Axl and VEGFRs exhibit variations in membrane localization and heterogeneity across monolayer and spheroid high-grade serous ovarian cancer models

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Abstract

Vascular endothelial growth factor receptors (VEGFRs) and Axl are tyrosine kinase receptors (RTK) that are targeted in ovarian cancer therapy. Two-dimensional monolayer culture and three-dimensional spheroids are common models for RTK-targeted drug screening: monolayers are simple and economical while spheroids include several genetic and histological tumor features. RTK membrane localization dictates RTK signaling and drug response, however, it is not characterized in these models. We quantify plasma membrane RTK concentrations and show differential RTK abundance and heterogeneity in monolayers vs. spheroids. We show VEGFR1 concentrations on the plasma membrane to be ten times higher in OVCAR8 spheroids than in monolayers; OVCAR8 spheroids are more heterogenous than monolayers, exhibiting a bimodal distribution of a low-Axl (6,200/cell) and a high-Axl subpopulation (25,000 /cell). Additionally, plasma membrane Axl concentrations differ by 100 times between chemo-sensitive (OVCAR3) and chemo-resistant (OVCAR8) cells and by ten times between chemo-resistant cell lines (OVCAR5 vs. OVCAR8). These systematic findings can guide ovarian cancer model selection for drug screening.

Introduction

Ovarian cancer is the most lethal gynecologic cancer. It is estimated that over 80% of ovarian cancer patients develop drug resistance to the first-line treatment, which combines tumor removal surgery and chemotherapy.[1] In search of alternative and second-line therapies, tyrosine kinase receptors (RTKs), the key regulators of cell proliferation, migration, and survival, are being investigated to reduce cancer aggressiveness or improve cancer chemosensitivity, which is the susceptibility to the cytotoxic effects of chemotherapy.[2,3]

The vascular endothelial growth factor receptor (VEGFR) family is a validated RTK target in many cancers[4] and is being explored as a target for treating ovarian cancers.[5] VEGFR-targeted therapies block the formation of the blood vessels supporting ovarian tumor growth[6] because VEGFRs are the frontline angiogenic regulators. VEGFR1 and VEGFR2 bind the most potent angiogenic ligand, VEGF-A_{165a} (herein described as "VEGF-A"). VEGFR1 binds VEGF-A with ten times higher affinity than VEGFR2 while inducing much lower kinase activity.[7] Therefore, VEGFR1 is conventionally described as a decoy receptor, sequestering VEGF-A ligand from the more potent, "pro-angiogenic," VEGFR2. VEGFR2 is also pro-lymphangiogenic along with VEGFR3 upon VEGF-C or VEGF-D binding. Altogether, the interactions between VEGF ligands and their cognate receptors direct healthy and pathological vascular growth and development.[8–11]

The presence of VEGFRs on ovarian cancer cells can serve as a biomarker and target for advanced ovarian cancers. Ovarian cancer metastases show higher VEGFR1 expression than primary cancer lesions;[12] primary ovarian tumor cells, and some, but not all, ovarian cancer cell lines show VEGFR2[13] and VEGFR3[14] protein expression and phosphorylation. Further, VEGFR-targeted therapies have shown reduced ovarian cancer cell migration, cell survival, and chemo-resistance in the VEGFR-expressing ovarian cancer cell lines.[13,14] Thus, VEGFR-targeted

therapies can have anti-angiogenic and anti-cancer effects on ovarian cancers, while the anti-cancer effects depend on the VEGFR presence on the ovarian cancer cells.

The presence of the important RTK, Axl, can serve as a biomarker and target for chemo-resistant ovarian tumor and metastasis.[15] Axl is upregulated in metastases and advanced-stage ovarian tumors but not in the normal ovarian epithelium.[16] Axl expression is associated with chemo-resistance in many cancers, including ovarian, prostate, breast, colorectal, and lung cancers.[15] In ovarian cancer, Axl abundance may further serve as a biomarker of chemo-resistance: in chemo-resistant ovarian cancer patients, Axl protein expression on ovarian cancer cells is significantly higher than that in chemo-sensitive and nonrecurrent patients.[17] Several studies have shown that inhibiting Axl improves chemosensitivity in Axl-expressing ovarian cancer cell lines.[18–20] Thus, the efficacy of Axl-targeted therapies depends on Axl presence and abundance on ovarian cancer cells.

