction sites. OVCAR-8 cells were transduced and selected with 2 µg/ml puromycin. Cells were treated with Apelin-13 peptide (Bachem, H-4568) for the duration of the experiment as specified. The inhibitors, ML221 (Tocris, 47–481-0), GSK2126458 (denoted as GSK458; MedChem Express, HY-10297), STATTC (MedChem Express, HY-13818) and U0126 (MedChem Express, HY-12031) were resuspended in DMSO according to manufacturer's protocol.

Patient Tissue Specimens

Patient samples were obtained from the Peggy and Charles Stephenson Cancer Center, OUHSC. The tissue microarray (TMA) comprised of 124 HGSOC samples from patients. Written informed consent was obtained from all women enrolled in the study. Institutional Review Board approval was provided by OUHSC. A gynecological pathologist (SH) graded APJ staining (Abcam, ab84296) on a scale of 0–3 (0: no staining, 3: strong staining). The median score was recorded from three repeats of each tissue and association between APJ intensity and patient survival was assessed. Covariates (age at diagnosis, race, stage) were considered in the multivariate analyses using Cox model. Two-way interactions between APJ expression (high vs. low) and each of the covariates were assessed. Backward selection was used to obtain the final model.

Gene expression data were obtained from publicly available HGSOC datasets in GEO (Gene Expression Omnibus). GSE14407 (11) and GSE18520 (12) specifically provide gene expression signatures in serous OvCa epithelial cells isolated by laser capture microdissection and normal OSE. Differential APJ gene expression in primary tumor and metastases samples was analyzed with Oncomine (version 3.0; www.oncomine.org) in three

publicly available OvCa datasets (13,14). APJ expression in 16 human OvCa cell lines was obtained from the CCLE.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted using HP E.Z.N.A kit from Promega. cDNA synthesis was performed using a Maxima cDNA synthesis kit (ThermoFisher). qRT-PCR assays were performed using ssoFast Evagreen supermix (BioRad) and analyzed using Biorad CFX96. Primer sequences are listed in Supplementary Table S1.

ELISA Assay

Assay was performed following manufacturer's protocol for Extraction Free EIA Kit (Phoenix Pharmaceuticals, Burlingame, CA). In brief, 50 µl of conditioned media was collected from OvCa cell lines cultured under normoxic and hypoxic conditions (1% oxygen; 5% carbon dioxide and 94% nitrogen), for 24 h in duplicates. Concentration of apelin in the samples was determined from the standard curve of apelin ranging from 0.01–100 ng/ml, and according to manufacturer's protocol.

Immunoblot Analysis

Whole cell lysates were generated as previously described (15). Briefly, RIPA (Sigma) lysis buffer and T-PER™ Tissue Protein Extraction Reagent (Fisher Scientific) was used to extract whole cell lysates from cells in culture and tumor tissue respectively. Protein concentration was determined using DC Protein reagents (Biorad). Equal amounts of lysates (10–15 µg) were electrophoresed and transferred to nitrocellulose membranes. Ponceau S Stain (Sigma) was used to stain total protein on membrane to obtain a non-specific band. Membranes were blocked in 5% BSA in TBST for 1 h post-transfer, and incubated overnight at 4°C with primary antibody. After secondary antibody incubation, membranes were analyzed using FluorChemFC2. For cell lysates from suspended cells, 250,000 cells were plated in poly-HEMA (Sigma, 12 mg/ml in 95% ethanol)-coated plates for 24–48 h. Cell clusters were centrifuged to obtain a pellet. Tumor tissue was weighed and homogenized prior to addition of lysis buffer. The antibody sources and dilutions are listed in Supplementary Table S2.

Human phospho-antibody array analysis

The phospho-antibody array analysis was performed using the Proteome profiler antibody array kit (R&D systems™, ARY003B) as per manufacturer's protocols. Briefly, OVCAR-5-EV and APJ cells were plated for 48 hrs, lysed with lysis buffer 6 (R&D systems™), agitated for 30 min gently at 2–8°C, and protein concentration was determined as described previously. The nitrocellulose membranes were blocked with 5% BSA in TBST and then treated with samples overnight on a rocking platform at 4°C. The membranes were washed with the 1X wash buffer to remove the unbound protein and incubated with the mixture of biotinylated detection antibodies and streptavidin-HRP antibodies. Chemi-Reagent mix was applied for detection of the spot densities, and quantified using ImageJ.

Cell proliferation assay

Proliferation assays were performed by cell counting and colony formation assays. Total cell numbers were counted using the LUNA-II™ cell counter. Briefly, 10,000 OVCAR-4 or OVCAR-5 cells and 7500 OVCAR-8 cells were plated in 6-well plates and treated with 10–100 ng/ml apelin-13 and/or 15–50 μ M ML221. After incubation for 24–96 h, cells were trypsinized, and suspended in equal volumes of media. An average of two replicates per sample was used for the analysis. For colony formation assays, 2500 cells/well were seeded in 6-well plates and cultured for ~11 days with media replacement every 3–4 days. Apelin-13 and drugs were added at the time of plating as indicated in the text. On day ~11, the colonies were fixed using 70% ethanol and stained using 0.4% crystal violet (CV). Images were taken using a bright field microscope (Leica, CFD365-FX). The assay was analyzed by counting colonies using ImageJ or measuring absorbance at 570 nm.

Cell adhesion assay

Cell adhesion assays were performed on 96-well Fibronectin/Laminin I- and Collagen IV-coated plates (Biocoat, Fisher). The plates were blocked with 1% BSA in PBS for 1 h at 37°C, followed by washes with 1X PBS. Five replicates of 40,000 cells/well were plated and incubated at 37°C for 2–2.5 h. Apelin-13 and drugs were added to cells at the time of plating. The wells were washed thrice with 1X PBS to remove non-adherent cells, fixed with ice-cold methanol for 10 min at room temperature, and stained with 0.05% CV. The cells were destained using 10% glacial acetic acid, followed by measurement of absorbance at 570 nm corresponding to “adhered” cells.

Anoikis resistance assay

Cells (50,000–150,000/well) were plated on poly-HEMA coated 12-well plates and treated with apelin-13 or drugs. Cells were retrieved after 24 h (48 h for OVCAR-8 cells), centrifuged for 5 min at 1000 rpm, and replated on adherent 24-well tissue culture plates for 5–6 h. Post adherence, cells were fixed with 10% formalin and stained with 0.05% CV. Bright-field images were taken using Leica either before or after CV staining, followed by destaining. Absorbance corresponding to “cells with increased anoikis resistance” was measured at 570 nm.

