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Original Articles

Prolactin signaling drives tumorigenesis in human high grade serous ovarian cancer cells and in a spontaneous fallopian tube derived model

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ABSTRACT

The pathways responsible for tumorigenesis of high grade serous ovarian cancer (HGSOC) from the fallopian tube epithelium (FTE) are still poorly understood. A human prolactin (PRL) like gene, *Prl2c2* was amplified > 100 fold in a spontaneous model of FTE-derived ovarian cancer (MOE^{high} - murine oviductal epithelium high passage). *Prl2c2* stable knockdown in MOE^{high} cells demonstrated a significant reduction in cell proliferation, 2-dimensional foci, anchorage independent growth, and blocked tumor formation. The overall survival of ovarian cancer patients from transcriptome analysis of 1868 samples was lower when abundant PRL and prolactin receptors (PRL-R) were expressed. A HGSOC cell line (OVCAR3) and a tumorigenic human FTE cell line (FT33-Tag-Myc) were treated with recombinant PRL and a significant increase in cellular proliferation was detected. A CRISPR/Cas9 mediated PRL-R deletion in OVCAR3 and FT33-Tag-Myc cells demonstrated significant reduction in cell proliferation and eliminated tumor growth using the OVCAR3 model. PRL was found to phosphorylate STAT5, m-TOR and ERK in ovarian cancer cells. This study identified *Prl2c2* as a driver of tumorigenesis in a spontaneous model and confirmed that prolactin signaling supports tumorigenesis in high grade serous ovarian cancer.

1. Introduction

High grade serous ovarian cancer (HGSOC) is the most lethal histotype of ovarian cancer (OVCA) [1], and growing evidence indicates that most HGSOC arises in the fallopian tube epithelium (FTE) [2–4]. Although mouse models with targeted genetic alterations in the FTE (typically called the oviduct in mice) developed HGSOC [5], few models characterize the spontaneous pathogenesis of HGSOC from the fallopian tube, which is essential to identify novel targets of ovarian tumor formation and to understand the disease etiology.

The laying hen has been identified as a spontaneous model of ovarian carcinoma, which develops all four histotypes as humans. While these carcinomas possess similar gene expression profile as seen in humans, it is challenging to study FTE-derived HGSOC in this model, because the oviduct primarily function as a shell gland [6,7]. Spontaneous cellular models of ovarian cancer were previously developed by serially passaging the ovarian surface epithelial (OSE) cells [8]. Using rat OSE cells, the ROSE model was made, which formed ovarian tumors and contained similar recurrent chromosomal alterations to those of OVCA [9]. Two other models of OVCA were derived using murine OSE, called MOSEC and STOSE (spontaneously transformed OSE) [10,11]. Both MOSEC and STOSE formed intraperitoneal tumors. A cDNA microarray on STOSE identified modifications in the expression of several genes that are also modified in HGSOC [10,11]. Although, these models defined the origin and progression of epithelial tumors from the ovary, the spontaneous events that happen in the FTE that can lead to HGSOC remain unexplored.

We previously created a spontaneous model of FTE-derived OVCA by serially passaging murine oviductal epithelial cells (MOE^{high}). These cells formed subcutaneous tumors in mice and transcriptomic analysis of the MOE^{high} cells revealed several pathways that were altered similar to HGSOC such as FOXM1, c-myc, and loss of Cdkn2a [12]. However, several other transcripts were significantly altered that may play an essential role in tumorigenesis of HGSOC, such as *Prl2c2* and *Wnt7b*

Abbreviations: HGSOC, High grade serous ovarian cancer; FTE, Fallopian tube epithelium; MOE, Murine oviductal epithelium; PRL, Prolactin; PRL-R, Prolactin receptors

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[12]. *Wnt7b* (Wingless – type MMTV integration site family, member 7B) is a secreted morphogen that was enriched 36-fold in MOE^{high} cells [12]. WNT7B expression levels are enriched in human OVCA and in breast cancer when compared to benign tissues [13,14]. *Prl2c2*, that encodes for proliferin, was amplified > 100 fold in the spontaneous model [12]. Enhanced expression of *Prl2c2* induced malignant transformation of murine fibroblasts and was a critical driver of murine lung adenocarcinoma [15,16]. *Prl2c2* is a murine hormone, without a direct human homolog; however, *Prl2c2* belongs to the prolactin superfamily (prolactin family 2 subfamily c member 2) [17].

Prolactin (PRL) is secreted by the pituitary gland and is best known to stimulate milk production [18]. However, prolactin is also produced locally by many tissues, and increasing evidence supports the hypothesis that the local accumulation of PRL can contribute to tumorigenesis of cancers, such as breast and colorectal [19]. PRL was a component of a multiplex immunoassay called Ovasure, used for early detection of OVCA [20]. While this product is no longer FDA approved, the role of prolactin as a biomarker suggests it might also play a significant role in disease formation and/or progression. Prolactin receptor (PRL-R) activation by its ligand activates several kinases that are well known to stimulate cellular proliferation [18]. Chronic exposure of PRL induced the transformation of immortalized OSE cells and resulted in tumor formation in mice [19]. Since most human HGSOC are now thought to originate from the FTE, and not the OSE, exploring the signaling mechanism of PRL in human FTE and FTE-like HGSOC cells is necessary.