Since the presence and abundance of VEGFRs and Axl on ovarian cancer cells can affect cancer cell aggressiveness and responses to chemotherapies, establishing their plasma membrane levels can advance new insights into dysregulated signaling and drug response. Indeed, studies of VEGFR quantities have shown that VEGFR quantities can differentiate cell subtypes: endothelial VEGFR2 is present at high concentrations on tip cells,[21,22] the highly specialized, leading cell of the vascular sprout, while VEGFR1 is present at high concentrations on the trailing, stalk cells.[23] VEGFR quantities are also important for understanding drug resistance: > 35,000 plasma membrane VEGFR1/tumor endothelial cell was predicted to cause a net increase in VEGF within the breast tumor, which is predicted to negate the effect of the anti-VEGF drug, bevacizumab.[24]This prediction was generated by combining quantitative characterization of VEGFR concentrations on tumor endothelial cells with deterministic, kinetic computational models and further supported by clinical observations, where VEGFR1 protein, overexpression in tumor was strongly associated with a lack of overall survival benefit from bevacizumab.[25]

Given this and other[25,26] correlations between receptor abundance and cancer cell responses to chemotherapy, we measure the concentrations of plasma membrane VEGFRs and Axl on individual cells in in-vitro ovarian cancer models via an optimized quantitative flow cytometry approach (**Fig. 1**). We focus on high-grade serous ovarian cancer (HGSOC) since it is the most common and deadly ovarian cancer subtype. We examine four histologically validated HGSOC cell lines (OVCAR3, OVCAR4, OVCAR5, and OVCAR8)[27] to determine if receptor concentrations are cell-line specific. OVCAR5 was established from an untreated tumor, OVCAR3 and OVCAR4 were established from cisplatin-refractory patients, and OVCAR8 was established from a high-dose carboplatin-refractory patient. These cell lines are further insightful because OVCAR3 is significantly more sensitive to paclitaxel and carboplatin chemotherapies than OVCAR5 and OVCAR8 in vitro, but OVCAR4 chemosensitivity in-vitro has not been reported.[18] Thus, these HGSOC cell lines are expected to exhibit distinct levels of Axl and VEGF receptors.

We devised a workflow (**Fig. 1**) to quantitatively compare monolayers and spheroids, examining their average VEGFR and Axl abundance and the heterogeneity in receptor concentration measurements. This work is based on three physiologically grounded reasons: 1) ovarian cancer cells are often found in spheroid form rather than single-cell form in malignant ascites,[28,29] suggesting that ovarian cancer spheroid models are more physiologically relevant than monolayer models; 2) spheroid models are showing greater reproducibility and

predictive value for patient drug responses in ovarian cancer,[30–32] and 3) ovarian spheroids have been shown to resemble in-vivo protein expression levels.[33,34] Our study quantitatively identifies the variation in ovarian cancer receptors across monolayers and spheroids of four commonly used HGSOC cell lines, which should guide model selection for in-vitro VEGFR- and Axl-targeted drug screening.

Results

An effective spheroid dissociation method that preserves plasma membrane receptors. Measuring receptor concentrations on single cells requires effective tissue dissociation. Cells within spheroid environments, like ex-vivo tissue samples[35–37] and the spheroids studied here synthesize an abundance of extracellular matrix (ECM) proteins beyond the 2D basement membrane. Previous quantitative flow cytometry studies have shown that monolayers can be readily dissociated into single intact cells by a 7- minute incubation with a non-enzymatic cell dissociation solution (e.g., Cellstripper) without additional mechanical disturbance.[38–41]However, the non-enzymatic dissociation approach for monolayers is insufficient for breaking down the more complex spheroid environment comprising fibronectin, laminin, collagen, and glycosaminoglycans, among others.[42] We observed that OVCAR spheroid dissociation required a minimum of 20-minute incubation with an enzymatic dissociation solution (i.e., TrypLE) followed by mechanical disturbance (i.e., pipetting ~50 times with a p1000 pipette). We observed comparable viability between monolayer and spheroid-derived cells of all the four cell lines, and the monolayer cells are noticeably larger than spheroid cells according to the flow cytometry analysis (i.e., higher FSC) (**Supplementary Fig. 1**).

Receptor quantification also requires that the cell dissociation method preserves plasma membrane receptors. However, enzymatic dissociation alone presents the possibility of decreasing plasma membrane receptor concentrations. Indeed, prior work established that enzymatic dissociation significantly reduced neuropilin-1 quantities on the plasma membrane compared to using a non-enzymatic dissociation solution.[43] To identify whether the enzymatic spheroid dissociation or enzymatic + mechanical dissociation approach affects our plasma membrane RTK measurement, we tested four dissociation approaches: 1) non-enzymatic (gentlest), 2) short enzymatic (7 min), 3) a combination of enzymatic (7min) and mechanical means, and 4) a combination of prolonged enzymatic (20 min) and mechanical means (most vigorous) (see Materials and Methods). We observed that none of the methods, from the gentlest to the most vigorous, affected plasma membrane VEGFR or Axl concentrations (**Fig. 2**). Thus, we combine prolonged enzymatic dissociation and mechanical disturbance to dissociate both monolayers and spheroids in this study.