Cell migration assay

Migration assays were performed using transwell 8 μ m cell culture inserts (BD Falcon, 353097). 40,000 cells/well were plated in serum-free medium on transwell filter and allowed to migrate to medium containing 10% FBS. After 6–8 h, cells from above the membrane were wiped with cotton swabs, and cells at the bottom were fixed in 10% formalin and stained with 0.05% CV. Cell migration was analyzed by counting cells using a bright field microscope (Leica) and ImageJ, or measuring absorbance at 570 nm. Apelin-13 and drugs were added at the time of plating.

Cell invasion assay

Invasion assays were performed using 8- μ m transwell cell culture inserts (BD Falcon), after coating filters with 1:20 diluted matrigel (Fisher, CB40230) in serum-free medium. Cells

(100,000–200,000/well) were plated on the matrigel and allowed to invade to 10% FBS-medium for 16 h. Apelin-13 was added to the cells and to the medium at the bottom. Inhibitors were added to the cells at the time of cell plating. Cell invasion was analyzed similar to transwell migration assays.

Dose response assay for ML221

Dose response assays were performed using colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay kit (PR G3580). Briefly, 10,000 cells were plated in 96-well plates overnight, treated with different concentrations of ML221 and incubated for 48 h. MTS assay was analyzed according to manufacturer's protocol, with absorbance read at 490 nm and cell survival calculated as the percentage of control (DMSO treated) group.

Each *in vitro* experiment was independently and successfully repeated more than three times.

Animal experiments

All animal studies were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at OUHSC.

Orthotopic model: OVCAR-3 cells (10×10^6) and OVCAR-5 cells (5×10^6) in PBS were intraperitoneally injected in 6-to-8-week-old female athymic nude mice (Charles River). Mouse weights were measured weekly, and mice were checked for ascites formation every 4 days. While in some cases mice that received APJ-overexpressing cells appeared to be moribund with extreme weight loss, no statistical trends were observed in either orthotopic model. Mice were euthanized after 55 days of injection for the OVCAR-3 model (n=4–5 per group) and 22 days for the OVCAR-5 model (n=9 per group), when moribund and based on timelines established in the literature (16). The tumor colonies were counted and collected for further analyses.

Subcutaneous model: 5×10^6 OVCAR-3 cells (in sterile PBS) were injected in the left flanks of 6-to-8-week-old female athymic nude mice. OVCAR-3-APJ cells were pre-treated with 100 ng/ml apelin-13 for 48 h prior to injection, and 100 ng/ml apelin-13 was added to the cell suspension at time of injection. Tumor sizes and mouse weights were measured weekly. Tumor volume (mm^3) was calculated using the formula: $\text{length} \times (\text{width}^2) \times 0.5$ and tumor masses were counted when mice were euthanized according to IACUC protocols.

Immunohistochemical (IHC) staining for pSTAT3 and STAT3 and H&E stains were performed in tumor tissues isolated from mice. Quantification was done using ImageJ software. The antibody sources and dilutions are listed in Supplementary Table S2.

Statistics

GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA) was used for all statistical analyses. Two-tailed unpaired Student's *t*-test was used to compare pairs of conditions. One-way Analysis of Variance (ANOVA) non-parametric followed by Tukey's/

Dunnett's post hoc test was used to compare more than two conditions and two-way ANOVA was used to analyze animal experiments. The mRNA expression in normal OSE and serous OvCa epithelial cells was compared with the unpaired Mann-Whitney test. OS outcome was summarized using Kaplan-Meier curves and compared between groups using the log-rank test. The association of APJ expression with patient OS was evaluated using the Cox model. A *P* value of <0.05 denoted statistical significance.

RESULTS

Increased APJ expression correlates with worsened prognosis in HGSOc patients.

To interrogate the specific role of apelin receptor APJ in OvCa, we first screened a panel of human OvCa cell lines for APJ expression. We found that on the mRNA and protein levels, OvCa cells differentially express APJ independent of their classification, (e.g., high grade versus low grade serous carcinomas, *p53* mutation status), but at a similar or higher level than that in HOSE (human ovarian surface epithelial) cells or FTE188 (fallopian tube epithelial) cells (Fig. 1A). An ELISA assay showed that OvCa cells secreted variable levels of the pathway ligand apelin (Supplementary Fig. S1A), indicating that OvCa cells co-express the receptor APJ and its ligand. We also observed elevated expression of apelin in response to hypoxia, akin to what has been shown in other systems where HIF-1 regulates expression of apelin (17,18). Analysis of APJ expression in HGSOc using publicly available datasets, revealed that *APJ* expression was significantly higher in tumor tissues compared to in non-malignant tissues (Fig. 1B). These studies (11,12) were performed on cancer cells micro-dissected from tumor tissues, indicating that *APJ* is specifically upregulated in cancer cells, and not the surrounding tumor microenvironment. Further analysis in 16 human OvCa cell lines using the Cancer Cell Line Encyclopedia (CCLE) showed that *APJ* expression in immortalized cell lines cultured *in vitro*, was lower than levels expressed by malignant cells in the tumor tissues (Fig. 1B). This suggests that APJ expression may increase *in vivo*. Furthermore, using Oncomine, we found that *APJ* expression was significantly increased in metastases compared to primary tumors in multiple human OvCa patient datasets (Supplementary Fig. S1B-D). A meta-analysis (19) further showed that increased *APJ* expression correlated with worsened progression-free survival and post-progression survival in patients with serous ovarian cancer (Supplementary Fig. S1E,F).

We independently assessed the protein expression of APJ by performing immunohistochemical analysis in tumor tissue microarrays (TMA) consisting of 124 HGSOc tissues (Fig. 1C). Seventy-five samples (60%) had moderate to high APJ expression (APJ high). This high expression of APJ protein (represented by both membranous and diffused staining in the cytoplasm of cancer cells) correlated with significantly reduced median overall survival (OS) by 14.7 months (38.5 vs 53.2 months, *p*=0.006; Fig. 1D). Additionally, a multivariate Cox model revealed that high APJ expression was significantly associated with shorter OS (hazard ratio 2.1, 95% CI 1.3 – 3.3, *p*=0.0016), after adjusting for age at diagnosis and stage. Together, our data demonstrate the pathological significance of APJ in ovarian cancer.

