Role of IGF1R in Ovarian Cancer Metastasis

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

Research article

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2. **Deo, Abhilash**, et al. "Subtype specific biomarkers associated with chemoresistance in epithelial ovarian cancer." Indian Journal of Pathology and Microbiology 63.5 (2020): 64.

B. Book Chapter

1. Aniketh Bishnu, **Abhilash Deo**, Ajit Dhadve, Bhushan Thakur, Souvik Mukherjee, Pritha Ray. Non Invasive Imaging in Clinical Oncology: A Testimony of Current Modalities and a Glimpse into the Future.” *Molecular Medicines for Cancer: Concepts and Applications of Nanotechnology*, CRC Press, Taylor & Francis Group, 2018, pp. 217–262.

Conference abstracts

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2. "Understanding the influence of acquired chemoresistance on metastatic properties of ovarian carcinoma cells" at International Congress of Cell Biology, Hyderabad, 2018.
3. "Acquirement of chemoresistance augments metastatic properties in ovarian cancer cells steered by IGF1 receptor" at 38th annual convention of Indian Association for Cancer Research, Chandigarh, 2019.
4. "Chemoresistance progressively enhances the metastatic phenotype of ovarian cancer (OC) cells imparted through IGF1R signalling" at 2nd joint EACR-MRS Conference on Seed and Soil: Mechanisms of Metastasis, Berlin-Germany, 2019.

Abhilash Nitin Deo

DEDICATIONS

न गुरोरधिकं तत्त्वं न गुरोरधिकं तपः।

तत्त्वज्ञानात् परं नास्ति तस्मै श्रीगुरवे नमः।।

Neither is there any reality beyond the Guru,

Nor is there any austerity higher than the Guru,

There is no knowledge of truth beyond what comes from the Guru,

Salutations to that Guru.

I dedicate this thesis to all my Gurus who have been a boundless source of knowledge for me, who instilled in me the thirst for knowledge and made me worthy of what I am today.

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LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
CTC	Circulating tumor cells
DTC	Disseminated tumor cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor family receptors
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGFBP	IGF ligand binding proteins
IR	Insulin receptor
LPA	Lysophosphatidic acid
MET	Mesenchymal to epithelial transition
OC	Ovarian cancer
PDGFR	Platelet derived growth factor receptor
PFI	Platinum free interval
SNP	Single nucleotide polymorphisms
STIC	Serous tubal intraepithelial carcinoma
TNBC	Triple negative breast cancer cells
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

List of Abbreviations

FCM	Fibroblast conditioned medium
IOSE	Immortalized ovarian surface epithelial cells
IP	Intra peritoneal
NSCLC	Non-small cell lung carcinoma
SEM	Standard error mean
TGFR	Transforming growth factor receptor
FAK	Focal adhesion kinases
OS	Overall survival
DFS	Disease-free survival
HGSOC	High grade serous epithelial ovarian cancer

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Chapter 6. Summary and Conclusion

Emergence of chemotolerant distant metastases is a lingering plight of therapeutic strategies aimed toward management of recurrent cancers, which continues to remain without a defined course of therapeutic intervention [147, 420]. As much as 90% of cancer-related deaths are a result of clinical manifestation of metastases or the repercussions of therapies administered for the treatment of these chemoresistant metastases [5, 365]. Since Stephen Paget's "seed and soil" hypothesis (1889), several attempts have been made to delineate the selective forces that drive the colonization of chemoresistant tumor cells at distant sites, such as liver, bone, lungs, and brain, during relapse [163, 476]. Clinical observations documented over decades and recent pre-clinical experimental findings provide multiple lines of compelling evidence suggesting that systemic chemotherapy accelerates the metastatic dissemination of tumor cells to distant sites [312]. Consequently, the remedial effects of chemotherapy efficaciously rendered at the primary tumor site are counteracted by the pro-metastatic effects of chemotherapy that modulate the intrinsic metastatic properties of the "seed" (tumor cells) and the congenial nature of the "soil" (microenvironment of the distant organs), thus maneuvering the metastatic course of the disseminated tumor cells at these vital organs, leading to dismal clinical outcomes. Understanding the molecular networks underlying this complex phenomenon is imperative in order to impede the double-edged sword effects of chemotherapy and develop targeted intervention strategies for cancers exhibiting overwhelming incidence of metastatic recurrence, such as epithelial ovarian cancer (EOC).

Nearly 70% of patients with EOC show relapse after chemotherapy, owing to the acquirement of chemoresistance, wherein the occurrence of extraperitoneal metastasis is commonly observed in the clinics [163, 476, 477]. Although distant metastasis is a well-observed manifestation of recurrent EOC, the organotropic behavior of EOC cells

and the underlying differential metastatic properties that are perturbed due to acquired chemoresistance remain elusive. Moreover, in the clinical setting, certain limitations hinder the comprehensive investigation into the dissemination patterns of chemoresistant EOC cells. First, patients are immediately treated with second/third-line therapy with non-platinum agents after the confirmation of relapse. Second, biopsy of metastatic tissues located in vital organs, like liver and lung, is extremely difficult. Finally, for patients with multiple recurrences and distant metastases, long-term follow-up is challenging because of extremely poor prognoses. Therefore, the development of animal models in which the unusual progression of chemoresistant EOC cells can be tracked at the cellular and molecular levels is an urgent necessity.