We provide the spheroid image of each OVCAR cell line (**Fig. 3**) because spheroids can come with various shapes (e.g., round, grape-like, mass, etc.).[44] OVCAR3 and OVCAR8 formed more circular spheroids than OVCAR4 and OVCAR5 (**Fig. 3**). Nevertheless, both OVCAR4 and OVCAR5 spheroids formed dark cores that correspond to elevated cell compactness in a spheroid.[45] In addition, all the spheroids required the additional mechanical disturbance (i.e., ~50 times pipetting) to be fully dissociated due to cell-ECM interactions.

Axl plasma membrane levels are lower in OVCAR3, OVCAR5, and OVCAR8 spheroids compared to their monolayers. The high expression of Axl on ovarian cancer cells is associated with chemotherapy resistance and metastatic potential; thus, Axl is a target for reversing chemo-resistance and reducing cancer metastasis. Axl protein expression in OVCAR

monolayers has been qualitatively measured through western blots, where Axl protein was expressed at *low-undetectable* levels in OVCAR3 and OVCAR4 monolayer cells and was *high* in OVCAR5 and OVCAR8 monolayer cells.[17,46,47] Our plasma membrane Axl measurements mirror these qualitative whole-cell Axl patterns. Briefly, our data show that the *low-undetectable* cell lines have < 800 plasma membrane Axl/cell and the Axl-high cell lines have > 3,000 plasma membrane Axl/cell (**Table 1**). Our quantitative approach offers greater precision over prior OVCAR8 and OVCAR5 characterization, which has shown them to be high-Axl cell lines. While we do observe significant Axl levels on the plasma membrane, we further identify that OVCAR8 monolayer cells have ~ten times more plasma membrane Axl than OVCAR5 monolayer cells: 33,000 ± 1,200 and 3,900 ± 280 plasma membrane Axl/cell, respectively (mean ± SEM, **Table 1**). Additionally, for the first time, we report that plasma membrane levels of Axl in spheroids are significantly lower compared to the monolayers of OVCAR3, OVCAR5, and OVCAR8 cells. Axl is similarly low in OVCAR4 spheroids and monolayers (**Fig. 3**). Thus, the spheroid environment can induce a decrease in plasma membrane Axl concentrations on Axl-expressing HGSOC cells.

Cancer cells can exhibit considerable heterogeneity.[24,35,48–50] Immunohistochemistry studies have shown that ovarian tumors express Axl at varying levels.[17,51] To ensure that the ensemble measurements do not mask the heterogeneity in Axl plasma membrane concentrations, we perform cell-by-cell analysis using single-cell Axl measurements (**Fig. 4A-D**) and report population statistics, such as pooled cell numbers, median, and geometric mean values. (**Fig. 4)**. We also use quadratic entropy (QE) as a comparative measure of cell heterogeneity: homogenous in-vitro human endothelial cells and fibroblasts have shown QE within 0.2–0.7 for plasma membrane VEGFRs;[40] QE values of cytometric RTK measurements in heterogeneous cells have only been reported in xenograft-derived glioblastoma cells (QE ~1.0).[48] Thus, we describe QE<0.7 as low heterogeneity and QE>0.7 as high heterogeneity.

Cell-by-cell analysis shows that the spheroid environment can increase or decrease Axl heterogeneity compared to monolayers. More specifically, OVCAR5 monolayers exhibit high heterogeneity in Axl on the plasma membrane, as indicated by the high QE value (0.8), whereas OVCAR5 spheroids are less heterogenous (QE: 0.55, **Fig. 4**). On the contrary, OVCAR8 monolayer cells exhibit lower heterogeneity (QE: 0.26), while OVCAR8 spheroids exhibit higher heterogeneity in Axl plasma membrane concentrations (QE: 0.54). Interestingly, we observed at least two cell subpopulations in the OVCAR8 spheroids that exhibit differential membrane Axl levels.

To better characterize the OVCAR8 subpopulations, we performed a mixture modeling analysis — modeling the cell population as a mixture of normal distributions (see Methods and Materials).[39] As a result, OVCAR8 spheroids consist of two distinguishable normally distributed subpopulations with low and high plasma membrane Axl levels (66.9% and 33.1% of the spheroid cell population, respectively). The low-Axl subpopulation has an average of 6,200 plasma membrane Axl/cell, and the high-Axl subpopulation has 25,000 plasma membrane Axl/cell (**Fig.4E**). While it might appear that there are two peaks in OVCAR8 monolayer cells, our evaluation for bimodal distribution revealed that these peaks are of the same population, which we attribute to the closeness of the two peaks. Altogether, our data provide high-resolution information on the plasma membrane Axl quantities, heterogeneity, and subpopulation composition to help researchers visualize the quantitative differences across different Axl-expressing HGSOC cell types and models.