APJ enhances OvCa cell proliferation.

To determine the role of increased APJ expression in OvCa and based on the lower expression of *APJ* in OvCa cell lines compared to in human tumor tissue, we first stably overexpressed APJ in OVCAR-5 (OVCAR-5-EV, APJ) and OVCAR-3 (OVCAR-3-EV, APJ) cells (Fig. 2A (inset), Supplementary Fig. S2A). To determine the necessity of APJ to increase metastatic properties of OvCa cells, we stably knocked down (KD) APJ using two different shRNA constructs (shAPJ-1, shAPJ-2) in OVCAR-8 cells expressing high endogenous APJ (Fig. 2C (inset)). Additionally, we used OVCAR-4 cells that have high endogenous expression of APJ and secrete relatively lower levels of apelin (Supplementary Fig. S1A), to determine whether exogenous addition of apelin-13 (the most biologically active form (20)), can enhance APJ-induced activity in the cells.

Using cell counting assays (as a surrogate for proliferation), we found significantly increased cell numbers in OVCAR-5-APJ cells compared to EV cells (Fig. 2A). We observed similar effects of increased proliferation in OVCAR-3-APJ compared to EV cells, using colony formation assays (Supplementary Fig. S2B). OVCAR-4 cells treated with apelin-13 also exhibited increased cell counts compared to control (Fig. 2B); together indicating that APJ expression is sufficient to increase proliferation in OvCa cells. In contrast, APJ KD in OVCAR-8 resulted in decreased cell numbers compared to control (Fig. 2C). When treated with apelin-13, only the cells with high APJ expression (OVCAR-8-shNT cells) and not the APJ KD cells, showed increases in proliferation (Supplementary Fig. S2C), further indicating the specificity of this phenotype to APJ activation. We additionally used ML221, a small-molecule inhibitor of APJ (21) to determine if the increases in proliferation of overexpression cell lines were specific to APJ, and dose-response studies for ML221 were first performed to determine appropriate drug concentrations for use in the cells (Supplementary Fig. S3A, B). In both OVCAR-5 and OVCAR-4 cells, ML221 efficiently suppressed increased cell proliferation (Fig. 2A, B), and to a greater extent when APJ was overexpressed and/or activated compared to corresponding controls, especially at the later time points. Together these data demonstrate that APJ specifically enhances proliferation of OvCa cells *in vitro*.

APJ increases pro-metastatic phenotypes of anoikis resistance and cell adhesion.

Due to their prime position in the peritoneal cavity, OvCa cells “slough off” from the primary tumor site as multicellular structures, and survive in suspension in order to metastasize (22). We thus examined whether APJ expression affected anoikis resistance, which is the ability of epithelial cells to survive detached from the basement membrane; a property that would be crucial for survival in the peritoneum. We found that OVCAR-5-APJ cells had significantly increased survival in suspension compared to EV cells (Fig. 3A, D), which corresponded with significantly decreased levels of cleaved-parp (used as a marker for apoptosis; Supplementary Fig. S4A) indicating that APJ protects OvCa cells from anoikis-induced cell death. We observed the same phenomenon of increased survival in suspension in OVCAR-3-APJ cells (Supplementary Fig. S4B,C), as well as in apelin-13-treated OVCAR-4 cells (Fig. 3B,D), compared to their respective controls. Both pharmacological inhibition using ML221 in the overexpression cell lines (Fig. 3A,B,D), and

genetic inhibition via knockdown of APJ in OVCAR-8 cells (Fig. 3C,D) were able to restore anoikis sensitivity to different extents.

Modelling *in vivo* adhesion of OvCa cells to the peritoneal mesothelium (22), a phenotype that would aid in more efficient formation of metastases, we next tested whether APJ-expressing OvCa cells displayed increased cell adhesion to extracellular matrix (ECM) molecules *in vitro*. OVCAR-5-APJ had significantly increased cell adhesion to fibronectin (FN1)/laminin-coated plates compared to EV cells (Fig. 3E,H), but not to collagen IV (Supplementary Fig. S4D,G). Treatment of APJ-overexpressing cells with ML221 reverted the increased cell adhesion to FN1/Laminin plates to control levels (Fig. 3E,H). Interestingly, apelin-13-treated OVCAR-4 cells exhibited significant increases in adhesion to both FN1/laminin (Fig. 3F,H) and collagen IV (Supplementary Fig. S4E,G), which was suppressed by ML221 treatment (Fig. 3F,H; Supplementary Fig. S4E,G). In OVCAR-8 cells, KD of APJ significantly decreased adhesion to FN1/laminin (Fig. 3G,H) and collagen IV (Supplementary Fig. S4F,G) compared to control cells. Together these data indicate that increased APJ expression/activation confers increased anoikis resistance and cell adhesion to OvCa cells.

APJ increases migration and invasion of OvCa cells *in vitro*.

Using transwell chamber assays, we examined the effect of APJ on OvCa cell migration and invasion; phenotypes that mimic OvCa cell movement to secondary sites, and invasion into peritoneal walls to establish metastases, respectively (22). Migration assays were carried out for 6–8 hours, and invasion assays for 12–16 hours to exclude the effects of APJ on cell proliferation. We found that OVCAR-5-APJ and OVCAR-4 cells (in response to apelin-13), had significantly increased migration (Fig. 4A,B) and invasion (Fig. 4D,E) compared to their corresponding controls. The migratory and invasive phenotypes were specific to increased APJ expression and/or activation since treatment with ML221 was able to efficiently reverse the phenotypes *in vitro* (Fig. 4A,B,D,E). Similarly, compared to control shNT cells, OVCAR-8 cells with APJ KD showed significantly decreased migration and invasion (Fig. 4C, F). Thus, together these data demonstrate that APJ expression is both required and sufficient to increase migration and invasion of OvCa cells.

APJ functions via upregulation of STAT, AKT, and ERK signaling in OvCa cells.

Since APJ is a GPCR, we first examined the mechanisms involved in APJ signaling via phospho-kinase arrays using lysates from OVCAR-5-EV and APJ cells (Fig. 5A). The kinase array analyzed the levels of 43 individual phospho-proteins and 2 total proteins involved in cellular proliferation and survival. The relative phosphorylation levels of several proteins were higher in OVCAR-5-APJ cells compared to EV cells, and confirmed some known signaling pathways activated downstream of APJ such as ERK, AKT, and AMPK α 1 (23). Interestingly, we found increased phosphorylation of Src family kinase members such as Src, Hck and Fyn, and of some key STAT transcription factors including STAT5 and STAT3 (Fig. 5A).

