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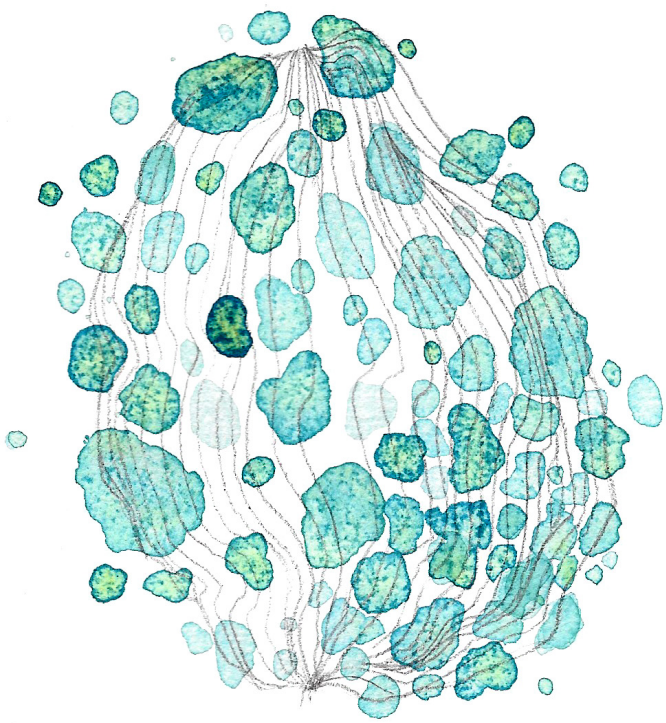
Receptor tyrosine kinase signaling and extracellular matrix cues in ovarian cancer metastasis and drug resistance



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RECEPTOR TYROSINE KINASE SIGNALING AND EXTRACELLULAR MATRIX CUES IN OVARIAN CANCER METASTASIS AND DRUG RESISTANCE

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Cover illustration: representation of an ovarian cancer tumoroid (cancer cells in teal, the color for ovarian cancer awareness). Artist: Júlia Moyano Galceran.

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By

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To my love, my friends and my family, for believing in me.

POPULAR SCIENCE SUMMARY OF THE THESIS

One out of 100 women develops ovarian cancer during her lifetime, and is usually diagnosed around the age of 60. Because there are no specific symptoms, in most women the cancer has already spread (metastasized) in the abdominal cavity and organs at the time of diagnosis. At this point, only 1 out of 3 women has chances of surviving for more than 5 years and in most of them the cancer will come back during this time (recurrence). Women with ovarian cancer are operated (tumors are surgically removed) and then treated with chemotherapy (platinum drugs) to eliminate the cancer cells that the surgeons could not see. Despite apparent treatment success, some of these cancer cells will not be affected by the chemotherapy and some other cells will become resistant to it.

This tragic situation has not changed much over the years and even though some new therapies are now available (such as PARP inhibitors or immunotherapy), they have not yet resulted in big improvements in patient survival. For this reason we should investigate how ovarian cancer metastasizes, responds to chemotherapy and develops into recurrent disease. Understanding the changes in the tumor and its surroundings at the molecular level will allow us to find markers to detect this type of cancers earlier and to predict how the patients will respond to the treatment. In addition, this knowledge will facilitate the choice or design of better anti-cancer therapies to combat ovarian cancer.

The aim of this thesis was to study how the ovarian cancer cells and their (micro)environment communicate, evolve and react to the chemotherapy. The findings from this thesis are presented in three papers:

In **Paper I** we discovered that the cancer cells that survive the treatment with platinum chemotherapy activate a mechanism of resistance that involves EphA2 (receptor that is abnormally activated in the cells) and GPRC5A (receptor that works together with EphA2). We used chemical compounds to find the protein responsible for the activation of this mechanism and noticed that it was RSK. We then tested RSK inhibitors and observed that these were effective in killing the cancer cells in the laboratory and in mice. In this paper we also learned that the expression of GPRC5A in the tumor could be used to predict the response of the patients to platinum chemotherapy, the time to cancer recurrence and the patient survival.

In **Paper II** we investigated ephrinA5 (one of the activating ligands of EphA2 receptor, produced by the gene *EFNA5*), which had previously been described as a marker to predict the survival of ovarian cancer patients. We found that ephrinA5 had a different function than ephrinA1, which is another ligand of EphA2 that has been more studied and is associated with anti-tumor activity. In addition, we discovered that ephrinA5 was expressed mostly by the cancer cells (compared to the surrounding cells) in the most aggressive ovarian cancer

type (high-grade serous carcinoma), and that upon chemotherapy treatment and cancer recurrence ephrinA5 expression increased even more.

In **Paper III** we studied the surroundings of ovarian cancer tumors, not focusing on the cells but on the proteins that they produce, particularly the group named matrisome. In the laboratory we recreated the extracellular matrix (protein network surrounding the cancer cells) and the different stiffness of ovarian cancer tumors. We found that cancer cells activated signaling mechanisms when sensing and adhering to some of the surrounding proteins and that in the most rigid condition (similar to the more advanced and aggressive tumors) the cancer cells grew and moved more, and were more resistant to chemotherapy. Among the matrisome proteins, collagen VI was increased after the treatment and its high expression correlated with shorter survival of ovarian cancer patients. When we provided collagen VI to the cancer cells in the laboratory, we observed that the cells that were a bit resistant to platinum chemotherapy and the cells obtained from patients with recurrent ovarian cancer became even more resistant and activated mechanisms to move and migrate.

Even though our promising results cannot directly help the patients (for example now for the first time an RSK inhibitor, like the ones used in **Paper I**, is in a clinical trial to check its safety and efficiency before being used to treat patients), they instead provide new knowledge that can be used by other scientists and that has the potential to improve the lives of many women.

ABSTRACT

Albeit many studies have contributed towards understanding the origin, development, treatment response and evolution of high-grade serous ovarian carcinoma (HGSC), the exact supporting mechanisms are still not fully understood, and the disease remains a clinical challenge with low 5-year survival and high recurrence rate.

The purpose of this thesis was to study the dynamic communication between the cancer cells and the tumor microenvironment (TME) to understand how these influence and are affected by disease progression and chemotherapy in the context of HGSC.

In **Paper I** we uncovered a robust platinum chemotherapy-induced ERK1/2-RSK1/2-EphA2-GPRC5A signaling switch associated with cancer cell intrinsic and acquired chemoresistance. Mechanistically, RSK inhibition halted this signaling switch and even restored the canonical, tumor-suppressive EphA2 signaling, resulting in the efficient sensitization of HGSC cells to platinum treatment *in vitro* and *in vivo*. In addition, we described GPRC5A association to chemotherapy response, overall and progression-free survival in HGSC patients. In **Paper II** we reported that the ligand ephrinA5 was highly expressed in the most aggressive ovarian cancer type (i.e. HGSC) and further upregulated both at the protein and mRNA levels upon disease progression. Functionally, ephrinA5 left unaffected or even impaired EphA2 tumor-suppressive signaling. In **Paper III** we characterized the matrisome of HGSC solid tumors (primary and different metastatic sites) and ascites, pre- and post-chemotherapy. We showed that extracellular matrix (ECM) stiffness promoted cell proliferation and spreading (via focal adhesion kinase and YAP/TAZ signaling), and protected HGSC cells against cisplatin-mediated, apoptosis-inducing DNA damage. In addition, we reported that different ECM components altered cell adhesion, migration and chemoresistance in diverse ways. In particular, collagen VI enhanced the chemoresistance of disease-recurrent, patient-derived organoids, was upregulated in HGSC patients upon chemotherapy and associated to poor survival.

In conclusion, the findings of this thesis help us to better understand how ovarian cancer cells activate specific signaling mechanisms and engage in altered ECM remodeling and TME interactions that enhance cancer cell growth, metastatic and chemoresistance capabilities.

LIST OF SCIENTIFIC PAPERS

I. **Moyano-Galceran L**, Pietilä EA*, Turunen SP*, Corvigno S, Hjerpe E, Bulanova D, Joneborg U, Alkasalias T, Miki Y, Yashiro M, Chernenko A, Jukonen J, Singh M, Dahlstrand H, Carlson JW, Lehti K.
Adaptive RSK-EphA2-GPRC5A signaling switch triggers chemotherapy resistance in ovarian cancer. *EMBO Mol Med.* 2020 Apr 7;12(4):e11177

II. Jukonen J, **Moyano-Galceran L***, Höpfner K*, Pietilä EA, Lehtinen L, Huhtinen K, Gucciardo E, Hynninen J, Hietanen S, Grénman S, Ojala PM, Carpén O, Lehti K.
Aggressive and recurrent ovarian cancers upregulate ephrinA5, a non-canonical effector of EphA2 signaling duality. *Sci Rep.* 2021 Apr 23; 11(1):8856

III. Pietilä EA, Gonzalez-Molina J, **Moyano-Galceran L**, Jamalzadeh S, Zhang K, Lehtinen L, Turunen SP, Martins T, Gultekin O, Lamminen T, Kaipio K, Joneborg U, Hynninen J, Hietanen S, Grénman S, Lehtonen R, Hautaniemi S, Carpén O, Carlson JW, Lehti K.
Co-evolution of matrisome and adaptive adhesion dynamics drives ovarian cancer chemoresistance. *Nat Commun.* 2021 Jun 23;12(1):3904

*Equal contribution

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Schoutrop E, El-Serafi I, Poiret T, Zhao Y, Gultekin O, He R, **Moyano-Galceran L**, Carlson JW, Lehti K, Hassan M, Magalhaes I, Mattsson J. Mesothelin-specific CAR T cells target ovarian cancer. *Cancer Res*. 2021 Jun 1;81(11):3022-3035.

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CONTENTS

LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
BReg	Regulatory B-cell
CA125	Cancer antigen 125
CAA	Cancer-associated adipocyte
CAF	Cancer-associated fibroblast
CAM	Cancer-associated mesothelial cell
CAR	Chimeric antigen receptor
COL	Collagen
DC	Dendritic cell
DDR	Discoidin domain receptor
DSB	Double-strand DNA break
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EOC	Epithelial ovarian cancer
Eph	Erythropoietin-producing hepatocellular receptor
FA	Focal adhesion
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FN	Fibronectin
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HGSC	High-grade serous ovarian carcinoma
HIF	Hypoxia-inducible factor
HR	Homologous recombination
HRD	Homologous recombination deficiency
HTS	High-throughput screening
i.p.	Intraperitoneal
IB/WB	Immunoblot
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
LAM	Laminin
LMR	Lemur receptor
LPA	Lysophosphatidic acid
LTK	Leukocyte tyrosine kinase
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell

MMP	Matrix metalloproteinase
MMT	Mesothelial-to-mesenchymal transition
MSC	Mesenchymal stem cell
MuSK	Muscle-specific kinase
NGFR	Nerve growth factor receptor
NK	Natural killer
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
OS	Overall survival
PARPi	Poly-ADP-ribose polymerase inhibitor
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDO	Patient-derived organoid
PDS	Primary debulking surgery
PDX	Patient-derived xenograft
PFI	Platinum-free interval
PFS	Progression-free survival
PKA	Protein kinase A
PTK7	Protein tyrosine kinase 7
PTP	Protein tyrosine phosphatase
Ret	Rearranged during transformation
Ror	RTK-like orphan receptor
RTK	Receptor tyrosine kinase
Ryk	Tyrosine-like kinase
SASP	Senescence-associated secretory phenotype
STIC	Serous tubal intraepithelial carcinoma
STYK1	Serine/threonine/tyrosine kinase 1
TAM	Tumor-associated macrophage
TAM	Tyro3-Axl-Mer family
TAN	Tumor-associated neutrophil
TCGA	The Cancer Genome Atlas
TGFβ	Transforming growth factor beta
TH	T helper cell
TIME	Tumor immune microenvironment
TIMP	Tissue inhibitor of MMPs
TK	Tyrosine kinase
TMA	Tissue microarray
TME	Tumor microenvironment
TReg	Regulatory T-cell
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VTN	Vitronectin

1 INTRODUCTION

Intrinsic and acquired treatment resistance are not only caused by genetic changes and phenotypic plasticity of the cancer cells but are also influenced by the tumor microenvironment (TME) (1,2). It has now become evident that both the tumor and the TME are affected by disease progression and therapy, however the exact mechanisms that are activated upon these processes are not completely understood (3,4). As such, deciphering and targeting these mechanisms may provide effective strategies to reduce tumor malignancy by halting the aggressive tumor-TME evolution and the development of therapy resistant relapses.

The purpose of this thesis was to study the dynamic communication between the cancer cells and the TME to understand how these influence and are affected by disease progression and chemotherapy in high-grade serous ovarian carcinoma (HGSC). Interactions between cancer cells and their surrounding TME are mediated by cell surface receptors, including receptor tyrosine kinases (RTK) (5). The biggest family of RTKs is the erythropoietin-producing hepatocellular (Eph) receptor family, from which the member EphA2 is frequently overexpressed in carcinomas and associated to disease aggressiveness and poor clinical outcome (6–9). EphA2 signaling is complex and context-dependent, resulting in both anti- versus pro-tumorigenic outcomes, generally associated to ligand-dependent versus ligand-independent activation of the receptor (6–8). Paradoxically, in HGSC patients high mRNA levels of ephrinA5 ligand are associated to cancer aggressiveness and poor survival (10–12).

Using established HGSC cell lines, a xenograft mouse model and patient-derived material, we uncovered a robust (cisplatin and carboplatin) chemotherapy-induced ERK1/2-RSK1/2-EphA2-GPRC5A signaling switch associated with cancer cell intrinsic and acquired chemoresistance. Mechanistically, blocking RSK kinase inhibited this signaling switch and even restored the canonical, tumor-suppressive EphA2 signaling, resulting in the efficient sensitization of patient-derived cells to *ex vivo* platinum treatment. We also found that the protein levels of the ligand ephrinA5 were high specifically in the most aggressive HGSC and further upregulated both at the protein and mRNA levels upon disease progression. Functionally, ephrinA5 left unaffected or even impaired EphA2 tyrosine phosphorylation, receptor internalization and degradation, which are associated to tumor-suppressive signaling. Finally, using transcriptomic and histological data, we identified the matrisome of HGSC tumors (primary and metastases) and ascites, as well as treatment-induced changes. By mimicking the increased tumor stiffness upon disease evolution, we revealed that activation of focal adhesion kinase (FAK) and YAP/TAZ signaling promoted cell proliferation and spreading, and that HGSC cells were protected from chemotherapy in stiff substrates. We also reported that diverse extracellular matrix (ECM) components differentially altered cancer cell adhesion, migration and chemoresistance. For instance, collagen VI (COL6), which was upregulated by chemotherapy in HGSC patients and associated to poor survival, enhanced the chemoresistance of disease-recurrent, patient-derived organoids.

The findings of this thesis help us to better understand how metastases and therapy-induced, adaptive changes in cancer and stromal cells provide unique niches for ovarian cancer cells to engage in altered ECM remodeling and TME interactions as well as activate signaling mechanisms that enhance cancer cell metastatic potential and survival upon anti-cancer drug treatments.

2 LITERATURE REVIEW

2.1 TUMOR AND THE TUMOR MICROENVIRONMENT

Neoplasms are tissue masses originated from the abnormal growth of cells and can be benign (not capable to spread) or malignant (with potential to invade, metastasize and colonize other tissues), the latter named tumors. Tumors are not only composed of cancer cells, but also encompass other cell types and acellular components defined as the TME (2). Together, cancer cells and the TME contribute to the development and evolution of cancer by sustaining proliferative signaling, enabling replicative immortality, promoting resistance to cell death, increasing genome instability, inducing angiogenesis and inflammation, allowing the evasion of growth suppressors and immune destruction, reprogramming the energy metabolism and activating cancer cell invasion and metastasis. These biological capabilities are known as the hallmarks of cancer (1,13) (**Figure 1**).