VEGFR1 plasma membrane concentrations are higher and more heterogeneous in OVCAR8 spheroids compared to OVCAR8 monolayers. VEGFR1 expressed in cancer cells was found to directly induce cancer cell migration and extracellular matrix invasion[52]. In HGSOC samples, higher VEGFR1 expression was seen in metastases compared to the primary tumors[12]. Our ensemble quantification results show that OVCAR8 cells have ~ten times more membrane VEGFR1 in spheroids (1,340 ± 200 R1/cell) than monolayers (110 ± 10 R1/cell) (mean ± SEM, **Fig 3D**). We also found that OVCAR5 cells have about twice as much plasma membrane VEGFR1 in monolayers (610 ± 110 R1/cell) as spheroids (260 ± 47 R1/cell). OVCAR3 and OVCAR4 have low VEGFR1 plasma membrane levels in monolayer and spheroid models (OVCAR3: 250 ± 20 R1/cell vs. 310 ± 40 R1/cell; OVCAR4: 740 ± 80 R1/cell vs. 650 ± 56 R1/cell) (**Fig 3** and **Table 1**).

While the heterogeneity in VEGFR1 plasma membrane concentrations was generally low in the examined OVCAR monolayer and spheroid models (QE <0.7), the heterogeneity in OVCAR4 spheroids was close to the high-QE cutoff 0.7 of glioblastoma cells (QE = 0.46 in monolayer vs. 0.67 in spheroid). The VEGFR1 plasma membrane heterogeneity was noticeably increased by 60% in OVCAR8 spheroids (QE =0.27 in monolayer vs. 0.43 in spheroid) (**Fig. 5**). Thus, among the four HGSOC cell lines, OVCAR4 and OVCAR8 spheroids may better capture the high-heterogeneity of tumor environment, compared to their monolayers. On the contrary, the VEGFR1 plasma membrane heterogeneity was identical across OVCAR3 monolayer and spheroid models (QE =0.41) and was remarkably similar across OVCAR5 monolayer and spheroid models (QE = 0.54 and 0.57, respectively) (**Fig. 5**). Overall, OVCAR8 spheroids exhibit the highest plasma membrane VEGFR1 levels (i.e., ten times higher) and heterogeneity in VEGFR1 plasma membrane concentrations (i.e., 60% higher) when compared to the monolayers. Thus, the OVCAR8 spheroid model may be more suitable for studying VEGFR1-targeted drug responses than the other examined HGSOC models.

VEGFR2 plasma membrane levels are higher and more heterogeneous in OVCAR5 spheroids compared to their monolayers. VEGFR2 protein in cancer cells was found to promote cell proliferation and survival in vitro[13]. It was shown that xenografts from breast tumor cells have ~1,000 plasma membrane VEGFR2/cell[35]. Previous whole-cell western blot analysis suggests low VEGFR2 plasma membrane levels in OVCAR8[53], and our qFlow measurements similarly show low plasma membrane VEGFR2 on OVCAR8 cells: 480 ± 93 and 380 ± 52 plasma membrane VEGFR2/cell in OVCAR8 monolayer and spheroid models, respectively (mean ± SEM). We also show that OVCAR3 and OVCAR5 have similarly low plasma membrane VEGFR2 levels in monolayers and spheroid (490 - 660 VEGFR2/cell), while OVCAR4 has higher plasma membrane VEGFR2: 1,030 ± 44 R2/cell in monolayers and 830 ± 30 R2/cell in spheroids (**Table 1**). Thus, according to the ensemble measurements, OVCAR4 monolayer cells have the highest plasma membrane VEGFR2 level.

However, the ensemble measurements masked the difference in VEGFR2 plasma membrane levels between OVCAR5 monolayer and spheroid models. Cell-by-cell analysis shows that OVCAR5 cells have twice the number of plasma membrane VEGFR2 per cell on spheroids than on monolayers, as described by the geometric population mean of 1,220 vs. 584 plasma membrane VEGFR2/cell, respectively (**Fig. 6**). In addition, OVCAR5 spheroids showed greater heterogeneity in VEGFR2 plasma membrane concentration, as indicated by the 35% higher QE in spheroids compared to monolayer cells (QE = 0.57 in spheroid vs. 0.37 in monolayer). On the other hand, the heterogeneity levels of OVCAR3, OVCAR4, and OVCAR8 are very similar when comparing monolayer and spheroid models (QE = 0.30~0.41) (**Fig. 6**). Together,

according to cell-by-cell analysis, the OVCAR5 spheroid model may be more suitable for studying VEGFR2-targeted drug responses.