Western blot analyses were performed to confirm a subset of phosphorylated proteins, including pERK, pAKT, and pSTAT, in OVCAR-5 (Fig. 5B) and OVCAR-3 cells

(Supplementary Fig. S5A,B). Cancer cells grown in suspension often have distinct gene and protein expressions compared to adherent cells, which can aid in their increased survival (24). Since we observed that APJ mediates such anoikis resistance, we also examined whether those phospho-proteins had differential expression under suspended conditions. In OVCAR-5-APJ cells compared with EV cells (Fig. 5B), increases in pSTAT3 were most substantial in cells in suspension cultures in comparison to pERK, while pAKT was significantly upregulated in both adherent and suspended conditions. In OVCAR-3-APJ cells compared to EV cells (Supplementary Fig. S5A,B), we found that pERK were elevated both in adherent and suspended conditions, whereas increases in pAKT and pSTAT3 were manifested mainly under adherent conditions. Interestingly, the overall levels of the phospho-proteins were higher in the OVCAR-3 suspended cells, compared to adherent conditions. Among the tested phospho-STAT proteins, only pSTAT3 was consistently regulated across all our model systems.

To interrogate which upregulated pathways downstream of APJ contributed to its pro-metastatic phenotype, we used pharmacologic inhibitors, U0126 (MEK/ERK inhibitor), GSK458 (AKT inhibitor), and Stattic (STAT3 inhibitor) in OVCAR-5 cells. The specificity of the drugs and appropriate drug concentrations were first confirmed by western blots for the respective phospho-proteins in adherent conditions (Supplementary Fig. S5C). Using these inhibitors in OVCAR-5-APJ cells (Fig. 5C-G), we observed that 1) ERK inhibitor was able to abrogate APJ-induced migration but no other phenotypes; 2) AKT inhibitor reversed the increased migration, invasion, cell adhesion, and anoikis resistance, but not clonal growth; and 3) STAT3 inhibition efficiently suppressed all five phenotypes downstream of APJ. In OVCAR-5-EV cells, these inhibitors showed either no effect, or a similar trend but to a lesser extent (Fig. 5C-G); the latter possibly due to their basal expression of APJ. These findings indicate that while multiple pro-metastatic signaling cascades are activated downstream of APJ, STAT3 consistently regulates the pro-metastatic phenotypes downstream of APJ.

Increased APJ expression increases intraperitoneal metastasis of HGSOC cells *in vivo*.

Since APJ expression in OvCa cells increased metastasis-related phenotypes *in vitro*, we determined whether APJ promotes later stages of metastasis of OvCa cells *in vivo*. Using OVCAR-3 cells, we found that compared to control mice injected with EV cells, mice injected with OVCAR-3-APJ cells had a significantly higher number of distinct large tumor masses (>1mm in diameter) on the peritoneal wall, gastrointestinal (GI) organs, and ovaries (Fig. 6A,B). No overt ascites formation was present in either group, and hence no differences were observed in the weights of the mice (Supplementary Fig. S6A). During a pilot study, we observed that when subcutaneously injected into the flank of mice, OVCAR-3-APJ cells produced modest increases in tumor volumes compared to control (Supplementary Fig. S6B), albeit with no statistical difference.

We confirmed the pro-metastatic role of APJ in another intraperitoneal model using OVCAR-5 cells. The most noticeable difference was increased area of miliary deposits on the peritoneum, and implantation into peritoneal organs (which are hallmarks of HGSOC), in mice injected with OVCAR-5-APJ cells (Fig. 6D) compared to control mice. While

miliary metastases on the abdominal walls were difficult to quantify, mice injected with OVCAR-5-APJ cells also had a significantly higher number of large non-miliary masses (>1 mm) compared to the control mice (Fig. 6D,E). Akin to the OVCAR-3 model, the weights of mice were fairly comparable in both groups (Supplementary Fig. S6C).

Further immunohistochemical analyses of tumor tissue taken from mice injected with APJ-overexpressing cells revealed significantly increased expression of pSTAT3 protein compared to control tumors, indicating that the STAT3 pathway is activated downstream of APJ *in vivo* as well (Fig. 6C,F). Notably, we observed massive upregulation of APJ protein expression in those excised tumor tissues compared to the EV control tumors, the extent of which was additionally multifold higher than that in cells grown in culture (Supplementary Fig. S6D), indicating that APJ expression is indeed increased *in vivo*. Taken together, these data demonstrate that increased APJ expression promotes the later stages of metastasis in ovarian cancer, which includes peritoneal implantation and growth within the peritoneum cavity.

DISCUSSION

HGSOC is the most common and lethal subtype of OvCa, accounting for around 80% of diagnosed cases in the United States (1). The high mortality rate associated with this disease is largely due to late stage diagnosis of the disease when tumors are widely metastasized, and the eventual development of resistance to conventional chemotherapeutics (22). Advanced HGSOCs are highly metastatic, and disseminate and seed extensively in the peritoneum. Hence, overall survival remains low, and long term survival is significantly worse (25). Thus, the identification of novel, targetable pathways to inhibit metastatic potential of HGSOC is urgently needed, and may lead to improvements in the outcomes of patients with this disease.

Herein, we demonstrated that the APJ pathway is pro-tumorigenic in HGSOC (Fig. 6G). We showed that APJ expression is significantly higher in malignant cells within HGSOC tissues, than in normal ovarian epithelial cells, and particularly higher in metastasized tumors compared to primary tumors. Ovarian cancer cells expressed variable levels of APJ, and the observed disparity of APJ expression on the mRNA and protein may be due to either post-translational modifications, or APJ protein stability in the individual cell lines, but remains to be determined. *In vitro*, using various model systems, we showed that APJ was both necessary and sufficient to increase aggressive phenotypes of OvCa cell lines including migration, invasion, and proliferation. We observed that not only did APJ promote anoikis resistance, cells with increased APJ expression also expressed high basal levels of PARP (Supplementary Fig. S4A). There have been reports (26,27) that show correlations between increased PARP levels and increased chemoresistance, and studies to elucidate this interplay are ongoing. The APJ-expressing cells also had differences in adhesion to the ECM proteins that may be attributed to the differences in cell types and/or the levels of APJ in the cells. Nevertheless, the consistent increase in adhesion to FN1/laminin indicates cross-talk between integrin (28) and APJ pathways, which remains to be explored.