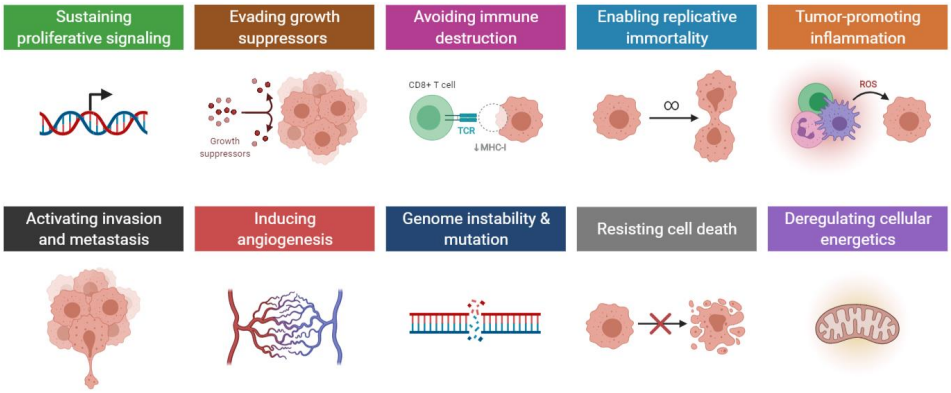


Figure 1. The hallmarks of cancer. *Adapted from* "Hallmarks of Cancer", *by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates*

The cellular components (i.e. stroma) of the TME include fibroblasts, endothelial cells, pericytes, adipocytes, mesenchymal stem cells (MSCs) and immune cells (2). These cell types encompass heterogeneous and plastic cell subsets that engage in a dynamic, bi-directional signaling crosstalk with cancer cells. The functions of the stroma are context-dependent and can be pro-tumorigenic and tumor-suppressive (3,13). The acellular elements of the TME consist of different ECM components and remodeling enzymes, inflammatory cyto/chemokines and growth factors, exosomes, and other secreted factors (2) (**Figure 2**).

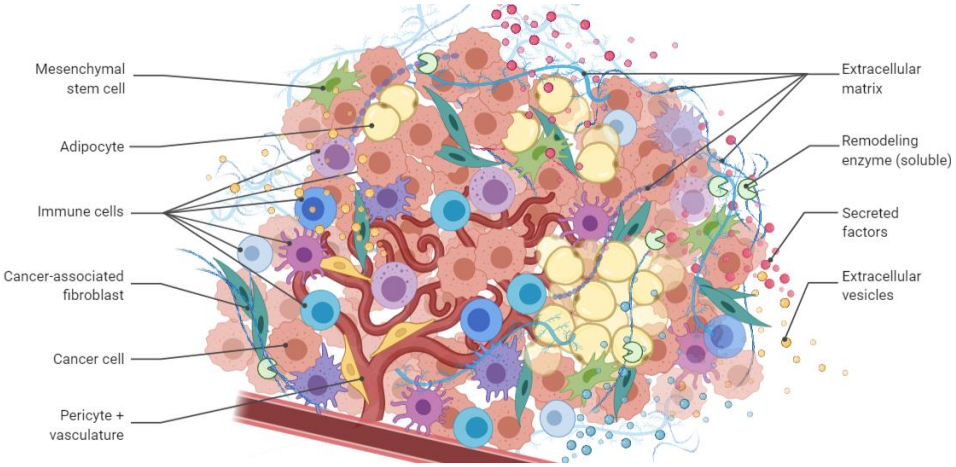


Figure 2. Illustrative scheme of the TME. *Adapted from "Tumor Microenvironment", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates*

2.1.1 Cellular components of the TME

Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are a major component of the TME and one of the most studied stromal cell types (14,15). However because of the heterogeneity in CAF origin and functions, the lack of specific CAF markers, and the difficulties in standardization of data generated by different approaches and in different cancer types, unifying functional and molecular definitions are still lacking (16).

It is generally accepted that fibroblasts surrounding early cancer lesions have tumor-suppressive effects. Secreted factors [e.g. transforming growth factor beta (TGFβ), lysophosphatidic acid (LPA), inflammatory modulators], cell-cell interactions, sensing of ECM changes as well as physiological and genomic stress can activate resident fibroblasts and other stromal cell types into CAFs (16). CAFs are the main source of ECM and greatly involved in its remodeling. By changing the biomechanical and physical properties of the TME through the production of linear collagen structures around the tumor, which are crosslinked by lysyl oxidase enzymes, CAFs promote a desmoplastic reaction associated with increased tumor stiffness. This increase in Rho-dependent cytoskeletal tension induces the assembly of integrin-based focal adhesions (FA), disrupts adherens junctions and perturbs tissue polarity, creating a pro-tumorigenic microenvironment (14,15,17). CAFs also secrete cyto/chemokines, growth factors and exosomes that alter stromal and cancer cell signaling networks to enhance invasion and metastasis, influence tumor angiogenesis and modulate immune infiltration (14,15,17).

Different CAF subtypes have been described, initially based on their origin [e.g. transformed tissue resident fibroblasts, fibrocytes, adipocytes, mesenchymal stem cells, pericytes, epithelial cells through epithelial-to-mesenchymal transition (EMT), endothelial progenitor

cells through endothelial-to-mesenchymal transition and mesothelial cells through mesothelial-to-mesenchymal transition (MMT)] and more recently based on single cell data analyses (mostly) from pancreatic and breast cancer (15–18). The two most accepted and better-defined subtypes are 'myoCAFs' and 'iCAFs': the former referring to CAFs with a myofibroblast phenotype characterized by ECM production and special contractile features (linked to TGFβ signaling and αSMA expression), and the latter referring to CAFs with a distinguishable immunomodulating secretome (most often associated with immunosuppressive outcomes) (19–21). Whether these CAF subtypes are stable, or CAFs can interconvert between them and other subtypes is still not fully understood. In addition, CAFs can become senescent, a phenotype that is associated to yet another distinct secretome named senescence-associated secretory phenotype (SASP, discussed more in detail in the next section 2.1.2) (16).

Many therapeutical strategies have been designed to target CAFs, as their abundance is associated to poor clinical outcome, and some of them are currently being tested in clinical trials. These approaches aim at preventing CAF activation or function, or at promoting CAF normalization or reprogramming towards an anti-tumorigenic phenotype (16). However, because of the incomplete knowledge on CAF phenotypes, functions and markers, too broad CAF targeting or deletion can actually result in enhanced tumor progression and should therefore be avoided (22,23). Instead blocking the signaling elicited by CAFs may be an alternative.

Endothelial cells and pericytes

Endothelial cells and pericytes contribute to the formation of new vasculature to supply the tumor with nutrients and oxygen and dispose of metabolic waste. These cells are stimulated by soluble factors found in the TME, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and chemokines (2).

Cancer-associated adipocytes

Cancer-associated adipocytes (CAAs) secrete adipokines and lipokines that support tumor growth, induce angiogenesis, and promote cancer cell migration, invasion, homing and seeding in distal organs (24,25). In addition, CAAs remodel the ECM [e.g. through matrix metalloproteinase-14 (MMP-14, MT1-MMP) activity] and promote treatment resistance in cancer cells (e.g. via FAPB4 upregulation) (24–26). Intra-abdominal tumors that metastasize to the omentum, such as pancreatic, gastric, colorectal and epithelial ovarian cancers (EOC), use omental CAAs as source of energy via lipolysis, resulting in adipocyte depletion and accumulation of fibroblast-like cells, eventually ensuing a desmoplastic stroma (24,25).

Mesenchymal stem cells

Mesenchymal stem cells are multipotent stromal cells residing in multiple tissues (including brain, thymus, muscle, bone marrow, lung, spleen, liver, kidney, pancreas, ovary and fat) that can self-renew and differentiate into bone, cartilage, muscle and fat cells, and connective

tissue (27). Upon recruitment to the tumor site, MSCs are transformed and can differentiate into other pro-tumorigenic components of the TME, suppress the immune response, promote angiogenesis, enhance EMT and stemness, increase cancer cell survival, and promote metastasis. However, in specific contexts MSCs can also inhibit tumor growth and metastasis by modulating immune responses, inhibiting angiogenesis, regulating cellular signaling, and inducing apoptosis (27).

Immune cells

The immune cellular compartment of the TME (TIME) is formed by adaptive immune cells (T- and B-lymphocytes) and innate immune cells encompassing natural killer (NK) cells, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs) and tumor-associated neutrophils (TANs) that can both inhibit tumor progression and drive tumor growth (2,28) (**Figure 3**).

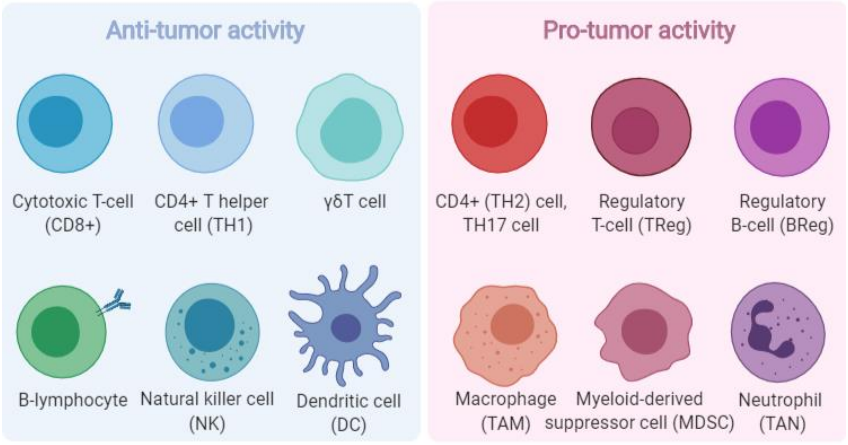


Figure 3. The TIME. *Created with BioRender.com*

2.1.2 Acellular components of the TME

Extracellular matrix and remodeling enzymes

The ECM is a tissue compartment consisting of insoluble collagen fibers that provide strength and resilience, highly viscous proteoglycans that shelter cancer cells, and soluble matrix proteins [e.g. laminins, fibronectin (FN)] that connect collagens and proteoglycans to cell surface receptors [e.g. integrins, syndecans, discoidin domain receptors (DDRs), hyaluronan receptor (CD44) and G protein-coupled receptors (GPCR)] (29,30). Besides serving as a physical scaffold and a reservoir of growth factors, cyto/chemokines and inorganic molecules, the ECM influences cancer cells through biochemical and biophysical cues (31). For example, the composition and organization of the basal membrane (a highly specialized sheet-like ECM) are important factors for maintaining epithelial cell polarity, while interstitial ECM stiffness is known to regulate cellular adhesion and signaling. Overall, the ECM influences cancer cell: (i) proliferation, by activating ERK, PI3K and Rac signaling

and by overriding the function of cell cycle regulators; (ii) survival, via modulation of pro- and anti-apoptotic regulators (such as BAX or Bcl-2 and NF-κB); (iii) invasion and migration, via TGFβ and RhoA/Rac signaling and by increased fiber organization and stiffness; (iv) stemness, by activating STAT3 and Wnt signaling; (v) therapy resistance, via activation of the above-mentioned anti-apoptotic and stemness signaling and by acting as a physical barrier for anti-cancer drug delivery (29–31).

Cancer and stromal cells can deposit, modify and degrade the ECM to create the optimal conditions for cancer cell invasion, proliferation and survival, allowing tumor growth and formation of metastases (30). In most solid tumors, cancer cells have to escape through the basal membrane and/or move through dense, desmoplastic matrices to be able to form distant metastases. The degradation of ECM is driven by matrix-degrading enzymes such as heparanases, cathepsins, hyaluronidases and metzincins. The latter comprises MMPs and adamalysins, a subfamily including a disintegrin and metalloproteinases (ADAMs) and ADAMs with thrombospondin motifs. The proteinase activity of these enzymes is controlled by various inhibitors, including tissue inhibitor of MMPs (TIMPs), cystatins and serpins (30,32).

The group of genes encoding for the core ECM proteins (collagens, proteoglycans and ECM glycoproteins) and ECM-associated proteins (secreted factors, ECM remodeling enzymes and proteins structurally resembling ECM proteins) is known as the matrisome (33) (**Figure 4**).

Figure 4. Composition of the matrisome.

In cancer, changes in the matrisome typically resemble those occurring in fibrosis, leading to the enhanced deposition and crosslinking of core ECM proteins and increased stiffness of the tumor, a characteristic used for the diagnosis of cancer in soft tissues by palpation during a clinical examination (32). Besides increased tumor stiffness, other physical factors such as interstitial pressure, pH, nutrient levels and oxygen availability are affected by tumor growth, particularly at the tumor core (34). A common feature of advanced cancers is thus hypoxia (i.e. limited availability of oxygen and nutrients), which is sensed via hypoxia-inducible factors (HIFs) in cancer and stromal cells (35). HIF-mediated signaling promotes ECM deposition (e.g. by controlling collagen expression) and remodeling (e.g. by inducing MMP expression and downregulating TIMPs), as well as an angiogenic switch to recruit endothelial cells and pericytes to form new vasculature (32,35).

Secreted factors and extracellular vesicles

Inflammatory cyto/chemokines and growth factors, exosomes and apoptotic bodies, as well as other soluble factors are also critical elements of the TME (2). These factors and vesicles can be produced by and affect both cancer and stromal cells, as they not only represent a way for communication but also allow horizontal genetic/biomaterial transfer (including DNAs, mRNAs, miRNAs, proteins and lipids) (4). Secreted factors [e.g. TGFβ, hepatocyte growth factor (HGF), Wnt ligands] and extracellular vesicles (e.g. exosomes containing mtDNA and miRNAs) can reprogram cancer cell energy metabolism, promote tumor invasion, enhance metastatic capabilities, and activate mechanisms of resistance to anti-cancer drugs (4,36).

Senescent cancer and stromal cells can also secrete a plethora of cyto/chemokines, growth factors and proteases collectively referred to as senescence-associated secretory phenotype (37,38). The functions of the SASP, which are still not fully elucidated, can be both tumor-suppressive and pro-tumorigenic and have autocrine and paracrine effects. For example, CCL2 and interleukin (IL)-6 secreted by senescent cancer cells have been reported to promote the recruitment of innate immune cells for tumor cell clearance (39,40). On the other hand, IL-1α, IL-6, IL-8, CXCL12 and MMPs secreted by senescent cancer and stromal cells create a pro-tumorigenic and immunosuppressive microenvironment that boosts cancer cell proliferation, enhances invasion and metastasis, promotes stemness and chemoresistance (38,41,42).

2.2 CANCER CELL-CELL AND CELL-ECM COMMUNICATION

Signal transduction, i.e. the transmission of signals from outside the cell (via several intermediate steps at the cell membrane and in the cytoplasm) to the nucleus ensuring appropriate cellular responses, allows cell-cell and cell-ECM communication (5,43). Cells can communicate via direct contact through communication junctions (gap junctions and ion channels), tight junctions and anchoring junctions mediated by cadherins (adherens and desmosomes) and integrins (FAs and hemidesmosomes) (**Figure 5**). Cells can also communicate via indirect mechanisms over short distances (paracrine signaling) and long distances (endocrine signaling) (5,43).