VEGFR3 plasma membrane levels are lower in OVCAR4 and OVCAR8 spheroids and higher in OVCAR5 spheroids compared to their monolayers. VEGFR3 signaling in ovarian cancer cells is associated with the upregulation of genes (e.g., BRCA gene) that regulate cell cycle arrest. Accordingly, VEGFR3 inhibition results in OVCAR8 growth inhibition.[14] However, to our knowledge, no VEGFR3 protein expression data allow comparison across OVCAR8 and other HGSOC OVCAR cell lines. Here we show that OVCAR8 cells have 3-10 times higher membrane VEGFR3 than the other examined OVCAR cell lines (**Table 1**). We also show that the spheroid environment decreases plasma membrane VEGFR3 levels on OVCAR4 and OVCAR8 but not on OVCAR3 and OVCAR5 cell lines, compared to their corresponding monolayers (**Fig. 3**). Overall, we see a cell-line specific difference in VEGFR3 plasma membrane concentrations indicating that OVCAR8 is an appropriate VEGFR3-protein-expression HGSOC model for screening VEGFR3-targeted drugs.

Cell-by-cell analysis further reveals increased VEGFR3 heterogeneity in OVCAR5 and OVCAR8 spheroids compared to corresponding monolayers, as indicated by the 34% and 28% higher QE in OVCAR5 and OVCAR8 spheroids, respectively, compared to monolayer cells (0.39 in monolayer OVCAR5 vs. 0.59 in spheroid OVCAR5 and 0.29 in monolayer OVCAR8 vs. 0.37 in spheroid OVCAR8) (**Fig. 7**). On the contrary, monolayer and spheroid cultures of OVCAR3 and OVCAR4 cells exhibit nearly identical heterogeneity (QE). Nonetheless, the VEGFR3 distributions are considered low heterogeneity (QE < 0.7) in all the examined HGSOC models.

Lastly, the cell-by-cell analysis reveals an increased membrane VEGFR3 level from OVCAR5 monolayer cells (561 VEGFR3/cell) to spheroid cells (1,009 plasma membrane VEGFR3/cell, geometric population mean) (**Fig. 7**). It is important to perform cell-by-cell analysis to supplement the ensemble measurements because this two-fold increase is masked by the average ensemble measurements of plasma membrane VEGFR3 on OVCAR5 monolayer cells and spheroid cells (**Fig. 7**). The two-fold increase could be meaningful because it implies that OVCAR5 spheroids are a more responsive model for VEGFR3-targeted drug screening, compared to OVCAR5 monolayer model.

Discussion

We advance quantitative RTK characterization as a vital tool for measuring ovarian cancer biomarkers and heterogeneity. The abundance of VEGFR and Axl on the plasma membrane influences RTK signaling and drug response;[54] however, prior studies have used non-quantitative immunoassays, such as western blot, immunohistochemistry, conventional flow cytometry, and immunofluorescence imaging. We show significant differences in OVCAR RTK heterogeneity that were not captured via these non-quantitative assays. The quantitative flow cytometry approach coupled with the cell-by-cell analysis has been used to measure plasma membrane VEGFR concentrations in ex-vivo from breast cancer xenografts (~2,000 VEGFR1 and ~1,000 VEGFR2 per tumor cell),[35] glioblastoma patient-derived xenografts,[48] and now HGSOC monolayer vs. spheroid models (varies between 100 and 5,000 VEGFR/cell). The cell-by-cell quantitative analysis is also more informative than qualitative protein analysis that has previously been performed via western blot.[18,46,47,53]

HGSOC in-vitro models have been used to study ovarian cancer cell response to RTK inhibition (e.g., VEGFR2 inhibitor,[53,55] VEGFR3 inhibitor,[14] and Axl inhibitor[56]) and growth factor

stimulation.[57] In this study, we established quantitative thresholds to differentiate *low-undetectable*, *intermediate*, *high*, and *very-high* plasma membrane Axl and VEGFR levels on HGSOC models (**Fig. 8**). Western blots studies have shown that Axl protein was expressed at *low-to-undetectable* levels in OVCAR3 and OVCAR4 monolayer cells and was *high* in OVCAR5 and OVCAR8 monolayer cells.[18,46,47] Our Axl measurements on the plasma membrane show that the Axl-undetectable cell lines have < 800 plasma membrane Axl/cell (**Table 1**). Western blot data also show that OVCAR3 is a low-VEGFR2 cell line, and we quantified ~500 plasma membrane VEGFR2/OVCAR3 cell. Thus, < 800 receptors/cell corresponds to the "low-undetectable" western blot expression. Further, although OVCAR5 and OVCAR8 were identified as high-Axl cell lines, we found that OVCAR8 has ~ten times higher plasma membrane Axl than the OVCAR5 (~30,000 vs. ~3,000 plasma membrane Axl/cell). We describe 3,000-30,000 receptors/cell as a "high" receptor concentration range. Altogether, we consider < 800 receptors/cell as a “low-undetectable” range; 800-3,000 receptors/cell as an “intermediate” range; 3,000-30,000 as a “high” range; and >30,000 as a “very high” range for HGSOC cells (**Fig. 8**).