Previously, we developed a cisplatin-paclitaxel dual chemoresistant model of A2780 cell line that mimics the distinct phases of acquired chemoresistance from sensitive (A2780) to onset of resistance (early resistant; A2780-dual^{ER}) to extreme resistance (late resistant; A2780-dual^{LR}) [284]. The striking characteristic of this cellular model is the oscillatory pattern of IGF1R expression, which was observed to escalate in the early stage of resistance with increased MAPK/ERK (A2780-dual^{ER}) signaling and declined in the late stage of resistance (A2780-dual^{LR}) with increased PI3K/AKT signaling [284]. Several researchers, including our group, have demonstrated that the inhibition of IGF1R signaling causes resistance reversal in ovarian cancer [271, 284]. Further, IGF1R signaling has also been implicated in the regulation of organ-selective colonization of several cancers to distant sites *in vivo* [281-283]. Since these studies underline the involvement of IGF1R signaling in the establishment of chemoresistance as well as organ-specific metastasis independently, IGF1R signaling may serve as a common regulatory axis that steers the organ-selective dissemination of EOC cells while governing the chemoresistant phenotype of these cells. Moreover, the role of

IGF1R in the propagation of chemoresistant EOC cells remains to be established. Therefore, in this study, we attempted to decipher the molecular mechanisms driving the evolving landscape of chemoresistant metastases regulated through IGF1R signaling using animal models of sensitive and chemoresistant A2780 EOC cells.

In order to investigate the impact of acquired chemoresistance on the organotropic behavior of EOC cells, we monitored the metastatic dissemination of A2780 EOC cells at various stages of chemoresistance using intra-peritoneal and orthotopic xenograft mouse models through non-invasive optical imaging. This *in vivo* mapping revealed a positive correlation between the chemoresistant stage of A2780 EOC cells and their propensity to metastasize distant sites such as kidneys, spleen, and notably lungs, which is a comparatively rare site of metastasis for EOC cells. A comparative analysis of the secondary sites colonized by wild type A2780 EOC cells and their IGF1R-KD counterparts showed that IGF1R signaling is involved in the colonization process of chemoresistant EOC cells at these sites particularly in the late stage (A2780-dual^{LR}). Further, *in vitro* investigation of the key events of metastasis using primary lung fibroblast cells revealed that the inhibition of IGF1R attenuates the adhesion of chemoresistant cells toward resident lung fibroblast cells also abating their responsiveness to the pro-invasive secretory cues of the lung fibroblast cells. Therefore, IGF1R signaling was found to play a central role in the establishment of initial key events of metastasis of chemoresistant EOC cells, involving the stromal components of the microenvironment at distant sites, like lungs. In addition, to compare between the metastatic properties of acquired and intrinsic resistant EOC cells, we also assessed the metastatic properties, governing the early events of metastasis, of the SKOV3 cell line (inherently resistant to cisplatin) and observed that the metastatic properties of SKOV3 cells resemble those of late-stage chemoresistant EOC cells (A2780-dual^{LR}).

Although the late-stage chemoresistant cells (A2780-dual^{LR}) exhibited relatively lower expression of IGF1R than the early stage chemoresistant EOC cells (A2780-dual^{ER}), IGF1R inhibition more severely affected the organ-selective behavior and the underlying metastatic properties of late-stage chemoresistant cells. Intrigued with this observation, which suggested a possibility of involvement of another signaling pathway, we explored the role of α6β4 integrin, which is known to co-operatively regulate IGF1R signaling by forming a tripartite complex with IGF1R in the presence of IGF1 ligand and activating downstream (MAPK/ERK and PI3K/AKT) pathways [415]. α6 integrin–IGF1R co-localization studies performed as a measure of this tripartite complex formation indicated that the IGF1-induced cooperativity between IGF1R and α6 integrin signaling was highest in the late-stage chemoresistant cells (A2780-dual^{LR}), which may have compensated for the low levels of IGF1R in these cells. Destabilization of this interaction through genetic silencing of either IGF1R or α6 integrin significantly reduced attachment of chemoresistant cells (maximum attenuation in the late stage) to primary lung fibroblast cells and decapacitated their invasiveness under co-culture conditions, indicating that IGF1R–integrin dual signaling is instrumental in establishing the key metastatic events with the resident fibroblast cells. On account of the IGF1R-mediated enhanced responsiveness of chemoresistant EOC cells to the pro-metastatic secretory cues of lung fibroblast cells, a comprehensive analysis of the secretome profile of EOC cell–primary lung fibroblast co-culture was undertaken. These data revealed exclusive secretion of S100A4 from late-stage chemoresistant (A2780-dual^{LR}) and SKOV3 cells, which reciprocally aided in the activation of lung fibroblast cells. Further, genetic and pharmacological inhibition of S100A4 revealed that this reciprocal function of S100A4 was vital for the organotropic

nature of chemoresistant EOC cells, underlining their colonization to distant sites, such as lungs, particularly in the late stage of chemoresistance.