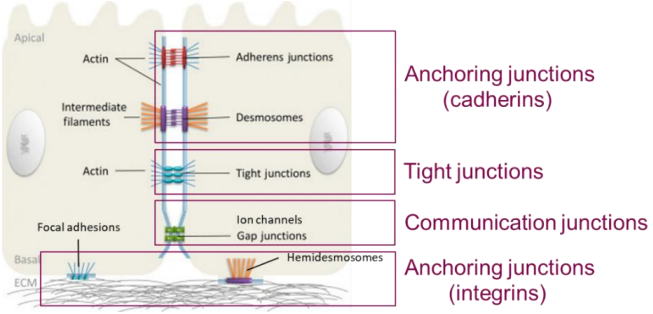


Figure 5. Cell-cell and cell-ECM direct communication. *Adapted from* Insights into the role of cell-cell junctions in physiology and disease. *Int Rev Cell Mol Biol. 2013. Wei Q and Huang H.*

Extra- and intracellular signals are sensed by receptors that can be found on the cell membrane (tyrosine, serine/threonine and histidine-specific protein kinases, GPCRs, integrins, ligand-gated ion channels and Toll-like receptors) or in the cytoplasm and nucleus (nucleic, steroid and retinoic acid receptors) (5,43).

2.2.1 Receptor tyrosine kinases

Receptor tyrosine kinases, key regulators of normal cellular processes, are frequently mutated or aberrantly expressed in cancer, promoting tumor formation and progression (44). RTKs are classified into 19 different families: EGFR (epidermal growth factor receptor)/ErbB receptor, insulin receptor, PDGFR (PDGF receptor), VEGFR (VEGF receptor), FGFR (FGF receptor), PTK7 (protein tyrosine kinase 7)/CCK4 receptor, NGFR (nerve growth factor receptor, including Trk receptors), Ror (RTK-like orphan receptor), MuSK (muscle-specific kinase) receptor, HGFR (HGF receptor)/c-Met receptor, TAM (Tyro3-Axl-Mer family) receptor, Tie receptor (angiopoietin receptor), Eph receptor, Ret (rearranged during transformation) receptor, Ryk (tyrosine-like kinase) receptor, DDR, Ros receptor, ALK/LTK (leukocyte tyrosine kinase) receptor, and STYK1 (serine/threonine/tyrosine kinase 1) receptor (44) (**Figure 6**). LMR (Lemur receptor) family, previously considered a family of RTKs, has recently been reclassified as a family of serine/threonine protein kinases (45).

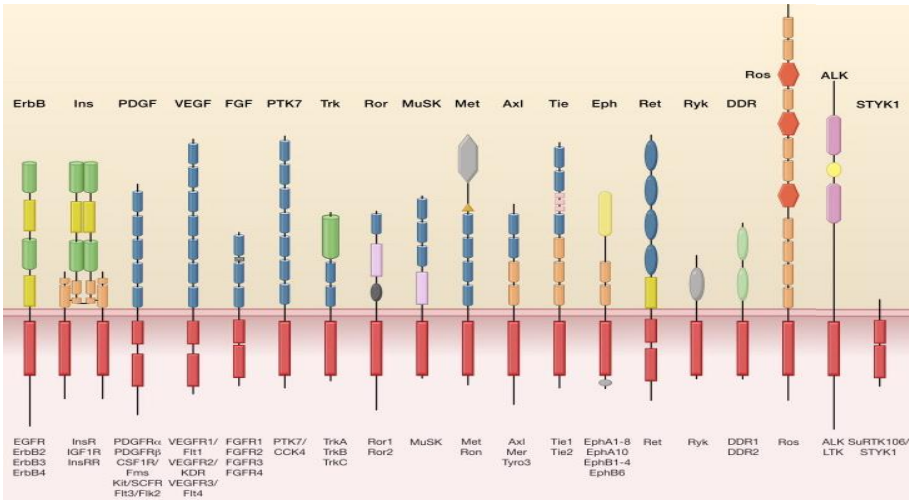


Figure 6. Receptor tyrosine kinases. *Adapted from* Cell Signaling by Receptor Tyrosine Kinases. *Cell. 2010. Lemmon MA and Schlessinger J.*

All RTKs share a molecular structure comprising extracellular ligand-binding domains, a single transmembrane helix, and a cytoplasmic tail where the protein tyrosine kinase (TK) domain, an additional carboxy (C-) terminal and juxtamembrane regulatory regions are found (44). It is generally accepted, albeit not completely understood at the mechanistic level for all RTKs, that ligand-binding to the extracellular domain promotes the dimerization of the receptors, bringing into proximity the TK and juxtamembrane regions and promoting the transphosphorylation of the TKs. These phosphorylated dimer structures function as docking

sites for numerous downstream signaling molecules from the Ras-mitogen-activated protein kinase (MAPK), PLC-PKC, FAK-Src, JAK-STAT, PI3K-Akt pathways (44). It is also known that most human RTKs can be activated by more than one ligand, resulting in different signaling outputs, and that some RTKs can form not only dimers but higher-order oligomers (e.g. Eph-ephrins) that control paradoxical cellular responses (46). Finally, some RTKs (ErbB3, EphA10, EphB6, and PTK7, Ror, Ryk and STYK1 receptor families) lack TK activity but may allosterically regulate other kinases or function as scaffolds for intermolecular interactions required for signaling propagation (47).

RTK activity is regulated by protein tyrosine phosphatases (PTPs) at the TK domain, by ubiquitin E3 ligases (e.g. Cbl, NEDD4) at the cytoplasmic regions of the receptors or by endocytosis of the receptor-ligand complexes (44,46,48). Internalization of these complexes can occur via clathrin- (e.g. EGFR) or caveolin-mediated mechanisms (e.g. VEGFR, PDGFR) and facilitates RTK dephosphorylation or ubiquitination, dissociation of the ligand in the acidic endosomes and ultimately lysosomal degradation (48). The endocytosed receptor-ligand complexes can still signal in the cytoplasm and even promote different signaling responses compared to the ones elicited by the membrane-anchored receptors. Besides lysosomal degradation of RTKs, the receptor-ligand complexes can be recycled back to the cell surface (44,48). To add more complexity to the still not fully understood RTK signaling mechanisms, RTKs can also signal upon proteolytic release of intracellular domain fragments mediated by sheddases (e.g. ADAM10, ADAM17) and gamma-secretase or by caspases. RTK fragments have been found in the nucleus controlling the activity of transcription factors, or in the mitochondria regulating apoptosis (49). Finally, shedded RTK ectodomains can sequester RTK ligands, which are mainly growth factors except for collagens (i.e. DDR ligands) and ephrins (i.e. Eph receptor ligands) (46) (**Figure 7**).

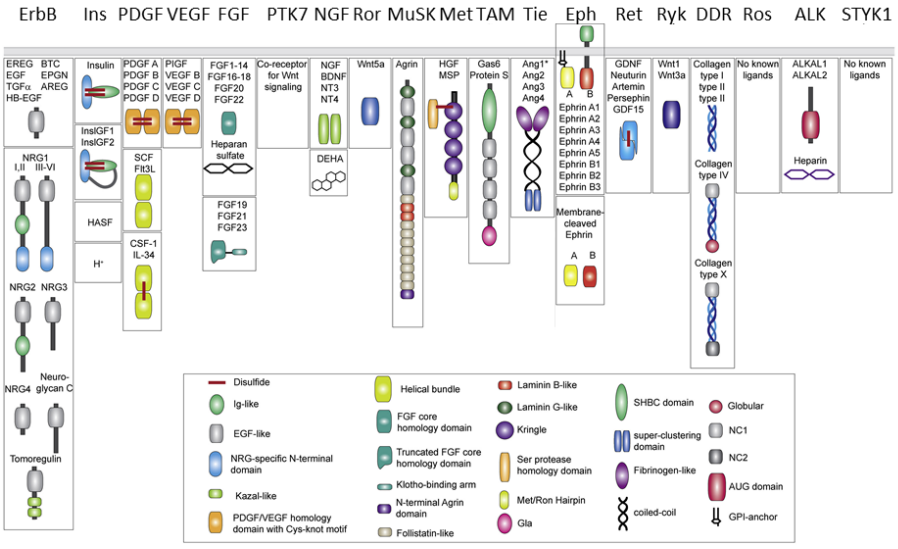
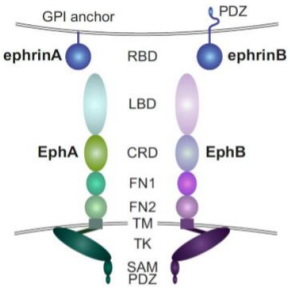


Figure 7. RTK ligands. *Adapted from* Receptor tyrosine kinase activation: From the ligand perspective. *Curr Opin Cell Biol. 2020. Trenker R and Jura N.*

2.2.2 Eph/ephrin signaling

The largest RTK family is the Eph family, which is comprised of: (i) a group of nine EphA receptors that bind to five ephrin-A ligands anchored to the cell membrane via glycosylphosphatidylinositol (GPI) membrane anchorage, and (ii) a group of five EphB receptors, which bind to three ephrin-B ligands that are type-I transmembrane proteins (6–8) (**Figure 8**).



The exceptions to such class specific binding include EphA4 binding to ephrin-Bs, and EphB2 binding to ephrinA5 (6–8). Albeit ephrin ligands are generally membrane-bound, a distinct characteristic from other RTK ligands, they can be cleaved and act as soluble ligands in pathological conditions such as cancer (7).

Figure 8. Eph receptors and ephrin ligands. *Reproduced with permission from* Eph- and ephrin-dependent mechanisms in tumor and stem cell dynamics. *Cell Mol Life Sci. 2014. Gucciardo E, Sugiyama N and Lehti K.*

The Eph/ephrin signaling system is characterized for its duality in: (i) signal transduction, i.e. the signal can be simultaneously transduced in the receptor- (forward signaling) and the ligand- (reverse signaling) expressing cells and (ii) its role in tumor progression, i.e. the outcome of Eph/ephrin signaling can promote and inhibit tumorigenesis (6–8). To add more complexity to this signaling system, Eph receptors can interact physically and/or at the intracellular signaling level with cadherins, MMPs, integrins, growth factor receptors and GPCRs, and elicit different cellular responses that are complex and context-dependent (7,50).

Eph receptors can be canonically activated via interaction with ephrin ligands, leading to dimerization of the receptor and transphosphorylation of the cytoplasmic tyrosine residues (6,7). This promotes further aggregation of multimeric receptor/ligand complexes and is associated with lateral receptor activation. Once the aggregated receptors are activated, SH2-domain containing proteins that mediate downstream signaling pathways are recruited to the kinase domain of the receptors. Eph receptors can then regulate PI3K-Akt, JNK-STAT, FAK and Src kinase-mediated signals. Activated Eph receptors can also recruit GEFs (guanine nucleotide exchange factors) that regulate Rho GTPase-mediated actin dynamics. Thus, Eph signaling can regulate cell repulsion, cell-cell adhesion, cell proliferation and apoptosis, tissue boundary formation and cell migration (6,7). Albeit being complex and context-dependent, Eph canonical signaling is most often associated with anti-invasive and growth-suppressive outcomes (**Figure 9**).

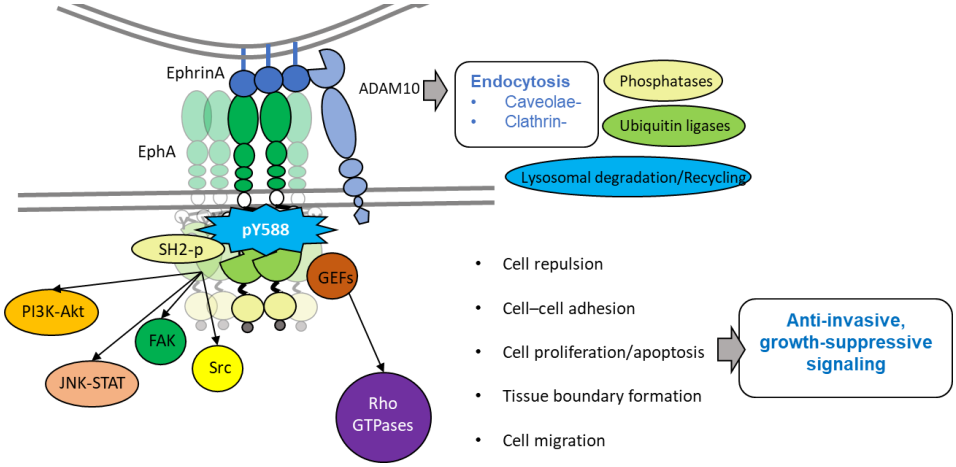


Figure 9. Simplified representation of ligand-dependent Eph receptor activation and elicited signaling.

Eph/ephrin complexes can be cleaved by metalloproteinases such as ADAMs and internalized via caveolae- or clathrin-dependent endocytosis. These complexes can still signal once endocytosed. The attenuation or termination of the signal occurs via receptor interaction with PTPs (dephosphorylation) and E3 ubiquitin-protein ligase Cbl (degradation), or upon lysosomal degradation or recycling of the receptor (7,8) (**Figure 9**).

The expression of Eph receptors and ephrin ligands is dysregulated in cancer, where non-canonical Eph signaling has been observed (6,9). This non-canonical signaling occurs via Eph receptor crosstalk with growth factor receptors, which in EphA2 promote the phosphorylation of S897 residue via AGC family kinases such as Akt, protein kinase A (PKA) and RSK (51–53). Another non-canonical mechanism of EphA2 receptor signaling involves the joint activity of this receptor, MT1-MMP (MMP14) and Src-kinase. In the presence of low levels of ephrin ligands and abundance of MT1-MMP, limited EphA2 activation coincident with receptor cleavage by MT1-MMP results in Src-mediated EphA2 intracellular translocation (54,55). The signaling elicited by ligand-independent activation of Eph receptors promotes alterations in cell-cell contacts and ECM adhesion or degradation to support anchorage-independent growth, invasion in collagen-rich TME, as well as stem-like properties and treatment resistance (50,54,55) (**Figure 10**). Albeit some reports have suggested that certain Eph receptors may suppress the expression of the corresponding ligands and the associated ligand-dependent, tumor-suppressive signaling [e.g. EphA2-ephrinA1 pair (56,57)], it is reasonable to think that the ligand-dependent, canonical Eph receptor signaling coexists with the ligand-independent, non-canonical signaling at the cellular and tissue level.

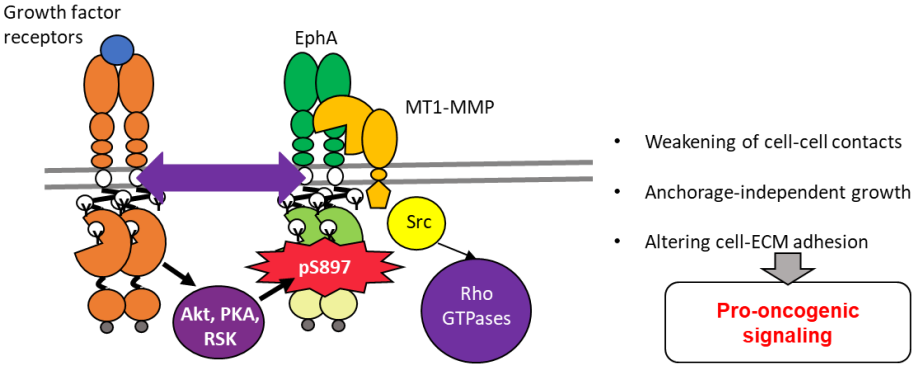


Figure 10. Schematic representation of ligand-independent Eph receptor activation and elicited signaling.