For the first time, we show that plasma membrane Axl and VEGFRs are differentially regulated in spheroid environments compared to corresponding monolayers -- the receptor concentrations are cell line-specific. We hypothesized that plasma membrane concentrations of Axl and VEGFR would be higher on spheroids than on corresponding monolayers because overexpression of these receptors is a hallmark of ovarian cancer chemo-resistance and metastasis,[14,18,19,58] and spheroids have been shown to better recapitulate the high chemo-resistance of ovarian cancers compared to monolayers.[31] Thus, we quantitatively characterized the variability and heterogeneity of VEGFR and Axl plasma membrane concentration on monolayer and spheroid models of four commonly used HGSOC cell lines (i.e., OVCAR3, OVCAR4, OVCAR5, and OVCAR8).

We revealed four key quantitative findings: (1) OVCAR8 cells exhibit the highest plasma membrane Axl levels, while on OVCAR8 spheroids the plasma membrane Axl is ~3.5 times lower than OVCAR8 monolayer cells and exhibits a bimodal distribution characterized by a low-Axl and a high-Axl subpopulation. (2) OVCAR8 cells have the highest plasma membrane concentrations of VEGFR1 among the HGSOC cell lines, and OVCAR8 spheroids exhibit about ten times higher plasma membrane VEGFR1 levels than OVCAR8 monolayers. (3) OVCAR5 spheroids exhibit the highest VEGFR2 plasma membrane concentrations and VEGFR2 heterogeneity. Lastly, (4) OVCAR8 monolayers have the highest plasma membrane concentrations of VEGFR3. The differential receptor presentation in the monolayer vs. spheroid models could be regulated at the transcriptional, translational, or trafficking levels, and there are several assays available to screen such effects. Differential transcription can be examined by a variety of gene expression analysis assays[59], and protein regulation can be further probed via several assays including western blot and immunofluorescence, with consideration of subcellular localization. These findings highlight the variability and heterogeneity in plasma membrane RTK concentrations across different ovarian cancer models and the importance of quantifying the receptor levels when selecting preclinical cancer models, as discussed below.

Suggested Axl-targeted HGSOC model: OVCAR8 monolayers and spheroids

OVCAR8 monolayers and spheroids are good models for testing Axl-targeted drugs. Axl inhibition is a strategy for ovarian cancers, and a lower plasma membrane Axl concentration

may lead to a weaker cell response to Axl-targeted drugs. The OVCAR8 monolayers offer an excellent first-line in vitro model for drug screening due to their remarkably high plasma membrane Axl protein concentration. Nevertheless, OVCAR8 spheroids are a more complex option that reflects the heterogeneity in a tumor environment. We show that the plasma membrane concentration of Axl is significantly lower on spheroids compared to monolayer models in 3/4 of examined HGSOC cell lines. We are not the only research group to have observed decreased membrane receptor levels in spheroids. For instance, insulin growth factor binding protein 5 (IGFBP-5) gene is fivefold lower in mesothelioma tumor spheroids when compared to monolayers.[60] We further observed two subpopulations in OVCAR8 spheroids, suggesting that cell responses to Axl-targeted drugs will differ throughout an OVCAR8 spheroid: the low-Axl subpopulation may be less responsive to Axl-targeted drugs compared to the high-Axl subpopulation. Characterizing cell subpopulations with different plasma membrane receptor levels provides necessary insights into the heterogeneous drug responses in tumor spheroids.

Suggested VEGFR1-targeted HGSOC model: OVCAR8 spheroids

OVCAR8 spheroids may better represent VEGFR1-protein expressing ovarian cancer metastases than OVCAR8 monolayers. Plasma membrane VEGFR1 levels are significantly higher in OVCAR8 spheroids compared to OVCAR8 monolayer cells and the other examined HGSOC models. The higher plasma membrane VEGFR1 levels in OVCAR8 spheroids may be associated with a higher metastatic capacity because (1) VEGFR1 promotes cancer cell migration and invasion;[61] (2) HGSOC metastases express higher VEGFR1 protein and gene levels than the matched primary tumors;[12] and (3) OVCAR8 cells form metastatic ascites more readily than OVCAR3, OVCAR4, and OVCAR5 in mouse models.[27] Thus, OVCAR8 spheroids could be used to test VEGFR1 inhibition strategies that reduce metastasis.