In our *in vitro* assays while OVCAR-4 cells exhibited increased metastatic properties with exogenous addition of the ligand, such addition was not required for OVCAR-5- and OVCAR-3-APJ-expressing cells. Since OvCa cells secrete apelin, we speculate that the endogenous apelin secreted by the OVCAR-3 and OVCAR-5 cells is sufficient to activate the pathway. Alternatively, it is possible that the APJ pathway is constitutively active in the APJ-overexpressing cells, which negates the requirement for additional ligand. Nonetheless, both autocrine (in the case of APJ-overexpressing cells) and paracrine (in OVCAR-4 cells) activation of the pathway in OvCa cells increased pro-metastatic phenotypes. These phenotypes were suppressed by both, APJ KD and pharmacological inhibition using ML221, further indicating the necessity and specificity of APJ for increasing aggressive phenotypes. While we observed some effects of ML221 in the EV cells, we speculate that this may be due to the basal level of APJ expressed in those cells. Moreover, ML221 effectively inhibited some phenotypes such as cell migration and invasion at low concentrations, but higher concentrations were required to suppress others, indicating the varying sensitivity of phenotypes to the drug. Coupled with poor solubility and metabolic stability issues (29), our data further indicate the need for better APJ antagonists. Nevertheless, our *in vitro* data demonstrate that expression/activation of APJ by cancer cells is functional, and pro-tumorigenic in OvCa.

APJ as a GPCR is known to activate several pathways, such as the ERK, AKT, and AMPK cascades to regulate a multitude of physiological processes (23). Notably, we found that STAT3 signaling was activated in APJ-overexpressing OvCa cells, the inhibition of which had the most profound effects on APJ-mediated pro-metastatic phenotypes *in vitro*. Importantly, we demonstrated that pSTAT3 protein expression was significantly increased in APJ-overexpressing tumors indicating that the STAT3 pathway is also activated downstream of APJ *in vivo*. While the mechanism by which APJ regulates STAT3 remains to be elucidated, to our knowledge, this is the first study to demonstrate STAT3 activation downstream of APJ. Thus together, our data indicate that a combination of inhibitors against STAT3 (which itself is emerging as an important player in OvCa tumorigenesis (30)), and other efficacious APJ pathway antagonists such as F13A and MM54 (31–33), will aid in achieving increased tumor inhibition in ovarian cancer.

Our *in vivo* models revealed that high APJ expression promotes peritoneal dissemination of HGSOC, which includes seeding and growth of cancer cells within the peritoneal cavity. The most striking difference was in the number of large tumor masses in mice that were injected with APJ-overexpressing cells compared to control. This indicates that APJ confers survival and/or proliferative advantages to cancer cells (which is in line with our *in vitro* data) and thus promotes peritoneal dissemination. Notably, we observed that APJ protein expression was significantly increased in the cells grown *in vivo* compared to those grown *in vitro*, which is in line with the observed patient data (Fig. 1B), demonstrating that APJ expression is indeed increased *in vivo*. While apelin is secreted by cancer cells themselves, and by endothelial cells (34,35) in the tumor microenvironment, apelin is also an adipokine secreted by adipocytes, and its serum level is elevated in some non-cancer related disease states (3,36). Thus, *in vivo*, it is possible that there are multiple additional sources for apelin, given that the tumor microenvironment for OvCa is replete with fat reserves consisting of adipocytes. *In vivo* activation of the pathway may also occur via other forms of apelin,

including apelin-12 and apelin-36 (37) that are not well-studied in cancer, or via elabela (38), an additional ligand for APJ, which has recently been shown to play an important role in clear cell ovarian carcinoma (39), but this remains to be determined.

Importantly, we showed that high expression of APJ in HGSOC patient tumors was significantly associated with shorter overall survival by 14.7 months, suggesting a pathological significance of APJ pathway in ovarian cancer. While the APJ pathway has been shown to increase pro-metastatic phenotypes *in vitro* in prostate cancer and cholangiocarcinoma (10,40), and correlate with worsened prognosis in oral squamous cell carcinoma (41), many studies have focused on the role of this axis in tumor angiogenesis (42–44). Thus, to our knowledge, our study is the first to demonstrate a pro-metastatic role of APJ in HGSOC, a malignancy where no effective therapies are currently available, and are hence desperately needed to improve patient outcome.

In summary, we have identified that increased APJ expression in HGSOC promotes tumor progression and metastasis. While not examined in this study, given the role of APJ in increasing angiogenesis (45,46), it is plausible that inhibition of the APJ pathway will cause more complete suppression of OvCa tumor progression by curbing both, the metastatic phenotypes in the tumor cells, and angiogenesis. Thus, our studies present the APJ pathway as a universal, novel therapeutic target in this deadly and highly metastatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank Drs. Kar-Ming Fung and Muralidharan Jayaraman, and Ms. Sheeja Aravindan for their help with the immunohistochemistry experiments, as well as the OUHSC Histology and Molecular Imaging Cores for their service and technical assistance.

This work was supported in part by research grants P20GM103639 (S Woo) from the National Institute of General Medical Sciences, NIH, DHHS, Research Scholar Grant RSG-16-006-01-CCE (S Woo) from the American Cancer Society, and Gynecology Oncology Drug Development Fund (S Woo) from Stephenson Cancer Center.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018;68(1):7–30 doi 10.3322/caac.21442. [PubMed: 29313949]
2. Chapman NA, Dupre DJ, Rainey JK. The apelin receptor: physiology, pathology, cell signalling, and ligand modulation of a peptide-activated class A GPCR. *Biochemistry and cell biology = Biochimie et biologie cellulaire* 2014;92(6):431–40 doi 10.1139/bcb-2014-0072. [PubMed: 25275559]
3. Bertrand C, Valet P, Castan-Laurell I. Apelin and energy metabolism. *Front Physiol* 2015;6:115 doi 10.3389/fphys.2015.00115. [PubMed: 25914650]
4. Yang Y, Lv SY, Ye W, Zhang L. Apelin/APJ system and cancer. *Clin Chim Acta* 2016;457:112–6 doi 10.1016/j.cca.2016.04.001. [PubMed: 27083318]
5. Berta J, Kenessey I, Dobos J, Továri J, Klepetko W, Jan Ankersmit H, et al. Apelin Expression in Human Non-small Cell Lung Cancer: Role in Angiogenesis and Prognosis. *Journal of Thoracic Oncology* 2010;5(8):1120–9 doi 10.1097/JTO.0b013e3181e2c1ff. [PubMed: 20581707]
6. Sorli SC, Le Gonidec S, Knibiehler B, Audigier Y. Apelin is a potent activator of tumour neoangiogenesis. *Oncogene* 2007;26(55):7692–9 doi 10.1038/sj.onc.1210573. [PubMed: 17563744]