Eph/ephrin signaling in physiology

Eph/ephrin signaling is involved in many physiological situations ranging from boundary formation during embryogenesis to homeostasis in the adult organism (8,9):

- In neural development, short distance cell-cell communication via Eph/ephrins regulates self-renewal and differentiation (plasticity), proliferation, adhesion and migration, as well as apoptosis (survival) of neural progenitors. EphA4, EphA7 and all EphB receptors, together with ephrinA2/A3/A5 and ephrinB3 have been identified as mediators of these processes (58).
- In vascular development, Eph/ephrin signaling defines boundaries between veins (expressing Eph receptors) and arteries (expressing ephrins), and is also required for the formation of vascular valves in the lymphatic system and for the stabilization of vessel networks. EphB4 and ephrinB2 are the most well characterized receptor-ligand pair in angio- and lymphogenesis (59).
- In axon guidance, cell-cell repulsion and adhesion mediated by Eph/ephrin signaling are involved in the extension of growth cones (i.e. the distal tip of a developing or regenerating neuron seeking its synaptic target), the formation of synapses and the determination of dendritic branching patterns. Ephrin reverse signaling as well as ephrin-mediated recruitment of specific receptors (not limited to Ephs) and other factors control these processes (60).
- In tissue repair, EphA4 and EphB receptors together with ephrinB1/B2 regulate epithelial cell migration for wound closure (re-epithelialization). EphA2-ephrinA1 signaling can also regulate wound closure (*in vitro* evidence) and control the vascularization of the granulation tissue (61).
- In immunity, Eph/ephrins have been implicated in immmune cell activation, differentiation, proliferation, adhesion, migration and survival. Albeit many Eph receptors and ephrin ligands are expressed in T- and B-cells, little is known about the specific mechanisms and the exact implication of certain Eph/ephrins in these processes (62).

- In adult physiology, Eph/ephrin signaling regulates the stability of neuronal synapses, the equilibrium between bone resorption and deposition, cell metabolism as well as stem cell niches (8,9).

Eph/ephrin signaling in cancer

Chromosomal alterations, epigenetic modifications, changes in mRNA stability and variations in transcriptional regulation influence Eph and ephrin expression in cancer cells (6). Both Eph receptors and ephrins may also be expressed by other cells in the TME. *In vitro*, Eph/ephrin signaling affects the growth, migration and invasion of cancer cells, and *in vivo* promotes tumor growth, invasiveness, angiogenesis, metastasis, stemness and treatment resistance (6–9). Indeed, many studies have linked the expression of Eph receptors and ephrins with cancer progression, metastasis and patient survival (6,9).

The widely studied Eph receptor in cancer, EphA2, is rarely mutated but frequently overexpressed in solid tumors and associated with increased metastasis, poor prognosis and decreased patient survival (50,63). Thus, EphA2 has been recognized as a putative target to block disease progression and multiple strategies targeting this receptor have been developed, including agonistic EphA2-targeting agents alone or conjugated to anti-cancer drugs, EphA2 siRNA and miRNA encapsulated in nanoliposomes and small molecule inhibitors (63,64). Despite encouraging pre-clinical data showing reduced tumor growth and increased chemotherapy response after EphA2 depletion/degradation/inhibition in several studies (65–67), these findings have not been successfully translated into clinical use and currently only one clinical trial (in phase 1) is testing the direct EphA2 activity inhibition in advanced metastatic cancers (NCT01591356, https://clinicaltrials.gov). At present, the developed molecular EphA2-targeted therapies lack proof for specificity and efficacy (63,64).

2.2.3 Integrins and GPCRs

Integrins are transmembrane receptors that link the ECM to cell cytoskeleton, acting as signaling hubs between the cells and their microenvironment (68). Integrins can sense not only the ECM biochemical properties (composition) but also the ECM physical/mechanical properties, which are responsible for mechanotransduction (i.e. transmission of mechanical stimuli that ultimately drive cytoskeleton reorganization, alter gene expression and promote ECM remodeling) (69). The integrin family comprises 24 transmembrane heterodimers formed by combinations of 18α integrin and 8β integrin subunits (68). Integrin activation is mediated by “inside-out” signaling that recruits proteins such as talin to the β integrin tail, changing the closed, inactive conformation to an extended conformation with high affinity for ECM ligands. Upon integrin-ECM binding, “outside-in” signals recruit a complex, dynamic and tightly regulated array of signaling, scaffolding and cytoskeletal proteins named the adhesome, which is responsible for regulating cell survival, migration, polarity and differentiation through “inside-in” signaling. One of the kinases orchestrating the “outside-in” signals is FAK, which assembles focal adhesions and binds Src tyrosine kinase. The FAK/Src complex does not only regulate the turnover of focal adhesions, but also the activity of Rho

family small GTPases, which in turn control actin polymerization, actomyosin contractility and force transmission to regulate the formation of cellular protrusions, cell spreading as well as motility and proliferation (68–70) (**Figure 11a**).

The signaling generated by integrins sensing soft ECM (low resistance) promotes low actomyosin contractility and inactivation of the mechanotrasducers YAP/TAZ located in the cytoplasm (69,71). The increase in ECM resistance generated by e.g. enhanced ECM rigidity is sensed by integrins (among other adhesive proteins), promoting integrin clustering, FA formation via FAK/Src activity and increased intracellular tension that allow the release of YAP/TAZ from inhibitors and the translocation to the nucleus. Nuclear YAP/TAZ act as transcriptional co-regulators by using TEAD factors as DNA-binding platforms to regulate a myriad of cellular processes and capabilities, including cell proliferation, stemness, metabolism and plasticity (69,71) (**Figure 11b**). YAP/TAZ mechanotransduction signaling mechanisms are however not yet fully understood.

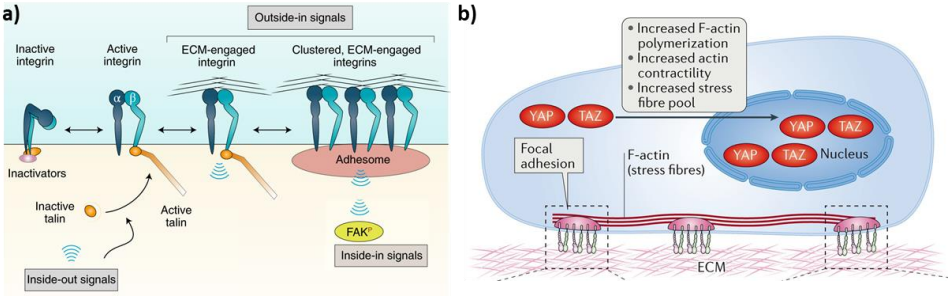


Figure 11. Schematic representation of a) integrin signaling and b) YAP/TAZ mechanotransduction. *Adapted from* ***a)*** Integrin trafficking in cells and tissues. *Nat Cell Biol. 2019. Moreno-Layseca P, Icha J, Hamidi H et al. and* ***b)*** Mechanobiology of YAP and TAZ in physiology and disease. *Nat Rev Mol Cell Biol. 2017. Panciera T, Azzolin L, Cordenonsi M et al.*

Integrin signaling and Eph receptor signaling share effector molecules such as FAK, Src and Rho GTPases. There is evidence suggesting that these pathways overlap to a certain extent and can even regulate each other (e.g. cell-cell repulsion upon ligand-dependent Eph receptor activation promotes cytoskeletal rearrangements that can disrupt integrin-mediated cell adhesion to ECM, resulting in integrin inactivation) (72,73). However, direct interaction between integrins and Eph receptors has been scarcely reported and the effects of such crosstalk (either physical or at the signaling level) have not been fully elucidated (6,7).

Other membrane receptors that can mediate adhesion signaling via FAK/Src/Rho GTPases are the seven-transmembrane receptors or GPCRs (74). This large family of cell surface receptors, encoded by >800 genes, is divided into five families (**Figure 12**):

i. Glutamate family (previously known as class C): includes metabotropic glutamate receptors, calcium-sensing receptors and γ-aminobutyric acid receptors.

ii. Rhodopsin family (previously known as class A): includes receptors for small molecules, peptides and hormones.
iii. Adhesion family: formed by receptors that have exceptionally long extracellular regions containing domains that facilitate cell-ECM interactions.
iv. Frizzled/Taste2 family: receptors in the Wnt signaling pathway.
v. Secretin family (previously known as class B): includes receptors for polypeptide hormones.

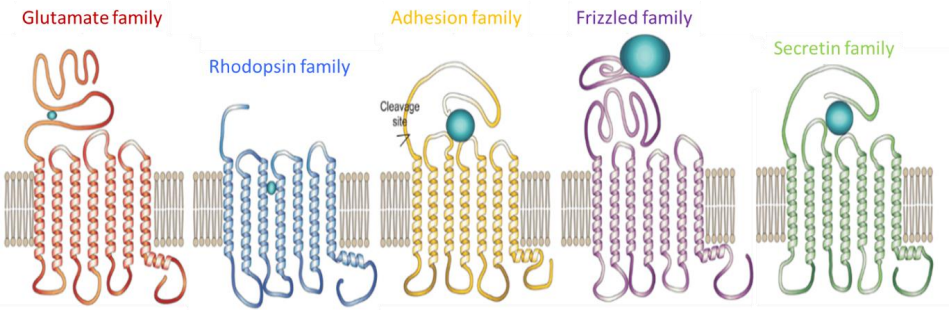


Figure 12. GPCR families. *Adapted from* Comprehensive Analysis of Non-Synonymous Natural Variants of G Protein-Coupled Receptors. *Biomol Ther. 2018. Kim HR, Duc NM and Chung KY.*

In addition, GPCR diversity is increased upon transcriptional and post-translational modifications and by the enormous repertoire of ligands and intracellular proteins that the GPCR receptors can engage with (75). Thus, GPCRs are involved in many physiological functions ranging from behavior and mood regulation to control of inflammation and modulation of homeostasis, but can also contribute to cancer growth and metastasis (75,76).

GPCR receptors are activated upon agonist ligand binding, promoting the interaction with a heterotrimeric guanine nucleotide-binding protein (G protein) that consists of an α-subunit containing the guanine nucleotide-binding pocket and the intrinsic GTPase activity, and the tightly associated β- and γ-subunits (75,76). Activation of GPCRs drives the exchange of bound GDP to GTP of the α-subunit and the independent release of this subunit from the β- and γ-subunits, triggering different downstream signals. For instance, the α-subunit can activate adenylyl cyclase that converts ATP to cAMP, which in turn activates PKA to regulate MAPK networks, controlling cell growth, proliferation, differentiation and apoptosis (76). In addition to signaling through G proteins, GPCRs can signal through arrestins, which serve as negative regulatory proteins by blocking the activated receptors from binding to G proteins (signaling desensitization) and by targeting ligand-occupied GPCRs for endocytosis (75,76). Moreover, GPCRs can also form GPCR-RTK signaling platforms which promote GPCR ligand-dependent transactivation of RTKs and RTK–G protein partnerships (77).

Recently, the orphan GPCR Class C, Group 5, Member A (GPRC5A) has been described as a direct binding partner of EphA2 (78). GPRC5A is highly expressed in many epithelial cancers including breast, pancreatic, gastric, ovarian, prostate and testicular cancers; however

GPRC5A function is disease-dependent, acting as an oncogene in some cancers and as a tumor suppressor in others (79). Before this thesis, GPRC5A function in ovarian cancer had remained unknown.

2.3 EPITHELIAL OVARIAN CANCER

Epithelial ovarian cancers comprise an heterogeneous group of adenocarcinomas with different histological subtypes: Clear cell, endometrioid, mucinous, serous (including low- and high-grade) and undifferentiated carcinomas (80). The most prevalent subtype is HGSC, particularly among advanced stage EOC, thus being responsible for most EOC-related fatalities. HGSC is characterized by high chromosomal instability and mutations in *TP53* in over 96% of cases. Moreover, a few other genes regulating DNA damage and repair and cell cycle are recurrently mutated, such as *NF1*, *BRCA1*, *BRCA2*, *RB1*, *CDK12* and *CCNE1* (81). Originally described as arising from the ovarian surface epithelium, there has been a paradigm shift and it is now widely accepted that most HGSC cases instead develop from precursor lesions in the fallopian tube epithelium named serous tubal intraepithelial carcinoma (STIC), and in some cases from primary peritoneal lesions (80,82) (**Figure 13**).

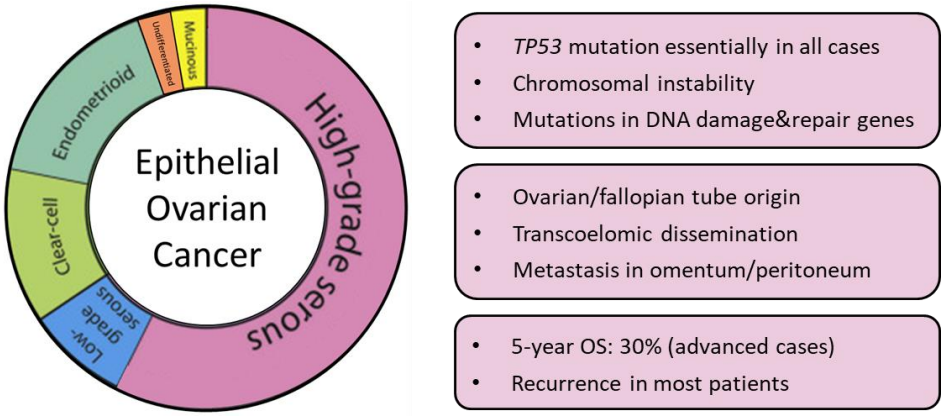


Figure 13. EOC classification and characteristics of HGSC. *Adapted from* The Origin of Ovarian Cancer Species and Precancerous Landscape. *Am J Pathol. 2021. Shih IM, Wang Y and Wang TL.*

Transcoelomic spread is the most common mechanism of OC cell dissemination, although lymphatic and hematogenous spread may also occur. OC cells and clusters exfoliate from the primary tumor into the peritoneal cavity and accumulate in the peritoneal fluid (83). In addition, malignant cells may also accumulate in pleural effusions in the thorax (being the most common extra-abdominal manifestation of EOC). Although not yet fully understood, obstructed lymphatics and increased vascular permeability induced by the cancer cells may be a cause for the formation of a fluid TME (i.e. ascites) rich in pro-tumorigenic factors and inflammatory cyto/chemokines where cancer cells thrive (84,85). In this microenvironment, cancer cells interact with other cells and ECM components and are exposed to physical and mechanical stimuli that promote cancer cell proliferation, evasion of anoikis, stemness and

adhesion to mesothelial-lined abdominal tissues, including the peritoneum (resulting in carcinomatosis) and the omentum (83–85). Under the mesothelial layer and basal membrane, the omentum is mainly composed of adipocytes enclosed in a collagen matrix, with scattered resident immune cells, fibroblasts and endothelial cells. Metastatic OC cells invade the omentum and together with the stroma promote a desmoplastic reaction, transforming this fatty organ into a fibrotic tissue (83,86) (**Figure 14**).