Suggested VEGFR2-targeted HGSOC model: OVCAR5 spheroids

OVCAR5 spheroids may be a better model than the existing OVCAR3 spheroid model[55] for screening VEGFR2-targeted drugs, based on the plasma membrane VEGFR2 measurements. Inhibiting VEGF:VEGFR2 signaling in OVCAR3 spheroids has been shown to induce cancer apoptosis,[55] and we found OVCAR3 monolayers and spheroids have, similarly, sparse numbers of plasma membrane VEGFR2 (~550 plasma membrane VEGFR2/cell). OVCAR5 spheroids and OVCAR4 monolayers may generate a more significant response because they have higher plasma membrane VEGFR2 (~1,000 VEGFR2/cell) than OVCAR3 and OVCAR8 cells. Further, OVCAR5 spheroids may serve as a better anti-VEGFR2 model than OVCAR4 monolayers because the spheroid structural and physical elements help cells recreate the in-vivo VEGFR2 inhibition responses.

In addition, VEGFR2 gene expression is cancer-dependent: VEGFR2 gene expression is downregulated in prostate cancers and upregulated in kidney cancers.[62] However, the variability of VEGFR2 protein expression is not well established. qFlow measurements allow the comparison of plasma membrane VEGFR protein levels across different cancer types. For instance, our qFlow data reveal that the plasma membrane VEGFR2 concentrations on OVCAR5 spheroids and OVCAR4 monolayers are comparable to xenografted breast tumor cells (~1,000 plasma membrane VEGFR2/cell).[35]

Suggested VEGFR3-targeted HGSOC model: OVCAR8 spheroids

OVCAR8 monolayers and spheroids have the highest plasma membrane levels of VEGFR3 among the examined HGSOC models. Thus, the profound VEGFR3 inhibitory effects seen in the exiting OVCAR8 model[14] might not hold in other HGSOC models with lower plasma membrane VEGFR3 levels. Further investigations are needed to understand the correlation between the number of targeted receptors and the cell responses to the targeting drugs—such understanding will be important for establishing the predictive values of receptor abundance on the targeted cells.

Conclusion:

We believe that these findings of differential VEGFR and Axl plasma membrane levels can shift perspective in four key areas. (1) Quantitative proteomics: we optimized a plasma membrane receptor quantification method for spheroid-derived single cells. This method allows precise, absolute measurements of plasma membrane receptors on the cells that are recovered from compact spheroids. (2) Monolayer vs. spheroid biology: our data reveal that the quantity and distribution of plasma membrane receptors are affected by monolayer and spheroid environments. (3) Model selection: Many VEGFR and Axl inhibitors are being explored for treating cancers, including ovarian cancers (Cediranib, Semaxanib, Sunitinib, Vatalanib, Pazopanib, and Cabozantinib).[63,64] In-vitro drug testing is usually the first step in cancer drug development. The recommendations we offer for testing VEGFR and Axl-targeted cancer therapies can guide in-vitro model selection. (4) Predictive medicine: the data on receptor quantities and heterogeneities can be used to parametrize computational models that recapitulate drug- or ligand-receptor interactions and predict the signaling and therapeutic outcomes in silico.

Altogether, our study provides an important quantitative approach and evidence for the variations and heterogeneity of Axl and VEGFR membrane concentrations across high-grade serous ovarian cancer monolayer and spheroid models.

The Bigger Picture

RTK plasma membrane concentration is a key factor regulating RTK signaling and RTK-targeted therapeutic outcomes. We demonstrate the variations in RTK abundance and heterogeneity in monolayer vs. spheroid ovarian cancer models and in chemo-sensitive vs. chemo-resistant HGSOC cell lines. Our approach integrates single-cell analysis of protein distribution and heterogeneity and thus provides finer-grained protein characterization than conventional western blot and qualitative methods. Our data provide important guidance for RTK-targeted drug screening model selection. Additionally, this study presents the importance and usefulness of quantitative protein data and paves the path for collective efforts to establish a biologically faithful, reproducible, and sharable protein database in the RTK research community. Our approach can be applied to other plasma membrane proteins, including other RTKs and G protein-coupled receptors (GPCRs). Our approach can be examined across various cell lines, and potentially other cell model configurations (e.g., organoids and multi-cell co-cultures).

Materials and methods:

Cell lines. Four ovarian cell lines were selected for their histologically validated high-grade serous ovarian cancer characteristics: OVCAR3, OVCAR4, OVCAR5, and OVCAR8.[27] Cells were

cultured in RPMI-1640 medium (Gibco™ 11875085) supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin (Sigma-Aldrich, P4333), and 10 µg/mL insulin (Sigma-Aldrich, I6634). Cells were kept at 5% O_2 at 37 °C.

3D spheroid formation. Uniform formation of ovarian cancer spheroids was achieved by using 96-well ultra-low attachment concave-bottom microplates (Corning™ 4515). For all cell lines, 5,000 cells in 100 µl of cell medium were seeded in each well. Spheroids were allowed to form over four days, generating spheroids of approximately 500 µm in diameter. On Day 3, 100 µl of fresh medium was added to each well to replenish the nutrient-depleted medium. Monolayers of each cell line were cultured in the same medium over the same 4-day period.