7. Kälén RE, Kretz MP, Meyer AM, Kispert A, Heppner FL, Brändli AW. Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis. *Developmental Biology* 2007;305(2):599–614 doi 10.1016/j.ydbio.2007.03.004. [PubMed: 17412318]
8. Chen T, Liu N, Xu GM, Liu TJ, Liu Y, Zhou Y, et al. Apelin13/APJ promotes proliferation of colon carcinoma by activating Notch3 signaling pathway. *Oncotarget* 2017;8(60):101697–706 doi 10.18632/oncotarget.21904. [PubMed: 29254197]
9. Picault F-X, Chaves-Almagro C, Progetti F, Prats H, Masri B, Audigier Y. Tumour co-expression of apelin and its receptor is the basis of an autocrine loop involved in the growth of colon adenocarcinomas. *European Journal of Cancer* 2014;50(3):663–74 doi 10.1016/j.ejca.2013.11.017. [PubMed: 24316062]
10. Hall C, Ehrlich L, Venter J, O'Brien A, White T, Zhou T, et al. Inhibition of the apelin/apelin receptor axis decreases cholangiocarcinoma growth. *Cancer letters* 2017;386:179–88 doi 10.1016/j.canlet.2016.11.025. [PubMed: 27894959]
11. Mok SC, Bonome T, Vathipadiekal V, Bell A, Johnson ME, Wong KK, et al. A gene signature predictive for outcome in advanced ovarian cancer identifies a survival factor: microfibril-associated glycoprotein 2. *Cancer Cell* 2009;16(6):521–32 doi 10.1016/j.ccr.2009.10.018. [PubMed: 19962670]
12. Bowen NJ, Walker LD, Matyunina LV, Logani S, Totten KA, Benigno BB, et al. Gene expression profiling supports the hypothesis that human ovarian surface epithelia are multipotent and capable of serving as ovarian cancer initiating cells. *BMC Med Genomics* 2009;2:71 doi 10.1186/1755-8794-2-71. [PubMed: 20040092]
13. Tothill RW, Tinker AV, George J, Brown R, Fox SB, Lade S, et al. Novel Molecular Subtypes of Serous and Endometrioid Ovarian Cancer Linked to Clinical Outcome. *Clinical Cancer Research* 2008;14(16):5198–208 doi 10.1158/1078-0432.ccr-08-0196. [PubMed: 18698038]
14. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 2004;6(1):1–6. [PubMed: 15068665]
15. Neelakantan D, Zhou H, Oliphant MUJ, Zhang X, Simon LM, Henke DM, et al. EMT cells increase breast cancer metastasis via paracrine GLI activation in neighbouring tumour cells. *Nat Commun* 2017;8:15773 doi 10.1038/ncomms15773. [PubMed: 28604738]
16. Mitra AK, Davis DA, Tomar S, Roy L, Gurler H, Xie J, et al. In vivo tumor growth of high-grade serous ovarian cancer cell lines. *Gynecol Oncol* 2015;138(2):372–7 doi 10.1016/j.ygyno.2015.05.040. [PubMed: 26050922]
17. Glassford AJ, Yue P, Sheikh AY, Chun HJ, Zarafshar S, Chan DA, et al. HIF-1 regulates hypoxia- and insulin-induced expression of apelin in adipocytes. *Am J Physiol Endocrinol Metab* 2007;293(6):E1590–6 doi 10.1152/ajpendo.00490.2007. [PubMed: 17878221]
18. Heo K, Kim YH, Sung HJ, Li HY, Yoo CW, Kim JY, et al. Hypoxia-induced up-regulation of apelin is associated with a poor prognosis in oral squamous cell carcinoma patients. *Oral Oncology* 2012;48(6):500–6 doi 10.1016/j.oraloncology.2011.12.015. [PubMed: 22285858]
19. Gyorffy B, Lanczky A, Szallasi Z. Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. *Endocr Relat Cancer* 2012;19(2):197–208 doi 10.1530/ERC-11-0329. [PubMed: 22277193]
20. Kakizawa S Chapter 31 - Apelin A2 - Takei, Yoshio In: Ando H, Tsutsui K, editors. *Handbook of Hormones*. San Diego: Academic Press; 2016 p 277–e31–3.
21. Maloney PR, Khan P, Hedrick M, Gosalia P, Milewski M, Li L, et al. Discovery of 4-oxo-6-((pyrimidin-2-ylthio)methyl)-4H-pyran-3-yl 4-nitrobenzoate (ML221) as a functional antagonist of the apelin (APJ) receptor. *Bioorganic & Medicinal Chemistry Letters* 2012;22(21):6656–60 doi 10.1016/j.bmcl.2012.08.105. [PubMed: 23010269]
22. Lengyel E Ovarian cancer development and metastasis. *Am J Pathol* 2010;177(3):1053–64 doi 10.2353/ajpath.2010.100105. [PubMed: 20651229]
23. Chaves-Almagro C, Castan-Laurell I, Dray C, Knauf C, Valet P, Masri B. Apelin receptors: From signaling to antidiabetic strategy. *Eur J Pharmacol* 2015;763(Pt B):149–59 doi 10.1016/j.ejphar.2015.05.017. [PubMed: 26007641]

24. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta* 2013;1833(12):3481–98 doi 10.1016/j.bbamcr.2013.06.026. [PubMed: 23830918]
25. Chen M, Jin Y, Bi Y, Yin J, Wang Y, Pan L. A survival analysis comparing women with ovarian low-grade serous carcinoma to those with high-grade histology. *Onco Targets Ther* 2014;7:1891–9 doi 10.2147/OTT.S67812. [PubMed: 25342912]
26. Ganesan S MYC, PARP1, and chemoresistance: BIN there, done that? *Sci Signal* 2011;4(166):pe15 doi 10.1126/scisignal.2001946. [PubMed: 21447796]
27. Cheng H, Zhang Z, Borczuk A, Powell CA, Balajee AS, Lieberman HB, et al. PARP inhibition selectively increases sensitivity to cisplatin in ERCC1-low non-small cell lung cancer cells. *Carcinogenesis* 2013;34(4):739–49 doi 10.1093/carcin/bgs393. [PubMed: 23275151]
28. Strobel T, Cannistra SA. Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. *Gynecologic oncology* 1999;73(3):362–7 doi 10.1006/gyno.1999.5388. [PubMed: 10366461]
29. Maloney PR, Khan P, Hedrick M, Gosalia P, Milewski M, Li L, et al. Functional antagonists of the Apelin (APJ) receptor. Probe Reports from the NIH Molecular Libraries Program. Bethesda (MD)2010.
30. Saini U, Suarez AA, Naidu S, Wallbillich JJ, Bixel K, Wanner R, et al. STAT3/PIAS3 levels serve as “early signature” genes in the development of high-grade serous carcinoma from the fallopian tube. *Cancer Res* 2018 doi 10.1158/0008-5472.CAN-17-1671.
31. Lee DK, Saldivia VR, Nguyen T, Cheng R, George SR, O’Dowd BF. Modification of the terminal residue of apelin-13 antagonizes its hypotensive action. *Endocrinology* 2005;146(1):231–6 doi 10.1210/en.2004-0359. [PubMed: 15486224]
32. Liu Y, Zhang J, Wang Y, Zeng X. Apelin involved in progression of diabetic nephropathy by inhibiting autophagy in podocytes. *Cell Death Dis* 2017;8(8):e3006 doi 10.1038/cddis.2017.414. [PubMed: 28837139]
33. Harford-Wright E, Andre-Gregoire G, Jacobs KA, Treps L, Le Gonidec S, Leclair HM, et al. Pharmacological targeting of apelin impairs glioblastoma growth. *Brain : a journal of neurology* 2017;140(11):2939–54 doi 10.1093/brain/awx253. [PubMed: 29053791]
34. Eyries M, Siegfried G, Ciomas M, Montagne K, Agrapart M, Lebrin F, et al. Hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. *Circ Res* 2008;103(4):432–40 doi 10.1161/CIRCRESAHA.108.179333. [PubMed: 18617693]
35. Novakova V, Sandhu GS, Dragomir-Daescu D, Klabusay M. Apelinergic system in endothelial cells and its role in angiogenesis in myocardial ischemia. *Vascul Pharmacol* 2016;76:1–10 doi 10.1016/j.vph.2015.08.005. [PubMed: 26254105]
36. Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, et al. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 2005;146(4):1764–71 doi 10.1210/en.2004-1427. [PubMed: 15677759]
37. Kleinz MJ, Davenport AP. Emerging roles of apelin in biology and medicine. *Pharmacol Ther* 2005;107(2):198–211 doi 10.1016/j.pharmthera.2005.04.001. [PubMed: 15907343]
38. Wang Z, Yu D, Wang M, Wang Q, Kouznetsova J, Yang R, et al. Elabela-apelin receptor signaling pathway is functional in mammalian systems. *Sci Rep* 2015;5:8170 doi 10.1038/srep08170. [PubMed: 25639753]
39. Yi Y, Tsai SH, Cheng JC, Wang EY, Anglesio MS, Cochrane DR, et al. APELA promotes tumour growth and cell migration in ovarian cancer in a p53-dependent manner. *Gynecologic oncology* 2017;147(3):663–71 doi 10.1016/j.ygyno.2017.10.016. [PubMed: 29079036]
40. Wan Y, Zeng ZC, Xi M, Wan S, Hua W, Liu YL, et al. Dysregulated microRNA-224/apelin axis associated with aggressive progression and poor prognosis in patients with prostate cancer. *Hum Pathol* 2015;46(2):295–303 doi 10.1016/j.humpath.2014.10.027. [PubMed: 25532941]
41. Heo K, Kim YH, Sung HJ, Li HY, Yoo CW, Kim JY, et al. Hypoxia-induced up-regulation of apelin is associated with a poor prognosis in oral squamous cell carcinoma patients. *Oral Oncol* 2012;48(6):500–6 doi 10.1016/j.oraloncology.2011.12.015. [PubMed: 22285858]

42. Feng M, Yao G, Yu H, Qing Y, Wang K. Tumor apelin, not serum apelin, is associated with the clinical features and prognosis of gastric cancer. *BMC Cancer* 2016;16(1):794 doi 10.1186/s12885-016-2815-y. [PubMed: 27733135]
43. Rayalam S, Della-Fera MA, Kasser T, Warren W, Baile CA. Emerging role of apelin as a therapeutic target in cancer: a patent review. *Recent Pat Anticancer Drug Discov* 2011;6(3):367–72 doi 10.2174/157489211796957856. [PubMed: 21612576]
44. Zhang L, Takara K, Yamakawa D, Kidoya H, Takakura N. Apelin as a marker for monitoring the tumor vessel normalization window during antiangiogenic therapy. *Cancer Sci* 2016;107(1):36–44 doi 10.1111/cas.12836. [PubMed: 26475217]
45. Wu L, Chen L, Li L. Apelin/APJ system: A novel promising therapy target for pathological angiogenesis. *Clin Chim Acta* 2017;466:78–84 doi 10.1016/j.cca.2016.12.023. [PubMed: 28025030]
46. Kidoya H, Takakura N. Biology of the apelin-APJ axis in vascular formation. *J Biochem* 2012;152(2):125–31 doi 10.1093/jb/mvs071. [PubMed: 22745157]

Implication:

The APJ pathway is a viable target in high-grade serous ovarian carcinoma.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Expression and pathological significance of APJ in ovarian cancer.