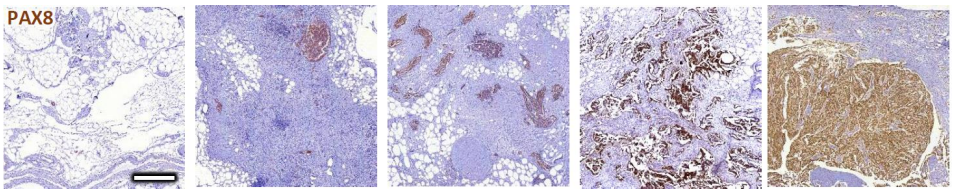


Figure 14. Representative images of omental tissues from patients with HGSC stage III-IV, where metastatic OC cells were stained with PAX8. *Reproduced with permission from* Modelling TGFβR and Hh pathway regulation of prognostic matrisome molecules in ovarian cancer. *iScience. 2021. Delaine-Smith RM, Maniati E, Malacrida B et al.*

Most HGSC cases are diagnosed at advanced stages due to lack of sensitive and reliable detection methods for the often mild and insidious symptoms that the patients develop (82,87). The standard of care for HGSC is debulking surgery followed by platinum-based chemotherapy, frequently combined with taxane (87,88). For patients in advanced stages (III with residual disease and IV), the first-line treatment can also include the antiangiogenic drug bevacizumab, aiming to prevent the formation of new vessels that irrigate metastatic tumors and to facilitate anti-cancer drug release by increasing the vascular permeability (87,88). Moreover, a subset of patients can receive poly-ADP-ribose polymerase inhibitors (PARPi, such as olaparib and niraparib) as maintenance. These patients are mainly selected based on mutations of enzymes involved in DNA repair pathways (e.g. *BRCA1/2* mutations) that diminish the efficiency of homologous recombination (HR) repair and boost the activity of base-excision repair, mediated by PARP enzymes (87–90). PARPi given in combination with chemotherapy lead to accumulation of DNA damage and eventually to cell death in a process of synthetic lethality (89). BRCA mutations are not the only cause of HR deficiency (HRD); instead, phenotypes that mimic the loss of BRCA, known as "BRCAness", can also arise due to mutations and/or epigenetic modifications of HR effector genes (e.g. *ATM, ATR, CHEK1/2, DSS1, RAD51, FANC, CDK12*). Thus, rather than screening patients for *BRCA1/2* mutations or assessing the genomic scar caused by chromosomal instability (e.g. loss of heterozygosity), functional HRD assays (e.g. detection of RAD51 foci upon radiation-induced DNA damage) have been developed to better identify patients that will benefit from PARPi (87–91).

Despite overall good response to first-line treatment, most patients experience recurrence of the disease, which can still be sensitive to chemotherapy, but will ultimately progress and become resistant to treatment (87,88). The current treatment options for patients with recurrence include surgery, platinum re-challenge (in the platinum-sensitive scenario) or non-

platinum chemotherapy with paclitaxel, gemcitabine, vinorelbine, etoposide, topotecan and/or PEGylated liposomal doxorubicin (in the platinum-resistant cases). In the maintenance setting, bevacizumab and PARPi (including rucaparib) are also recommended, the latter for platinum-responsive patients. Finally, patients can enroll in clinical trials to be treated with therapies and anti-cancer drugs currently being developed (87–89). However, the rather small benefit of these combinatorial treatments and the emergence of resistance to both platinum and non-platinum drugs (e.g. to PARPi) really highlights the need to find better first-line treatment strategies to avoid the recurrence of the disease (87–89).

Albeit HGSCs are potentially immunoreactive (the presence of tumor infiltrating lymphocytes, a requisite to respond to immune checkpoint blockade, correlates with improved HGSC clinical outcome), to date immunotherapy has only shown modest or negative results (87,92,93). Several immunosuppressive factors and inhibitory signals in the TME suppress T-cell activation, thus single agent approaches (mostly immune checkpoint inhibitors) have not proven successful. Currently, rational immunomodulatory combinations with antiangiogenic drugs (e.g. bevacizumab), chemotherapy, PARPi, vaccines, other immunomodulatory agents (e.g. radiation therapy, cytokine immunotherapy), and adoptive T-cell therapies [e.g. chimeric antigen receptor (CAR)-expressing T cells] are under investigation (92).

2.3.1 The TME in HGSC

HGSC has multiple TMEs, including the primary site, the ascites fluid, the early metastasis at the mesothelial layer covering the peritoneum and omentum as well as the late metastatic site (94), from which the ascites (fluid TME) and the metastatic omentum (solid TME) have been more extensively characterized (**Figure 15**).

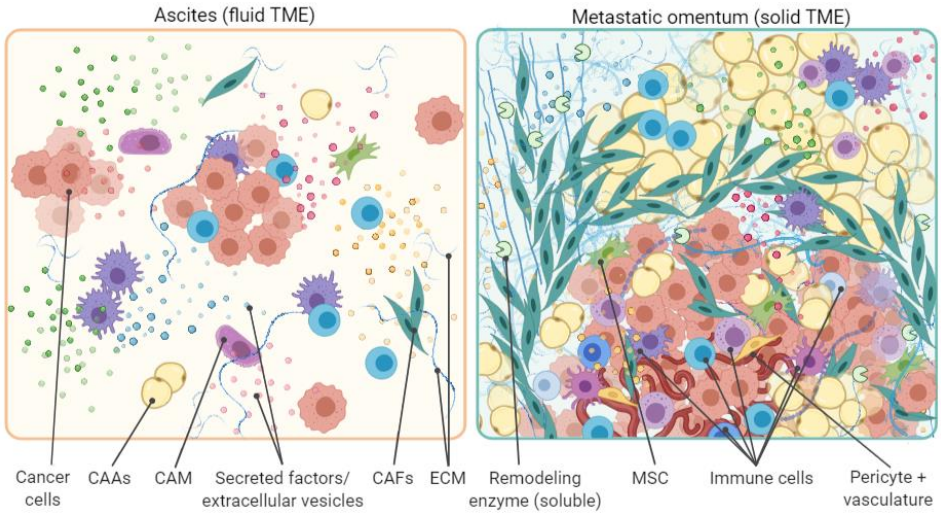


Figure 15. The TMEs of HGSC. *Created with BioRender.com.*

The fluid TME (i.e. ascites) contains immune cells, including macrophages and T-cells (cytotoxic and regulatory), and to a variable extent also CAFs, MSCs, CAAs and cancer-associated mesothelial cells (CAMs) (84,94,95). These stromal cells together with cancer cells and peritoneal fibroblasts, adipocytes, and mesothelial cells secrete cyto/chemokines [e.g. IL-1β, IL-6, IL-8, IL-10, leptin], growth factors (e.g. HGF, LPA, TGFβ, VEGF), extracellular vesicles (e.g. exosomes) and ECM components [e.g. vitronectin (VTN), FN] that allow cancer cell immune evasion, growth and survival, promote increased permeability of the peritoneal membrane and prime the omental metastatic niche (84,94,95).

Mesothelial cells lining the omentum become activated by cues from the fluid TME and undergo MMT (18). Once transformed, CAMs secrete oncogenic factors such as IL-8 to promote cancer cell adhesion (via integrins and CD44), migration and invasion through the peritoneal basal membrane [composed of COL1, COL4, FN and laminin (LAM)], and produce and remodel the ECM by secreting MMPs (18,83,94). Embedded between the mesothelial layer and the adipocytes, the omentum contains "milky spots", which are immune structures similar to secondary lymphoid tissues, consisting of lymphocytes, macrophages and DCs irrigated by blood and lymphatic vessels (96). Cancer cells are recruited to these "milky spots" and trapped in neutrophil extracellular traps, which are chromatin structures secreted by neutrophils recruited to the omentum (97).

The metastatic TME (i.e. omentum) comprises CAAs, CAFs, endothelial cells and pericytes, MSCs and immune cells (95,98,99). CAAs secrete cytokines with tumor-promoting effects (e.g. IL-6 and IL-8) and are subjected to lipolysis by cancer cells, releasing free fatty acids that can be used by the cancer cells as energy source thanks to their metabolic plasticity (25,26). CAFs produce ECM components and modifying enzymes as well as an ample repertoire of cyto/chemokines, growth factors and extracellular vesicles (e.g. IL-6, CCL5, TGFβ, exosomes) that promote cancer cell proliferation, invasion and migration, stemness and platinum resistance, as well as immune inhibition and angiogenesis (98,100). VEGF and angiopoietins found in the ascites and produced by the cancer cells and stromal cells in the omentum promote the formation of new vessels through the recruitment of endothelial cells and pericytes to the metastatic niche (98). Resident and recruited MSCs also increase tumor cell migration, proliferation and platinum resistance and can be reprogrammed towards a CAF-like phenotype (101,102). The depletion of adipocytes coincident with increased collagen and glycosaminoglycan deposition by CAFs and CAF-like stroma results in a fibrotic TME (86). This microenvironment is also characterized by leaky and ruptured vasculature that reduces oxygen perfusion and generates hypoxic areas where cancer and stromal cells contribute to collagen deposition and crosslinking, ultimately facilitating cancer cell invasion (103). Clinically, abundance of CAFs, pericytes and MSCs in the tumor is associated with advanced stage HGSC, higher frequency of metastases, increased lymphatic and microvessel density, treatment resistance as well as poor overall (OS) and progression-free (PFS) survival (98,99).

Cells of both innate and adaptive immunity are found in the solid TME of HGSC, albeit the functions of some cell types (e.g. NK cells and B-cells) are not yet fully understood (104). TAMs secrete cyto/chemokines and growth factors (e.g. IL-1β, IL-10, CCL22, TGFβ, tumor necrosis factor alpha, VEGF) that enhance angiogenesis, cancer cell migration, invasiveness and stemness and that have immunosuppressive effects, such as inhibiting T-cell proliferation and function, and recruiting TRegs (105,106). In addition, TAMs can remodel ECM by secreting MMPs and other proteases to facilitate cancer cell migration (107). At the metastatic niche, resident TRegs and MDSCs induce stemness and increase the tumorigenic properties of cancer cells, and together with DCs can also inhibit T-cell activity (106,108). Clinically, high amounts of infiltrated TAMs, TRegs, MDSCs and DCs are associated to poor HGSC prognosis and short survival as well as to early relapse of the disease, whereas high anti-tumoral T-cell infiltration (i.e. CD4+ TH1 and CD8+) is associated with delayed recurrence and a survival benefit (98,99,104).

2.4 MODELING EOC/HGSC

Albeit HGSC clinical care has improved in the last decades and many reports have contributed towards trying to understand the origin, development, treatment response and evolution of HGSC to recurrent disease, the underpinning mechanisms are still not fully elucidated and the 5-year survival of patients with advanced disease still remains as low as 30% (109). Thus, there is still a need for additional studies to address biological and clinical challenges, and to do so, the development of reliable experimental models is of critical importance.

Currently, there are over 50 established OC cell lines available for research (110). These cancer cells can be cultured in a simple, fast, and economical manner, and thus are commonly used in the laboratory. However, these OC cell lines are grown for many passages and for long periods of time, and thus represent (quite) homogeneous populations (110,111). Moreover, recent studies have brought into light striking findings on the use of cells that do not recapitulate the mutational landscape of specific OC subtypes, such is the case of HGSC: up to 90% of published studies using “HGSC” cell lines are actually based on cells that are *TP53* wild-type, have characteristic mutations of other OC subtypes or do not fully recapitulate HGSC (112). Hence, the selection of OC cell lines and the analysis of the obtained results should be consciously made depending on the purpose of the study.

Patient-derived cell lines can also be generated, yet usually with low efficiency and with culture conditions that promote the survival of the best adapted cell clones, leading to loss of tumor heterogeneity, copy number variations and mutations. Particularly, culture of primary cells directly on top of cell culture plastics and with media containing inadequate growth factors (e.g. FBS supplement) can introduce artificial changes in morphology and behavior (e.g. mesenchymal phenotype) (94,110,111). However, a small number of studies have reported specific culture media recipes and conditions that allow the establishment of cell lines with unchanged genetic landscape and unaltered molecular profiles (113). In general,

replacing the long-term, adherent cell cultures with spheroid and organoid models will be useful to maintain the characteristics of the patient-derived cells (**Figure 16**).

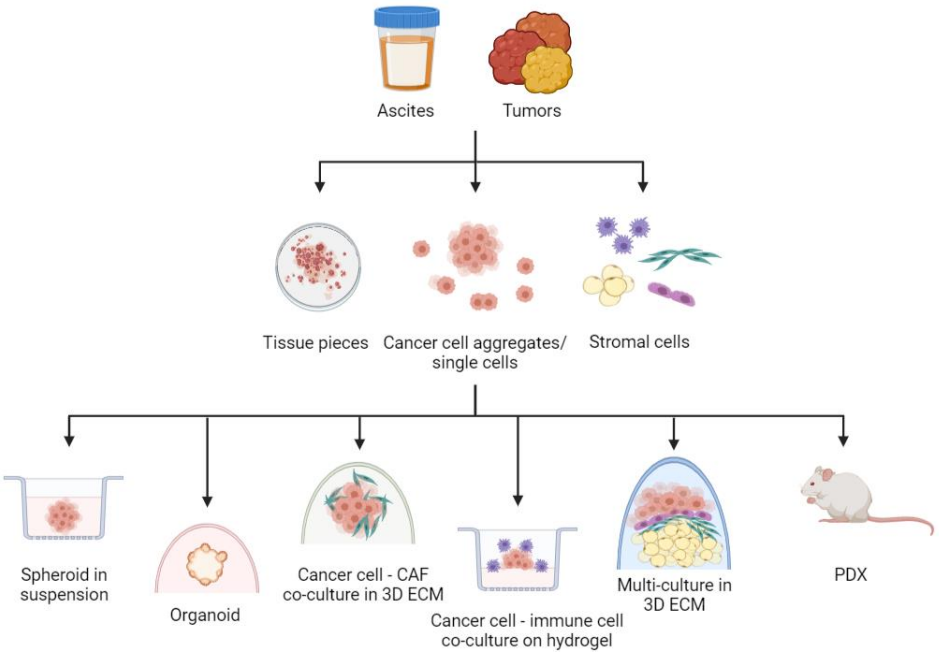


Figure 16. Patient-derived HGSC models. *Created with BioRender.com.*

Spheroids are obtained by dissociating and isolating/sorting tumor cells and culturing them in suspension in ultra-low attachment plates, where cells grow maintaining a 3D sphere-like structure (110). This method enriches the cultures for cells with stem-like properties, which are frequently used for anti-cancer drug testing and studying metastatic potential *in vivo* (114). Due to the similarities between ascites clusters and tumor spheroids in culture, most cultures are based on ascites-derived cells rather than metastatic omental tumors (110).

The next level of complexity is presented in the patient-derived tumor organoid cultures (PDOs), which are described as "mini-organs" exclusively composed by epithelial cells (110,111). These cultures are established from as little as single cells by using defined cocktails of growth factors, small molecules and hydrogels and result in organized 3D structures that recapitulate the anatomy and functions of the tumor from which they are derived. Patient-derived tumor organoids can be grown for long periods of time without compromising the fast cellular growth, can be cryopreserved and successfully recovered, and even genetically modified. Thus, PDOs can be used for studying tumor heterogeneity, HRD and anti-cancer drug responses in a high throughput manner (115,116).

Despite the structural complexity, spheroids and organoids are composed by epithelial cancer cells only, and thus still lack the contribution of the TME to fully recapitulate the tumor characteristics. To address this issue, organotypic cultures of different cell types and ECM components have been described (94). These cultures are not limited to adherent conditions

but rather are in suspension or based on 3D structures generated with help of different ECM components. Some examples are: culture of OC suspended spheroids in contact with mesothelial cells to study the clearance of the mesothelial cell layer; growth of OC spheroids on top of collagen and surrounded by macrophages to investigate the influence of cancer cells on the phenotype of these immune cells; spheroid co-cultures of OC cells and CAFs in 3D ECM structures for the study of invasion; tri- and tetra-cultures of adipocytes, CAFs, mesothelial cells and OC cancer cells in collagen (with possibility to add other ECM components such as fibronectin or other cell types such as platelets), which recapitulate the different interactions between the tumor and its microenvironment, and can be used, for instance, for quantitative high-throughput screening (HTS) of anti-cancer drugs (94,117–119).