Single cell preparation. Both monolayers and spheroids were dissociated into single cells by 20-min incubation with TrypLE™ Express Enzyme (Gibco™), followed by ~50 times gentle pipetting with a p1000 pipette. At least five spheroids were pooled for each receptor measurement per replicate. Although monolayers detach more quickly (~7 min) than spheroids (~20 min) and do not require additional pipetting, the same dissociation procedures were carried out for 2D monolayer and 3D spheroids to minimize possible changes in plasma membrane protein concentration associated with this dissociation step.

To assess whether the enzymatic cell dissociation solution (TrypLE Express) causes plasma membrane protein levels to decrease, we compared the receptor levels on the 2D cells dissociated by 7-min TrypLE incubation versus 7-min Cellstripper (non-enzymatic) incubation. In addition, to assess whether the prolonged incubation and mechanical disturbance caused plasma membrane protein levels to decrease, we compared the receptor levels on the monolayer cells dissociated by 7-min TrypLE incubation versus 7-min or 20-min TrypLE incubation followed by 50 times gentle pipetting with a p1000 pipette.

Flow cytometry. Single cells were labeled using phycoerythrin (PE)-conjugated VEGFR1 antibody (R&D Systems), PE-conjugated VEGFR2 antibody (Biolegend), or PE-conjugated VEGFR3 antibody (R&D Systems), or PE-conjugated Axl antibody (Biolegend) at respective saturating concentration. The saturating concentrations of PE-VEGFR1, -VEGFR2, and -VEGFR3 antibodies were previously determined (14 µg/mL)[40,65] and confirmed at the beginning of this study. The saturating concentration of the PE-Axl antibody was determined at the beginning of this study (saturating range: 2.5-3.5 µg/mL). Samples were incubated in the dark for 40 minutes at 4 °C, washed twice with 2 mL of stain buffer (1X PBS supplemented with 0.5% bovine serum albumin, 0.09% Sodium Azide, and 2 mM EDTA), centrifuged at 400 g at 4 °C for 5 min, and re-suspended in 100 µl of stain buffer. To assess the cell integrity, 1 µl Sytox Blue was added to each sample before flow cytometry. Fluorescence Minus One (FMO) controls are flow cytometry samples stained with all the fluorophores in a panel, minus one of them. FMO controls are used to determine the spillover effects in other fluorescence dimensions on a particular channel of interest, thus identifying true positive cell populations.[41,66] PE FMO samples provide PE background signal levels.

Flow cytometry was performed on either of two available instruments: LSR Fortessa (BD) or CytoFLEX S Flow cytometer (Beckman Coulter). Samples were vortexed immediately before placement in the flow cytometer. Before sample acquisition, PE voltage settings were finalized, and Quantibrite™ PE beads (BD, Cat. No. 340495) were collected. A detailed protocol for plasma membrane protein quantification is available.[41]

Quantitative flow cytometric data analysis. Flow cytometric data analysis was performed

using Kaluza analytical software (Beckman Coulter)**.** The levels of VEGFR per cell were acquired by converting PE fluorescence intensity to the number of PE molecules per cell using Quantibrite™ PE beads, as previously described.[8,35,36,38–40,43,67] Geometric mean values of PE intensities of individual samples and individual cells were exported to Excel.

Statistical analysis. Ensemble-averaged receptor levels on the plasma membrane were shown as the number of receptors per cell (mean ± standard error of the mean SEM) from four to six samples (at least five spheroids per sample). Statistically, differences in plasma membrane receptor levels between monolayer and spheroid conditions were determined by two-sample t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p< 0.0001$) using Originlab Pro software. Cell-by-cell receptor distributions are constructed by pooling the single-cell data from all the samples using Matlab. To examine the cell heterogeneity based on each receptor, we calculated quadratic entropy (QE) for each receptor distribution as a quantitative measure of cell heterogeneity defined by receptor concentration. [39] Statistical analysis is not feasible and thus not performed to compare cell-by-cell distributions because each distribution consists of a large number of cells (N = 20,000~40,000) and any comparison test (e.g., Z test, Kolmogorov–Smirnov test, Mann Whitney Wilcoxon test, and Kruskal–Wallis test) come back as 'statistically significantly different' ($p<0.05$) for such a large N.[67] Cell (sub)populations in the cell-by-cell distributions were identified via mixture modeling using R studio, in which Bayesian information criterion (BIC)-assisted Gaussian mixture modeling was performed to obtain the number of subpopulations within each cell type from cocultures, as previously described.[39]

Authorship contribution statement

P.I. and Y.F. conceived of the presented idea. P.I. supervised the project. Y.F. carried out the experiment, analyzed the data, and prepared the manuscript in consultation with P.I. Y.F. and P.I. contributed to the editing and the final version of the manuscript.

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