(A) qRT-PCR and representative western blot of whole cell lysates (WCL) from a panel of ovarian cancer (OvCa) cells compared to HEK293T cells transiently transfected with APJ, fallopian tube epithelial cells (FTE188) and human ovarian surface epithelial cells (HOSE), α -tubulin – loading control. (B) Expression of *APJ* in OvCa tumor (laser micro-dissected) and non-tumor tissues from GEO databases, and OvCa cell lines from Cancer Cell Line Encyclopedia (CCLE) databases. (C) Immunohistochemical staining for APJ in tumor tissue microarray (TMA) containing high grade serous ovarian tumors (n=124). Representative images are shown of no or weak staining (APJ low) and moderate or high staining (APJ high). Scale bar - 200 μ m. (D) Kaplan-Meier survival plot for overall survival in APJ-expressing tumors in TMA, corresponding to panel C.

n = 3 for **A**; statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test for **A** (*: significance relative to HOSE, #: significance relative to FTE188); unpaired Mann-Whitney test for **B** and log-rank test for **D** where P value < 0.05 considered significant.

Fig. 2. APJ specifically increases OvCa cell proliferation.

Representative 4 day cell counting assay in (A) OVCAR-5-EV and APJ cells, (B) OVCAR-4 cells +/- 100 ng/mL apelin-13, and (C) OVCAR-8 shNT and shAPJ-1,2 cells. ML221 in the concentration range of 15–50 μ M was used. Insets: Representative western blot analyses of WCLs from (A) OVCAR-5 cells stably transfected with APJ and EV plasmids and (C) OVCAR-8 cells stably transfected with two different shRNA constructs, shAPJ-1, shAPJ-2 and non-targeting control shNT.

Results obtained from 3 independent experiments (Mean $\pm$ SEM). Statistical analysis was performed using two-way ANOVA followed by Tuckey's post hoc test for A-C. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Fig. 3. APJ-dependent increases in anoikis resistance and cell adhesion of OvCa cells. (A, B) Representative 24 h anoikis resistance assays in (A) OVCAR-5-EV and APJ cells (magnification-2.5X) and (B) OVCAR-4 cells +/- 100 ng/mL apelin-13 (magnification-5X). (C) Representative 48 h anoikis resistance assay in OVCAR-8 shNT and shAPJ cells (magnification-10x). (D) Quantification of assays in panels A, B and C represented as fold change over EV or shNT (set to 1). (E, F, G) Representative 2–2.5 h cell adhesion assays in (E) OVCAR-5-EV and APJ cells, (F) OVCAR-4 cells +/- 100 ng/mL apelin-13 and ML221 and (G) OVCAR-8 APJ KD and control cells, plated on fibronectin-1(FN1)/ laminin-coated

plates; magnification-5X. **(H)** Quantification of assays in panels **E**, **F**, and **G** represented as fold change over EV or shNT (set to 1). Scale bar: 200 μ m; ML221 in the concentration range of 30–50 μ M was used.

Results obtained from 3 independent experiments (Mean $\pm$ SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test in **D** and **H**. * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001.

Fig. 4. ML-221 and APJ knockdown decreases APJ-specific increases in OvCa cell migration and invasion.

(A-C) Representative transwell migration assay and quantification in (A) OVCAR-5-EV and APJ cells for 8 h, (B) OVCAR-4 cells +/- 100 ng/mL of apelin-13 and ML221 for 6 h, and in (C) OVCAR-8 APJ KD and control cells for 8 h. (D-F) Representative 16 h-transwell invasion assay and quantification in (D) OVCAR-5-EV and APJ cells, (E) OVCAR-4 cells +/- 100 ng/mL apelin-13 and ML221 and (F) OVCAR-8 APJ KD and control cells. Quantification is represented as fold change over EV or shNT (set to 1). ML221 in the

concentration range of 5–10 μM was used; Scale bar: 100 μm for A, D; 200 μm for B, C, E, F.

Results obtained from 3 independent experiments ($\text{Mean} \pm \text{SEM}$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test in all cases. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Fig. 5. STAT-3 pathway mediates pro-metastatic phenotypes downstream of APJ in OvCa cells.

(A) Phospho-kinase array performed in OVCAR-5-EV and APJ cells and corresponding quantification (as fold change over EV). (B) Representative western blot analysis in WCLs from OVCAR-5-EV and APJ cells grown in adherent and suspended conditions for 48 h, probed with specified antibodies; GAPDH- loading control. Representative (C) 8 h-migration, (D) 16 h-invasion, (E) 2 h-cell adhesion to FN1/laminin plates, (F) 24 h-anoikis resistance, and (G) 11 day-clonogenic assay performed in OVCAR-5-EV and APJ cells

treated with 10 μ M U0126 (MEK inhibitor), 2.5 μ M (**B-F**) and 0.5 μ M (**for G**) Stattic (STAT3 inhibitor), and 40 nM GSK458 (AKT inhibitor) or vehicle control. Results obtained from 3 independent experiments (Mean $\pm$ SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test in all cases. * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001; ns: not significant.

Fig. 6. Increased APJ expression promotes metastasis in orthotopic models of HGSOc.

6–8 week old female athymic nude mice received intraperitoneal injections of either (10×10^6 cells/mouse) OVCAR-3-EV and APJ cells (treated with 100 ng/mL apelin-13) or (5×10^6 cells/mouse) OVCAR-5-EV and APJ cells. Mice were euthanized when moribund and tumor tissue was collected and processed. (A) Representative images of the metastasized tumors in the peritoneal cavities of mice injected with OVCAR-3-EV and APJ cells (circled in yellow). (B) Total number of large metastasized nodules $\pm$ SD (>1 mm) in OVCAR-3-EV and APJ tumor groups. (C) Representative H&E stains and immunohistochemical (IHC)

stains for pSTAT3 and STAT3 in OVCAR-3 tumors with quantification. **(D)** Representative images of metastasized tumors in mice injected with OVCAR-5-EV and APJ cells (yellow arrows-miliary mets; yellow circles-non-miliary mets). **(E)** Total number of large metastasized nodules $\pm$ *SD* (>1 mm) in OVCAR-5-EV and APJ tumor groups. **(F)** Representative H&E stains and IHC for pSTAT3 and STAT3 in OVCAR-5 tumors with quantification. **(G)** Graphical summary of paper demonstrating that APJ-overexpressing OvCa cells can efficiently metastasize in the peritoneum. APJ overexpression results in activation of various pro-metastatic cascades including STAT3, ERK, and AKT, which promote pro-metastatic phenotypes in tumor cells. These phenotypes can be reversed using various inhibitors *in vitro* and may result in curbing tumor progression *in vivo*. Scale bar in **C**, **F**– 200 μ m; statistical analysis was performed using two-tailed unpaired *t*-test in **B**, **C**, **E**, and **F**.; **P*<0.05 ***P*<0.01; ****P*<0.001.