The *in vivo* models used to study EOC are mostly mouse models, albeit some rat and hen spontaneous OC models have also been described (120). The murine models include: (i) genetically engineered models, which are useful for studying EOC initiation and development in an immunocompetent and genetically defined system (e.g. *Trp53;Rb1* and *Trp53;Pten;Brca1/2* backgrounds for HGSC, *Arid1a;Pten;Apc* for endometroid cancer and *Arid1a;Pik3ca* for clear cell carcinoma); (ii) syngeneic models, which are based on the implantation of murine OC cells/tumors and are useful for studying anti-tumor immunity and TME interactions (but may greatly differ from humans to directly translate the results), and (iii) xenograft models, which rely on the injection of human OC cell lines or patient-derived cells (PDX) into immunodeficient mice, mimicking the 3D structure of the tumor, its microenvironment and the tumor-TME interactions (110,111,120,121). Depending on the engraftment procedure, xenografts/PDXs are classified as subcutaneous (simple model, tumor growth is limited to the injection site, not very physiologically relevant), intraperitoneal (i.p., injection in the peritoneal cavity, good model for studying cancer cell dissemination and late stage disease with metastasis and ascites formation) or orthotopic-like (injection in the ovarian bursa encapsulating the ovaries, relevant for studying tumor development and disease progression, however humans do not have ovarian bursa) (120). The most commonly used mice strains are nude (T-cell deficient), non-obese diabetic/severe combined immunodeficiency (NOD/SCID; T- and B-cell deficient) and NOD/SCID/IL2Rγ null (NSG; T-, B- and NK cell deficient) (120). As a result of these immunodeficiencies, the above-mentioned models allow the growth and metastasis of human OC tumors, and the formation of ascites as seen in patients; yet fail in recapitulating the interactions between cancer cells and immune cells. PDXs are rather expensive models and thus cannot be used for large-scale HTS; however, the responses to chemotherapy and targeted therapy seen in these PDX models correlate better to those seen in the donor patients (120,121).

3 RESEARCH AIMS

The overall aim of this thesis was to identify mechanisms activated upon tumor evolution and chemotherapy treatment responsible for the aggressiveness and chemoresistance of HGSC.

The specific aims of each paper were:

Paper I: To assess the effect of platinum treatment on EphA2 signaling mechanisms and their clinical implications in HGSC.

Paper II: To investigate *EFNA5* and the corresponding protein ephrinA5 clinically and functionally in HGSC.

Paper III: To understand how the ECM co-evolves with cancer cell functions in HGSC metastasis and chemoresistance.

4 MATERIALS AND METHODS

The methods used in this study are listed in Table 1 and described in detail in the Materials and Methods section of the respective publications. Here, some of the key models and methods are presented and discussed.

Table 1. Methods used in this study and corresponding papers.

Method	**Paper**
3D collagen matrices	I, III
cDNA overexpression	I
Culture of cancer cell lines	I, II, III
Cytotoxicity assay (CellTiter-Glo)	I, III
Chemotherapy and inhibitor treatments	I, III
Extracellular matrix array	III
Image acquisition and analysis	I, II, III
Immunoblotting (IB/WB)	I, II, III
Immunofluorescence (IF)	I, II, III
Immunohistochemistry (IHC)	I, II, III
In vivo ovarian xenograft model	I
Isolation and culture of primary cells	I, II, III
Live cell imaging	III
Polyacrylamide gels (hydrogels)	III
RNA sequencing and bioinformatic analysis	II, III
siRNA knockdown	I, II
Spheroid formation	I
Statistical analysis	I, II, III
Tissue microarrays	I, II

4.1 ISOLATION AND CULTURE OF PRIMARY CELLS

As mentioned previously in this thesis, cancer cell lines represent (quite) homogeneous populations that may greatly differ from the cancer cells found in a tumor. However, cancer cell lines are a simple and fast option/tool to test scientific hypotheses and thus with rational thinking and understanding of their limitations, these cell lines can be routinely utilized in the laboratory. In this study, we used cancer cell lines together with patient-derived cells (also called primary cells) to validate our findings in a more clinically relevant manner. In **Papers I-III**, the patient-derived cells were characterized and utilized freshly or in early passages to avoid phenotypic and genetic changes and the associated clonal selection that occurs upon culture of any heterogenous cell population. In **Paper III**, stable, late-passage, well characterized patient-derived cells (routinely used in our laboratory) were also utilized as examples of cancer or stromal cell populations, including cells from multiple patients to recapitulate the heterogeneity of HGSC.

In **Paper I**, ascites fluid and omental tumor samples from 12 treatment-naïve HGSC patients (and 1 clear cell carcinoma case) collected at the time of primary debulking surgery (PDS) at the Karolinska Hospital (Sweden) were used. The samples used in **Papers II and III** consisted of ascites fluid and tumor samples (including primary tumor, omental, peritoneal and mesenteric metastases) from a total of 63 HGSC patients (18 treatment-naïve patients and 45 patients treated with neoadjuvant chemotherapy) collected at the time of diagnosis or PDS, as well as from post-chemotherapy interval debulking surgery and from palliative punctures at relapse (ascites only). These samples were collected at the Turku University Hospital (Finland). Control omental tissues were obtained from Auria Biobank (Finland). In **Paper III**, samples from 3 patients included in **Paper I** as well as 2 additional samples from treatment-naïve patients taken at PDS at the Karolinska Hospital were used.

In **Papers I-III**, the isolation of HGSC cells from ascites was performed by centrifuging the fluid, lysing the red blood cells from the cell pellet and collecting the multicellular clusters from the resulting cell suspension. For CAF isolation, fresh omental tumors were dissected into small explants and cultured on collagen type I (COL1)-coated dishes in fibroblast medium. The patient-derived cells were characterized by immunofluorescence of epithelial and stromal markers immediately after isolation and during culture. Tumor tissues were formalin-fixed and paraffin-embedded to later be used for IHC or embedded in Tissue-Tek OCT compound and frozen for future IF stainings. In **Papers II and III**, ascites-derived cells and tumor tissues were also used for paired-end RNA-sequencing (**Figure 17**).

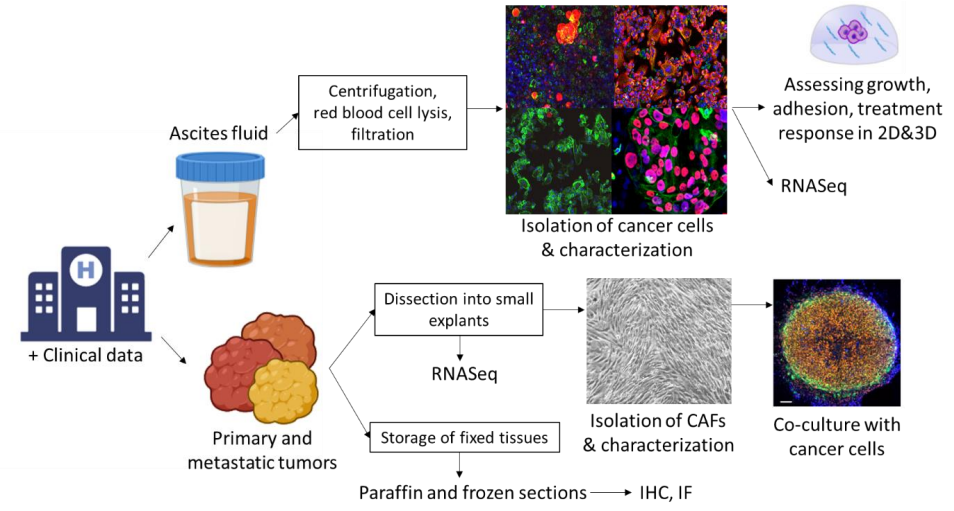


Figure 17. Workflow for processing HGSC patient-derived material and applications in this study.

4.2 HYDROGELS, SPHEROIDS AND 3D CULTURES IN EXTRACELLULAR MATRICES

Cells are most often cultured in 2D cell culture-treated plastic or glass, which are very stiff substrates that do not mimic the tumor stiffness and that can induce morphological changes (resembling EMT) in the epithelial, primary cells upon culture. In addition, cells in 2D cultures are equally exposed to nutrients and oxygen (and anti-cancer drugs when treated *in vitro*) since the barrier effect of the ECM and the gradients found in the tumors (periphery versus core) are missing in this culture setting. In addition, when maintained as mono-cultures, cells lack the interaction with other cell types and with the ECM in their microenvironment. In this study, we used simple 2D mono-cultures in combination with polyacrylamide hydrogels, 3D spheroid cultures (mono- and co-cultures of cancer cells with CAFs) and 3D ECM components to strengthen our findings in more physiologically relevant models.

In **Paper III**, hydrogels functionalized for cell adhesion were used as soft (2 kPa) and stiff (21 kPa) substrates, mimicking the physiological low and high stiffness range (0.40-33.13 kPa) reported in HGSC omental metastatic tissues (86). With these models, we were able to recreate the biomechanical signaling elicited by the increasingly desmoplastic and stiff omental microenvironment.

In **Papers I and III**, freshly isolated HGSC cancer cells growing in suspension as aggregates (named ascites-like cultures or short-term organoids) were embedded in 3D COL1 to mimic the fibrotic, metastatic omentum. This collagen was prepared from rat tail collagen type I after dissolution in 0.3% acetic acid to 4.5 mg/ml stock, dilution to a final concentration of 2.25 mg/ml in 2x MEM on ice and adjustment of the pH to 7.5-8 with sodium hydroxide. In **Paper III**, this collagen was supplemented with 50 µg/ml recombinant human COL6 or FN in certain experiments, and LAM rich hydrogels were prepared by diluting growth factor reduced Matrigel (20.67 mg/ml) with Hank's Balanced Salt Solution to 12 mg/ml on ice. In **Paper I**, besides the above-mentioned ascites-like cultures, mono- and co-culture spheroids were generated by seeding OVCAR8-RFP cells (expressing recombinant histone H2A-red fluorescent protein) or patient-derived HGSC cells alone or in combination with patient-derived CAFs (1:5 ratio) on ultra-low attachment 96-well plates and incubated for 48 h at 37°C before embedding in 3D COL1. These co-culture models increased the complexity of the simple 2D cultures by bringing the cancer-stroma interactions and the corresponding elicited signaling. By embedding cells and preformed spheroids in 3D matrices we further allowed the establishment of the ECM-mediated adhesion signaling as well as provided barrier-like structures for nutrient and anti-cancer drug cell exposure in different applications and assays.

4.3 *IN VIVO* OVARIAN XENOGRAFT MODEL

In **Paper I**, we generated a xenograft model of ovarian cancer metastasis to study the effect of platinum chemotherapy on EphA2 signaling as well as the efficacy of combinatorial RSKi-platinum treatment *in vivo*.

To produce retroviral particles with pMx-*Renilla* luciferase–GFP fusion reporter, 293-GPG cells were transfected with the plasmid using Lipofectamine 2000. The medium was changed 6 h after transfection, and the viral supernatants were collected after 4 d, passed through a 0.45-µm filter and used for transduction of OVCAR4 cells.

To mimic the spread of HGSC cells as clusters in ascites, a mixture of preformed OVCAR4 spheres and single cells in sterile saline was injected i.p. into 6-week old female ICR-SCID C.B-17 (IcrHan®Hsd-Prkdc^{scid}) mice (N = 10), which lack functional B- and T-cells. Tumor growth was followed by noninvasive bioluminescence imaging after i.p. injection of coelenterazine using Xenogen IVIS 100 imaging system. Carboplatin treatment (every 4 d, total of 4 doses) started on week 5 (25 mg/kg i.p. in sterile saline, N = 5). Control group (N = 5) received sterile saline injections. All mice received seven i.p. injections of an inert vehicle (30% PEG400 + 0.5% Tween80 + 5% Propylene glycol) used for an additional RSKi-treated group for which data was excluded from **Paper I** due to extensive inhibitor liver toxicity. Mice were sacrificed on week 7 (**Figure 18**).

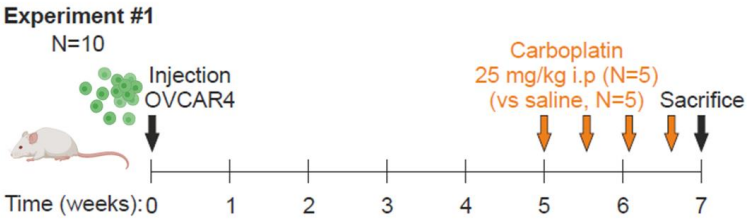


Figure 18. Experimental design #1.

In a separate experiment, the above model (CB17/IcrJcl-Prkdc^{scid}) was used to evaluate the MEKi Trametinib. Carboplatin treatment (every 4 d, total of 3 doses) started on day 25 (15 mg/kg i.p. in sterile saline, N = 4). Trametinib was additionally administered daily (1 mg/kg by oral administration in 4% DMSO + Corn oil, N = 5). Control group (N = 4) received injections of sterile saline and was also subjected to oral administration of 4% DMSO + Corn oil (this vehicle was also administered to carboplatin-treated mice). Mice were sacrificed on day 35 (**Figure 19**).

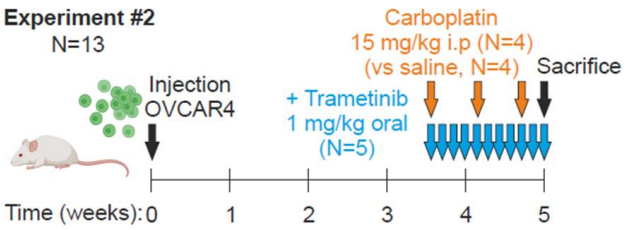


Figure 19. Experimental design #2.

A third independent experiment was conducted (CB17/IcrJcl-Prkdc^{scid}) to investigate the effects of RSKi BI-D1870 in a shorter time scale to mitigate liver toxicity. Carboplatin treatment (every 4 d, total of 2 doses) started on day 31 (15 mg/kg i.p. in sterile saline, N =

4). BI-D1870 was additionally administered on days 35-36 (25 mg/kg i.p. in 30% PEG400 + 0.5% Tween80 + 5% Propylene glycol, N = 5). Control group (N = 4) received injections of both vehicles. Mice were sacrificed on day 36, 5 h after the second injection of RSKi (**Figure 20**).

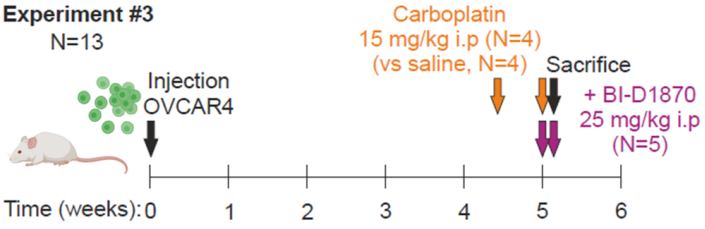


Figure 20. Experimental design #3.

4.4 ETHICAL CONSIDERATIONS

The collection and use of samples from patients with metastatic HGSC in **Paper I** were approved by the Swedish Ethical Review Agency (Etikprövningsmyndigheten: 2012/539-31/1, 2016/1197-31/1, 2016/2060-32). In **Paper II**, the approvals were given by the Ethics Committee of the Hospital District of Southwest Finland (ETMK: ETMK145/1801/2015) and the Hospital District of Helsinki and Uusimaa (HUS: HUS359/2017). The samples used in **Paper III** were under approval from the Ethics Committee of the Hospital District of Southwest Finland (ETMK: ETMK145/1801/2015), the National Supervisory Authority for Welfare and Health in Finland (Valvira) and the Swedish Ethical Review Agency (Etikprövningsmyndigheten: 2016/1197-31/1, 2016/2060-32). The clinical material in Finland was collected under the auspices of Auria Biobank. In all cases, written informed consent was received from participants prior to inclusion in the studies. All experiments were performed according to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

The tumor xenograft experiments in **Paper I** were approved by the National Animal Experiment Board in Finland (ESAVI/8983/04.10.07/2015) and the Animal Experiment Board in Osaka City University in Japan (19001) and performed in compliance with ethical regulations for animal experiments and welfare. In all experiments housing was in individually ventilated cages with 4-5 mice per EU-standard sized cage with aspen-bedding. Nest boxes and material were provided as enrichments. Temperature was set to 20-24°C, relative humidity to 45-65% and light rhythm to 12 h. Welfare was checked daily by the animal facility personnel and/or the study-conducting researchers.