We hypothesized that *Prl2c2* and *Wnt7b* are critical for enhanced cell proliferation and induction of ovarian tumors in the spontaneous MOE^{high} cells. This study found that both *Wnt7b* and *Prl2c2* were essential for increased cell proliferation of MOE^{high} cells *in vitro*, but only knockdown of *Prl2c2* suppressed MOE^{high} tumors *in vivo*. To translate these findings into human models, the role of PRL and WNT7B signaling in proliferation and tumorigenesis of human FTE and HGSOC cells was explored. Although PRL and WNT7B were sufficient to alter proliferation, PRL alone was pro-tumorigenic in human FTE and tumors, which could have significant implications for future studies aimed at suppressing PRL levels or using neutralizing antibodies to block PRL-R signaling in HGSOC.

2. Materials and methods

2.1. Cell culture

High passage murine oviductal epithelial cells (MOE^{high}) were established as previously described [12]. The human FT33-Tag-Myc cells were a generous gift from Dr. Ronny Drapkin, at the University of Pennsylvania, PA, and were maintained in the media as described earlier [21]. OVCAR3 and OVCAR8 (from ATCC) and OVCAR4 cells (from the National Cancer Institute), were maintained in media as described previously and verified by STR analysis [22].

2.2. Transient transfection and generation of stable cell lines

MOE cells were transiently transfected with pCDNA-Wnt7b (gift from Dr. Marian Waterman, University of California, CA; Addgene plasmid # 35915) [23] using TransIT LT1™ (Mirus Bio, Madison, WI) according to the manufacturer's instructions. For stable cell lines, MOE^{high} cells stably expressing shRNA targeting *Prl2c2*, *Wnt7b*, or a non-target shRNA control were produced by transfecting MOE^{high} cells with shRNA targeting each gene (Supplementary Table S1). FT33-Tag-Myc cells stably expressing a constitutively active PRL-R or empty vector control were produced by transfecting FT33-Tag-Myc cells with pCDNA-PRL-R$_{CA}$ (gift from Dr. Geula Gibori and Dr. Carlos Stocco, University of Illinois at Chicago, IL) and pCMV6-Myc-Neo (donated by Dr. Kwong Wong, M.D. Anderson Cancer Center, Houston, TX). Cell lines were generated by treatment with selection antibiotic and clonal selection. Guide RNAs (gRNA) for CRISPR/Cas9 were designed using CRISPOR (http://crispor.tefor.net/; Supplementary Table 2) [24]. The gRNAs (Integrated DNA Technologies, IA) were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (gift from Dr. Feng Zhang, Massachusetts Institute of Technology, MA; Addgene plasmid # 42230) [25]. The pX330 plasmid with gRNA was co-transfected with pPGKpuro plasmid (gift from Dr. Rudolf Jaenisch, Massachusetts Institute of Technology, MA; Addgene plasmid # 11349) [26]. Cells were treated with puromycin and single cell clones were isolated. Genomic DNA was extracted from cells using genomic DNA extraction kit (#G170, Sigma-Aldrich, MO) as per the manufacturer's instructions, and the targeted exon was amplified and sequenced (**primers in** Supplementary Table S2).

2.3. Quantitative reverse transcriptase PCR (qPCR)

RNA was extracted was using Trizol (product # 15596026, Life Technologies, Grand Island, NY) and precipitated with chloroform and isopropanol followed by ethanol washes and DNAse treatment. RNA (1 μg) was reverse transcribed using iScript cDNA synthesis kit (#1708890, Biorad, Hercules, CA) according to manufacturer's instructions. All qPCR measurements were performed using the ABI ViiA7 (Life Technologies, San Diego, CA) and SYBR green (#4913850001, Roche, WI). All qPCR primers (Supplementary Table S3) were validated for specificity by inspection of the melt curve.

2.4. Immunoblotting

Cells were lysed with RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) containing protease and phosphatase inhibitors (#4693159001, Roche, WI, #P0044, Sigma-Aldrich, St. Louis, MO), freeze-thawed, and centrifuged. Protein (25–30 μg) was separated by electrophoresis in SDS-PAGE gels and transferred to nitrocellulose membrane (#P188018, Thermo Fisher Scientific, Waltham, MA). Blots were then blocked with 5% milk or BSA in TBS-T and probed at 4 °C overnight with primary antibodies (Supplementary Table S4). Membranes were then washed and incubated with HRP-conjugated secondary antibodies raised against rabbit or mouse (#7076 and #7074, Cell Signaling Technology) for 30 min. After washing, membranes were incubated in SuperSignal West Femto substrate (#34095, Thermo Scientific, IL) before imaging on a FlourChem™ E system (ProteinSimple, Santa Clara, CA) [27]. Densitometric analysis was performed using ImageJ (imagej.nih.gov).

2.5. SRB proliferation assay

The sulforhodamine B (SRB) assay was used to measure cell proliferation as previously described [12,28]. Cells (500–25000, depending on cell line) were plated in 96 well plates and incubated for 0, 1, 3, 5 and 7 days. Cells were treated with PRL, small molecule inhibitors, or siRNA. The media was changed after 24 h to media with 2% FBS before the addition of PRL, inhibitors (Supplementary Table S5), or PRL-R siRNA (#EHU095011, Sigma-Aldrich, MO) to each well as indicated. MOE^{High} cells were treated with indicated concentrations of cisplatin for 4 days. Absorbance were read at 505 nm and normalized to its corresponding control (day 0 or vehicle control treatment) to determine relative cell proliferation.

2.6. 2D foci assay

The 2D foci assay was used to measure clonal expansion [12]. Cells were plated (200–500 cells per 60 mm plate), based on cell lines and incubated for 7–15 days according to the growth of cells. After incubation, the cells were fixed with 4% (w/v) paraformaldehyde and then stained with 0.05% crystal violet. Images were taken using FlourChem™ E system (ProteinSimple, Santa Clara, CA). Colonies were counted using ImageJ (imagej.nih.gov).