5 RESULTS

5.1 PAPER I: CHEMORESISTANCE VIA RSK-EphA2-GPRC5A

The aim of **Paper I** was to assess the effect of platinum treatment on EphA2 signaling mechanisms and their clinical implications in HGSC. For that we established *ex vivo* cultures from the ascites of treatment-naïve HGSC patients with metastatic disease and embedded them in 3D COL1 to mimic the collagen-rich TME of the metastatic HGSC lesions. Interestingly, EphA2 expression (assessed by IF) increased upon platinum treatment, particularly in the cultures that better resisted to cisplatin in 3D compared to when grown in suspension.

To investigate if platinum chemotherapy could also affect EphA2 signaling duality, we assessed EphA2 phosphorylation at residues Y588 and S897 (by WB), which are respectively associated to ligand-dependent versus ligand-independent activation of the receptor. Platinum treatment of 5 HGSC cell lines and 6 patient-derived HGSC cultures resulted in an EphA2 phosphorylation switch to increased EphA2 pS897/pY588 (ratio of pro-tumorigenic to tumor-suppressive signaling state). To define this effect *in vivo*, we generated OVCAR4 xenografts that recapitulated the human HGSC disease and the response to chemotherapy. In the residual, treatment-escaping metastatic lesions, EphA2 and EphA2-pS897 (assessed by IF) were increased after chemotherapy, suggesting that this signaling switch could be an adaptive resistance mechanism. To address the possible association between EphA2 signaling and platinum resistance, we used the HGSC cell line pair of parental and resistant subline TYK-nu/TYK-nu.R and found that in the resistant cells, EphA2 pS897/pY588 was higher than in the parental cells.

To identify the kinase mediating EphA2-pS897 in these HGSC cells, we used small molecule inhibitors against PKA, Akt and RSK, which had been reported to phosphorylate EphA2 at S897 in other cancers (51–53). The platinum-induced EphA2-pS897 was most effectively inhibited by RSKi (BI-D1870 and the more specific LJH685), which also inhibited RSK1/2, but not the upstream kinase ERK1/2 (both also activated upon platinum treatment). In addition, RSKi increased the canonical EphA2-pY588 in a subset of HGSC cell lines and patient-derived cells [expressing high levels of GPRC5A receptor, an interactor of EphA2 (78)], and resulted in enhanced sensitization to platinum chemotherapy (assessed by cell viability assay and by IF/IHC stainings for apoptosis) *in vitro* (in 2D and 3D mono- and co-cultures with patient-derived CAFs) and *in vivo*. This sensitization was not achieved by either total EphA2 silencing or by MEKi (kinase upstream of ERK1/2-RSK1/2) *in vitro* or *in vivo*, or by RSKi in normal cells, highlighting the specificity of the RSK-EphA2-pS897 mechanism and its inhibition (**Figure 21**).

Overexpression of RSK1 or GPRC5A, and to a lesser extent RSK2 or EphA2, enhanced platinum resistance of HGSC cells. Silencing of RSK1 in platinum treated cells resulted in reduced GPRC5A and increased PARP (assessed by WB), indicating increased apoptosis, whereas silencing of RSK2 reversed the platinum-induced EphA2 phosphorylation switch.

Thus, albeit RSK1 and RSK2 have overlapping functions, in this particular context RSK2 mediated the EphA2 phosphorylation switch and RSK1 controlled the EphA2-GPRC5A co-regulation at the cell membrane to altogether promote cancer cell evasion from platinum-induced apoptosis.

To investigate GPRC5A in a clinical setting, we first compared the expression of this orphan receptor in cancer cells versus stroma (by IF of HGSC tumor tissue sections and WB of patient-derived cells) and found specific GPRC5A expression in the cancer cells. To correlate the expression of this receptor to HGSC clinical outcomes, we evaluated a tissue microarray (TMA) with primary and metastatic tumors and reported the correlation of high GPRC5A expression with worse OS and shorter PFS (the latter in metastatic tumors only), in univariate and multivariate analyses. In addition, GPRC5A expression in primary tumors correlated to incomplete reduction of tumor burden at the end of treatment and to platinum resistance. These results were validated using publicly available data from The Cancer Genome Atlas (TCGA) HGSC dataset (also used to assess the co-expression of EphA2 and GPRC5A) and one independent mRNA cohort (122) (used to assess EphA2+GPRC5A expression as predictor of PFS).

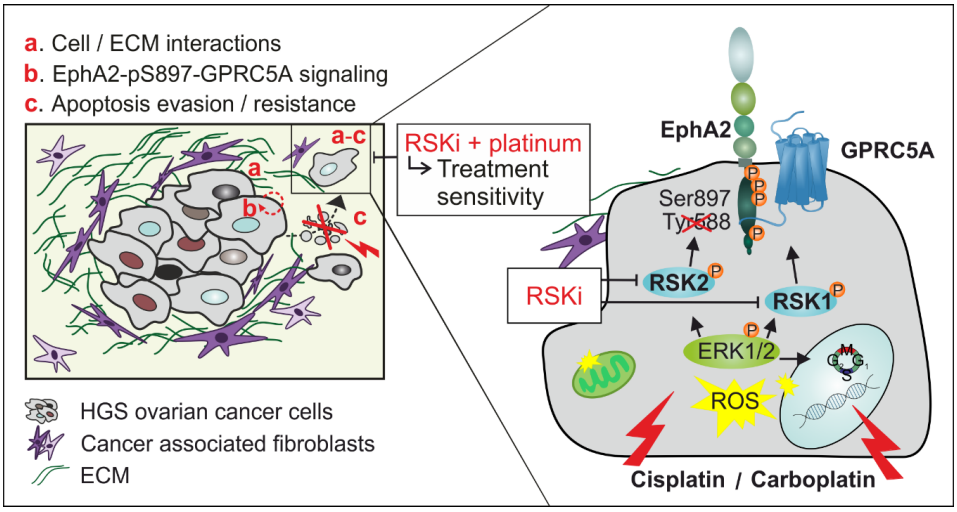


Figure 21. Graphical abstract for Paper I. *From* Adaptive RSK-EphA2-GPRC5A signaling switch triggers chemotherapy resistance in ovarian cancer. *EMBO Mol Med (2020)12:e11177. Moyano-Galceran L, Pietilä EA, Turunen SP et al.* ***a)*** *The interaction between cancer cells and their surrounding ECM, deposited for instance by CAFs, influences EphA2 signaling and chemoresistance in HGSC cells;* ***b)*** *Cellular stress generated by platinum treatment induces ERK1/2-RSK1/2-EphA2-pS897-GPRC5A signaling in the treatment-escaping cancer cells;* ***c)*** *This platinum-induced signaling in HGSC cells promotes apoptosis evasion. Combinatorial treatment with RSK inhibitors and platinum reverses the RSK-EphA2-GPRC5A signaling switch and restores treatment sensitivity in HGSC cells.*

5.2 PAPER II: EphrinA5 NON-CANONICAL SIGNALING FUNCTION AND CLINICAL POTENTIAL IN HGSC

The aim of **Paper II** was to investigate *EFNA5* and the corresponding protein ephrinA5 clinically and functionally in HGSC. First, we performed a systematic analysis of ephrin and Eph receptor mRNA expression association to HGSC clinical outcome using TCGA HGSC dataset and validated our findings with independent datasets from the *curatedOvarianData* database (123). Low *EPHA1*, and high *EFNB2*, *EPHA2*, *EPHB2* and *EPHB4* correlated to poor OS, as previously reported (124–127). From all 8 ephrin ligands and 14 Eph receptor genes, *EFNA5* was one of the three genes with strongest predictive power for poor OS and had the strongest association to poor OS among all ephrin ligands in multivariate analysis.

To further examine ephrinA5 in HGSC, we compared the effects of silencing *EFNA1* (the more studied, canonical EphA2 ligand) and *EFNA5* in HGSC cells and found that in contrast to the EphA2-pY588 reduction upon *EFNA1* knockdown (assessed by WB), silencing *EFNA5* left unaltered or even increased the canonical phosphorylation at Y588 (associated to ligand-dependent activation of EphA2). To better understand EphA2-ephrinA5 signaling, we next treated HGSC cells with recombinant ephrinA1 and ephrinA5 and noticed that opposite to the ephrinA1-mediated EphA2-pY588 and receptor internalization (assessed by WB and IF), ephrinA5 had a limited effect.

To investigate ephrinA5 in a clinical setting, we analyzed the expression of this ligand in cancer cells versus stroma (by IHC of a HGSC tumor tissue section), and in different malignant and benign tumor subtypes of EOC (by IHC of TMAs) and reported higher *EFNA5* expression in cancer cells of the most aggressive subtype: HGSC. Using patient-derived cells from longitudinal ascites samples, we found that ephrinA5 expression (both at the protein and mRNA levels) increased upon HGSC progression to recurrence of the disease (**Figure 22**). Likewise, *EFNB2* expression increased from the time of diagnosis to relapse of the disease, whereas *EFNA3* expression decreased, and *EFNA1*, *EPHA1*, *EPHA2*, *EPHA4*, *EPHB2* and *EPHB4* expression remained unaltered.

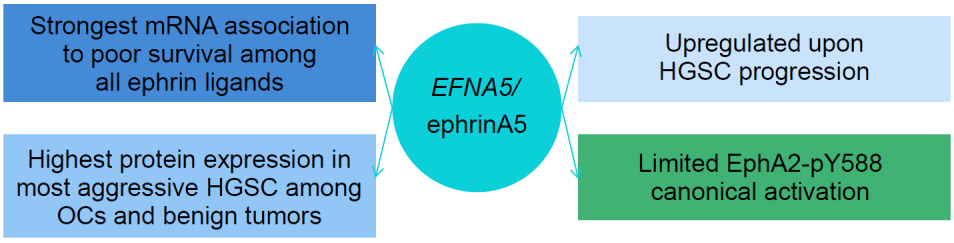


Figure 22. Summary of the findings in Paper II.

5.3 PAPER III: ECM COMPOSITION AND SENSING DRIVE HGSC CHEMORESISTANCE

The aim of **Paper III** was to understand how the ECM co-evolves with cancer cell functions in HGSC metastasis and chemoresistance. For that we performed RNA sequencing in pre- and post-chemotherapy samples from HGSC patients including ascites, primary tumor and metastatic tumors in omentum, peritoneum and mesentery. By analyzing the matrisome of the pre-chemo samples, we found that the expression of the core ECM components (e.g. COL and FN) was higher in the solid tumor tissues compared to the ascites samples and that the metastatic tissues were affected by a desmoplastic reaction (validated by IHC of HGSC tissues), as previously described (83,86). The identified matrisome signatures were distinct in each of the solid tumor tissues.

In addition, by comparing the matrisome of the pre- and post-chemotherapy samples, we found that platinum treatment promoted changes in the core matrisome and ECM-affiliated genes in solid tumors. In contrast, the matrisome-associated soluble factors were more affected by the treatment in the ascites. Enrichment analysis of metastatic versus primary tumor gene sets highlighted pathways associated to fibrotic signaling, ECM alterations and mononuclear leukocyte activity (assessed by Ingenuity Pathway Analysis). In the post-chemo metastatic tumors, the ECM appeared less dense and more fragmented, coincident with the presence of small cells similar to those detected as CD45+ immune cells (assessed by IHC of HGSC tissues), compared to the corresponding pre-chemo tissues. There were also differences in the matrisome of platinum-sensitive [platinum-free interval (PFI) > 6 months] versus resistant (PFI ≤ 6 months) patients, such as the upregulation of *CCL28, MUC4, BGN* and *S100A11* in post-chemo metastatic tumors of resistant patients, overall describing metastasis- and chemotherapy-induced changes in HGSC.

To understand how the changes in the ECM affect the cancer cells, we studied the biomechanical signaling elicited by the tumor stiffness (depending on the extent of collagenous ECM) and the adhesion signaling caused by the ECM composition (influenced by different substrates). Regarding the biomechanical signaling, we found that increasing stiffness (modelled *in vitro* with hydrogels) promoted OC cell proliferation and spreading, FA formation and nuclear localization of the transcriptional co-activator YAP/TAZ, as well as conferred protection against cisplatin-mediated, apoptosis-inducing DNA damage independent of the HR status (assessed by IF and live cell imaging). Concerning the effect of the ECM composition (evaluated with ECM arrays), we reported alterations in OC cell adhesion, migration and chemoresistance caused by different ECM components to be cell line-dependent and influenced by cancer cell growth (assessed by cell count, IF and cell viability assay).

To examine the ECM components of interest in a clinical setting, we analyzed the survival associations for VTN, FN and COL6 in our cohort as well as in TCGA HGSC dataset and found that high *COL6A2*, *COL6A3* and *FN1* associated to poor OS and that an increase in *COL6A1* and *COL6A2* in the solid tumor tissues upon chemotherapy correlated with shorter

PFI and PFS. In addition, chemotherapy induced *COL6A1-6A3* expression in the ascites and solid tumors of platinum-resistant patients. Using a publicly available dataset with epithelial HGSC and stromal samples (128), we found that *COL6* expression was higher in the stroma than in the cancer cells, and confirmed this finding in patient-derived cells and tumor sections (by WB and IHC).

To functionally investigate the putative contribution of COL6 to chemoresistance, we embedded 5 HGSC cell lines and 8 ascites-derived patient cells (4 pre-chemotherapy samples and 4 from relapsed disease) in 3D COL1-based matrices supplemented with COL6 and found that the addition of COL6 further enhanced the chemoresistance of the already resistant cell lines and relapsed patient-derived cells (assessed by cell viability assay). In addition, platinum treatment of these cells promoted FA formation, phosphorylation of myosin light chain and β1-integrin activity (assessed by IF), involved in actomyosin contractility and migration (**Figure 23**).

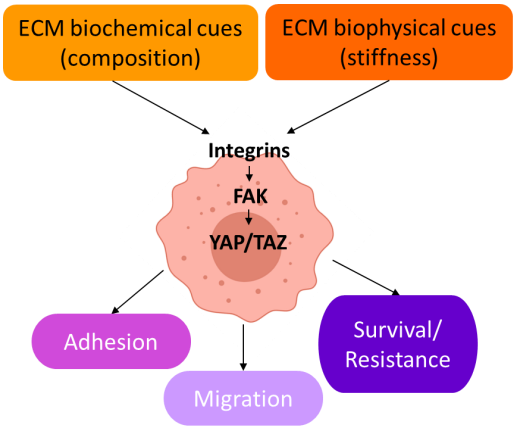


Figure 23. Summary of the findings in Paper III. *HGSC cancer cells sense the biochemical and biomechanical properties of the ECM via integrins (e.g. β1-integrin). Certain ECM components (e.g. COL6) as well as stiff substrates promote the formation of focal adhesions, activation of YAP/TAZ (nuclear translocation) and actomyosin contractility, enhancing cancer cell adhesion, migration and chemoresistance.*

6 DISCUSSION

The dynamic crosstalk between cancer cells and with the TME upon disease evolution and treatment regulates multiple mechanisms with the potential to enhance the metastatic and chemoresistance capabilities of cancer cells (3,4). The work in this thesis has focused on gaining a better understanding on some of the mechanisms activated by cell-cell and cell-ECM communication in the context of HGSC. This disease is diagnosed in advanced stages and, despite being successfully treated with surgery in combination with chemotherapy, often recurs and gradually becomes chemoresistant (87). One of the most pressing clinical challenges in HGSC is thus overcoming therapy resistance.

Our findings from **Paper I** revealed a protective effect of the collagenous ECM against platinum chemotherapy on HGSC cells. In the treatment-escaping cells, EphA2 receptor underwent a phosphorylation shift from the canonical EphA2-Y588 (associated to tumor-suppressive signaling) to the non-canonical EphA2-pS897 (linked to EphA2 ligand-independent activity and tumor-promoting signaling). This EphA2-S897 phosphorylation, which has previously been associated to stemness, invasion, and metastasis in different types of cancer (50), conferred platinum resistance to HGSC cells.

As EphA2 is frequently upregulated in solid malignancies including OC, multiple targeting approaches and clinical trials have been designed and conducted, yet with no clear success (63,64). Our results from **Paper I** indicate that rather than total EphA2 depletion or inhibition, it is the combined reduction of oncogenic S897 phosphorylation with the restoration or even enhancement of tumor-suppressive Y588 phosphorylation that sensitizes HGSC cells to platinum-induced apoptosis. This reversal of serine to tyrosine phosphorylation was achieved by inhibiting RSK, the kinase that mediates EphA2-pS897 in HGSC, but not Akt or PKA, which had been previously reported to phosphorylate EphA2 on S897 in other cancers (51,53). *In vivo*, RSKi also enhanced the platinum-induced apoptosis, consistent with previously reported increased OC cell sensitization to cisplatin treatment and reduction of tumor growth in a lung carcinoma model (129,130). Altogether, all these results emphasized the potential of combinatorial RSKi with platinum chemotherapy as a therapeutic strategy, but the cancer cell specificity and off target effects needed to be assessed. Therefore, we investigated the effect of RSKi on normal cells and CAFs, showing that the enhanced platinum-induced apoptosis was specific to the cancer cells. Despite these promising results, the RSK inhibitors available at the time when we performed the study had suboptimal *in vivo* pharmacokinetic properties and the FDA approved MEK inhibitors (targeting the upstream kinase of RSK), such as Trametinib, with broader effects (including cell cycle regulation) had no additive effect on apoptosis compared to platinum alone. Recently, the first-in-class oral RSKi (PMD-026) has entered a phase 1/1b clinical trial (NCT04115306) and shown initial, encouraging signs of efficacy in patients with metastatic breast cancer (131).

To a certain extent, the expression and functions of GPRC5A orphan receptor, as well as their regulation, have been described. GPRC5A is involved in facilitating HR DNA repair (by

regulating BRCA1), controlling cell proliferation and migration (via EGFR, GSK-3β and β1-integrin signaling), as well as regulating cell survival under hypoxia (via HIF-YAP axis) (132–134). The regulation of GPRC5A activity has been reported in the context of lung cancer, where receptor phosphorylation upon crosstalk with EGFR induces a switch from tumor-suppressive to oncogenic activities (135). However, GPRC5A has only been defined as a tumor suppressor in lung cancer, whereas in most other solid malignancies GPRC5A has been associated to poor clinical outcome and had not been studied in OC before this thesis (79). GPRC5A can be transcriptionally suppressed by *TP53*, which is mutated in essentially all HGSCs (112,136), potentially promoting aberrant GPRC5A expression in these cases. In our study we found that the expression of GPRC5A was exclusive to cancer cells, where this receptor was co-expressed and co-regulated with EphA2.

Our investigation of the complex and context-dependent Eph/ephrin signaling in HGSC continued in **Paper II**, where we focused on the cell-cell communication mediated by EphA2-ephrinA interactions. In this study we reported the differences between the canonical EphA2-ephrinA1 (more studied ligand) versus EphA2-ephrinA5 signaling: The latter can hinder the tumor suppressive signaling normally coupled with degradation of EphA2, thus allowing the oncogenic receptor to function at the HGSC cell membranes. We noticed that EphA2 expression was the highest among all Eph receptors in HGSC cell lines, whereas in HGSC patients EphA2 expression was more variable and/or low. This observation emphasizes the need to take into account the limitations of *in vitro* studies and cautiously distinguish between the function of a protein in a very defined (and sometimes artificial) context versus in complex and dynamic settings (e.g. in a tumor).

Changes in cancer cells and their TME upon chemotherapy treatment have been associated to increased disease aggressiveness, cancer cell stemness and EMT (137,138), but the cues and mechanisms that drive the cancer cell plasticity beyond genetic and epigenetic changes remain incompletely understood. In **Paper III** we investigated the effects of cell-ECM interactions on the cancer cell properties. We found that increasing ECM stiffness enhanced OC cell proliferation and spreading, FA formation and nuclear localization of YAP/TAZ, indicating enhanced adhesion signaling. All these ECM-driven changes in the cancer cells resulted in increased protection against cisplatin-mediated, apoptosis-inducing DNA damage independent of the cancer cell HR status. These results concur with previous findings on ECM stiffness conferring protection against DNA damage, as cells grown on soft compared to stiff substrates accumulate double-strand DNA breaks (DSB) upon inactivation of ubiquitin signaling required for the recruitment of DSB repair factors such as BRCA1 and 53BP1 (139). In **Paper III** we also showed that OC cancer cell adhesion, migration and chemoresistance were altered by diverse ECM components, such as COL6 (which enhanced all these properties). Previously, COL6 has been described as an anti-apoptotic factor and inhibition of COL6A3 cleavage product (endotrophin) sensitized resistant breast cancer cells to chemotherapy (140,141). We found that COL6 is mostly produced by the stroma in the metastatic niche and can support cancer cell growth and apoptosis evasion in early metastasis and later encapsulate the treatment-escaping cells. Altogether, our results emphasize the

importance of the dynamic interconnection between cancer cells and the TME on different stages of the disease as well as on cancer cell phenotypic plasticity.

In metastatic HGSC, the fluid TME created by the accumulation of lymph in the peritoneal cavity (i.e. ascites) is characterized by high levels of soluble factors with potentially immunosuppressive effects secreted largely by immune cells (and to a lesser extent by stromal and normal cells from the peritoneal cavity), whereas the solid TME is shaped by an extensive desmoplastic reaction that transforms the adipocyte-rich omentum into a stiff, collagenous tissue with hypoxic and immune-suppressed areas (86,103). In **Paper III** we performed a comprehensive analysis of the HGSC matrisome and reported the previously unknown matrisome genes that are responsible for the differences between primary tumor and desmoplastic metastases. By comparing gene expression of bulk HGSC tumors (composed of cancer and stromal cells) with ascites (containing only a minor fraction of the ECM-producing stroma), we also found that stromal cells have a greater contribution to ECM deposition than the cancer cells, as previously described in pancreatic carcinoma (142). In addition, we investigated the effect of chemotherapy on the matrisome of both the solid and the fluid TME and reported an enrichment in fibro-inflammatory factors, as well as ECM fragmentation and possibly immune cell infiltration in the solid TME.

One of the reasons why HGSC clinical outcome has not improved faster and further is the lack of biomarkers for diagnosis and prediction of therapy response. Currently, detection of cancer antigen 125 (CA125) in serum is the only generally approved diagnostic marker, which is used in combination with radiologic assessment (88). The predictive and prognostic abilities of CA125 are at debate, as studies have reported opposite findings and CA125 has been detected in benign conditions (143). At present, no predictive or prognostic biomarkers have been standardized for worldwide use and promising biomarkers such as “BRCAness” or functional HRD assays have even now been demoted by the FDA as predictors of PARPi (niraparib) response (87). Our results from **Paper I** indicate that GPRC5A expression (both protein and mRNA) is associated to abysmal HGSC outcome and poor chemotherapy response, and could also be used to stratify unresponsive patients for treatment sensitization through novel combinatorial therapies, such as the RSK1/2-EphA2-pS897 pathway inhibition. GPRC5A has also been suggested as prognostic marker in gastric cancer, hepatocellular carcinoma and esophageal cancer, where high expression of the receptor is associated to poor survival (144–146). In contrast, in lung cancer the survival association is the opposite, as expected for the tumor-suppressive properties of GPRC5A in this cancer type (147). In **Paper II** we showed that ephrinA5 was mostly expressed by cancer cells, that ephrinA5 protein expression was highest in HGSC among different EOC subtypes and benign tissues, and that both *EFNA5*/ephrinA5 expression were greatly increased upon disease progression in patient-derived cancer cells. Thus, we suggest the potential use of ephrinA5 as an indicator of disease subtype and progression stage, besides its relevance as a putative survival biomarker, as previously reported (10–12). In other cancers, such as gastric cancer and B-cell lymphoma, *EFNA5* has also been identified as part of gene expression signatures that predict patient survival (148,149). Finally, in **Paper III** we showed the

survival associations of different ECM components (at the mRNA level) in HGSC, including high *COL6A2*, *COL6A3* as well as *FN1* to poor OS. In addition, we reported that treatment-induced expression changes in *COL6* correlate to shorter PFI and PFS, altogether suggesting the potential of COL6 as a marker for chemotherapy response and survival. In other cancer types, including breast and pancreatic, COL6 has also been associated with tumor progression and poor survival (142,150).

Another unresolved issue to improve oncologic outcome in HGSC is overcoming platinum resistance. Despite successful debulking surgery and patient response to first-line platinum chemotherapy, almost all patients experience recurrence of the disease within 2 years, and either remain platinum-sensitive or become resistant (87). Albeit different strategies are available in the recurrent situation, the rather small benefit of these second-line treatments and the emergence of resistance to both platinum and non-platinum drugs highlights the need to find better first-line therapeutic strategies to avoid the recurrence of the disease (87–89). In **Paper I** we show the benefit of adding a platinum-sensitizing compound (RSKi targeting the platinum-induced, oncogenic EphA2-pS897) to platinum chemotherapy *in vitro* and *in vivo*, and we suggest that similar combinatorial strategies could be used to reduce tumor malignancy and the development of increasingly therapy resistant relapses. Similarly, in **Paper II** we propose the restoration of tumor-suppressive signaling (particularly for EphA2) by modulating the availability of different ligands. In **Paper III**, we discuss new approaches targeting the tumor-TME interactions, ranging from modulation of cancer cell adhesion signaling and normalization/reprogramming of the surrounding stroma to modifications in the ECM (34). Although the switch from a cancer cell centric view towards a whole tumor perspective has already provided insights into the effects of therapies used in the treatment of HGSC on certain stromal cells (151), further understanding of the cancer cell-TME dynamic interactions upon disease evolution and treatment will critically improve the design of future therapies.

7 CONCLUSIONS

The overall aim of this thesis was to identify mechanisms activated upon tumor evolution and chemotherapy treatment responsible for the aggressiveness and chemoresistance of HGSC. The main conclusions can be summarized as follows:

- Platinum chemotherapy induces an oncogenic feedback response via EphA2 tyrosine–serine phosphorylation switch in HGSC cell lines, patient-derived cells and *ex vivo* cultures as well as *in vivo*.
- EphA2-S897 phosphorylation is associated with platinum resistance and mediated by RSK activity. Inhibition of RSK and EphA2-pS897 enhances EphA2-pY588 and platinum-induced apoptosis *in vitro* (particularly in GPRC5A^{high} HGSC cells) and *in vivo*.
- High GPRC5A expression is associated with poor survival and chemotherapy resistance in HGSC patients. GPRC5A and EphA2 are co-expressed and their combined expression is a predictor of PFS in HGSC patients.
- High *EFNA5* expression correlates with poor HGSC patient survival. The encoded protein, ephrinA5, is not a canonical EphA2-pY588 signaling mediator in HGSC cells.
- EphrinA5 expression is higher in cancer cells compared to stroma, strongly associates with the most aggressive subtype of EOC (i.e. HGSC) and increases during disease progression (both protein and mRNA levels) in patient-derived HGSC cells.
- The HGSC matrisome is different in primary tumors, metastases and ascites, although in general core matrisome components are upregulated in the solid tumors while secreted factors are more abundant in the ascites. Chemotherapy treatment induces changes in the HGSC matrisome, which to a certain extent also differs between platinum-sensitive and resistant patients.
- ECM stiffness promotes cell proliferation and spreading, FA formation and nuclear localization of YAP/TAZ, and protects HGSC cells against cisplatin-mediated, apoptosis-inducing DNA damage independently of the cancer cells HR status. Different ECM components alter cell adhesion, migration and chemoresistance in diverse ways.
- COL6 expression (which is higher in the stroma than in the cancer cells) increases upon chemotherapy and is associated with poor HGSC patient survival. *In vitro*, COL6 enhances chemoresistance of resistant HGSC cell lines and patient-derived cells from relapsed disease (grown in 3D) and promotes actomyosin contractility and migration.

8 POINTS OF PERSPECTIVE

In this thesis we have described an adaptive resistance mechanism to platinum-based chemotherapy, reported several putative prognostic and predictive markers, showed the effect that different ECM components and chemotherapy have in cancer cells, and portrayed the matrisome changes caused by disease progression and chemotherapy in HGSC.

In **Papers I and II** we investigated the complex and context-dependent Eph/ephrin signaling in HGSC, and our results have shed light on EphA2 ligand-dependent and independent signaling. However, we should bear in mind that the EphA(/B)-ephrinA(/B) interactions are not exclusive (e.g. ephrinA5-EphB2 interaction occurs in neural development, and ephrinB2-EphA4 in the context of monocyte adhesion to endothelial cells), that signaling in both the cell with the receptor (forward) and the cell with the ligand (reverse) are triggered upon ligand-dependent Eph receptor activation, and that the spatial organization of Eph/ephrins can regulate Eph receptor activity and transcriptional responses (152–155). In addition, Eph receptors can crosstalk with other receptors (maybe even with other Eph receptors) and proteolytic ligand and/or receptor cleavages can occur (6). This complexity may be one of the reasons why therapeutic approaches targeting EphA2 have not yet proven successful (63,64), indicating that further research will be needed to better understand the Eph/ephrin signaling. Similarly, the context-dependent activity and opposite clinical associations of GPRC5A in different cancer types, influenced by the broad range of signaling interactors and by the multiple levels of regulation of this orphan receptor, warrant further research (79). Eventually, a deeper mechanistic understanding of this receptor may provide the assurance to use GPRC5A expression as a clinical biomarker and to even design GPRC5A-targeted therapeutic strategies.

In **Papers I and III** we explored the effects of chemotherapy on the treatment-escaping cancer cells, which are the cause of frequent recurrent disease in HGSC patients. We identified a mechanism of adaptive resistance induced by platinum chemotherapy and proposed a therapeutic approach to restore platinum response. The recent findings with a first-in-class oral RSKi with high selectivity in metastatic breast cancer (131) bring optimism for its potential future use in other cancers such as HGSC, where we have demonstrated the benefit of using RSKi in combination with platinum chemotherapy. In our studies we also uncovered changes in cancer cell properties and adhesion signaling that arise upon the stiffening of the TME and chemotherapy treatment, and that promote chemoresistance. As no breakthroughs have been made towards eradicating disease recurrence and therapy resistance in HGSC patients in the last decades, the need to identify common treatment resistance mechanisms and develop first-line therapeutic strategies that prevent them, rather than trying to reverse the surgery- and platinum-induced tumor aggressiveness in second-line treatments is obvious. Hence, more comprehensive approaches using old and new methods (e.g. single cell omics), complementary models of the disease as well as knowledge from other fields of research will be required.

By understanding HGSC origin and evolution at the biological level (considering the critical impact of the TME), we will be able to uncover diagnostic, prognostic and predictive biomarkers that together with improved therapeutic approaches will ultimately have a remarkable effect on many women's lives.

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