In addition to the role of IGF1R in the regulation of differential metastatic properties of chemoresistant EOC cells, we also measured the IGF1R expression in chemo-treated primary and metastatic tumors harvested from a small cohort of EOC patients with high grade serous subtype, in order to assess the prognostic merit of IGF1R. Although statistically insignificant, high levels of IGF1R expression in the primary or metastatic tumor tissues correlated with improved overall (OS) and disease-free survival (DFS). Interestingly, a positive correlation was observed between the expression of IGF1R and that of hCtr1 (a platinum influx drug transporter) in these tissues, which could rationalize its merit in predicting longer survival in these patients.

In conclusion, this study evinced IGF1R–α6 integrin–S100A4 signaling network as a critical determinant for the organ-specific metastases of platinum-taxol resistant EOC cells. Herein, S100A4 was identified as a key molecule that plays a central role in the colonization of chemoresistant cells to distant sites, such as lungs, which can be therapeutically targeted for improving the clinical outcome of recurrent EOC.

Chapter 6. Summary and Conclusion

Thesis summary

Recurrent metastatic epithelial ovarian cancer (EOC) is challenging and associated with treatment limitations, as the mechanisms governing the metastatic behavior of chemoresistant EOC cells remain elusive. Using orthotopic xenograft mouse models of sensitive and acquired platinum-taxol-resistant A2780 EOC cells, we studied the mechanistic role of insulin like growth factor 1 receptor (IGF1R) signaling in the regulation of organ-specific metastasis of EOC cells undergoing acquirement of chemoresistance. Biochemical assays and organ-specific fibroblast-EOC cell co-culture were used to study the differential metastatic characteristics of sensitive vs. chemoresistant EOC cells, and the key molecule/s underlying the organ-specific homing of chemoresistant EOC cells were identified through subtractive LC/MS profiling of the co-culture secretome. The role of the identified molecule was validated through genetic/pharmacologic perturbation experiments. Acquired chemoresistance augmented organotropism of EOC cells and enhanced lung homing, particularly for the late-stage chemoresistant cells, which was abrogated after IGF1R silencing. Escalation of chemoresistance (intrinsic and acquired) conferred EOC cells with higher adhesion toward primary lung fibroblasts, largely governed by the α6 integrin-IGF1R dual signaling axes. Subtractive analysis of the co-culture secretome revealed that interaction with lung fibroblast cells induced the secretion of S100A4 from highly resistant EOC cells, which reciprocally activated lung fibroblasts. Genetic and pharmacologic inhibition of S100A4 significantly lowered distant metastases and completely abrogated lung-tropic nature of late-stage chemoresistant EOC cells. These results indicate that chemoresistance exacerbates organotropic metastasis of EOC cells via the IGF1R-α6 integrin-S100A4 molecular network, of which S100A4 may serve as a potential target for the treatment of recurrent metastatic EOC.

Chapter 1

Introduction and Review of Literature

1.1 An insight into cancer metastasis: A therapeutic perspective

Metastasis is a fundamental trait of neoplastic cells, which allows them to escape the primary site and colonize foreign tissues where, at least initially, the space and nutrition are not constrained in comparison to their site of origin [1]. Almost all cancers, sooner or later, exhibit metastatic features, during the course of progression, that essentially determine their clinical outcome [2]. Despite considerable advances in diagnostics, surgical techniques, and systemic and targeted chemo/radiotherapies, mortality due to metastatic manifestation is observed in about 90% of cancer patients worldwide [3]. The basis for such a high mortality rate can be found in the approach used for the clinical management of recurrent metastatic cancers. First of all, surgical resection of metastases at relapse is rarely performed; second, using histology and toxicity profiles of the cancer, patients are administered with the standardized course of chemo/radiotherapy. The premise underlying this course of treatment is that there exist essential similarities between metastatic and primary tumors, and therefore the primary and metastatic tumors are treated alike [4]. However, a growing body of molecular and pre-clinical evidence is now proving this assumption false [5, 6]. It has come to light that the treatment modalities that suppress tumor growth may in fact ultimately promote metastasis. Such effects of chemo, radio-, and targeted therapies have been identified in several instances [5, 7-10]. Therefore, investigation of not only novel cancer therapies but also their effects on metastasis at the pre-clinical stage is imperative [11]. Vast research over the past four decades has led to an exponential growth in the knowledge regarding this field, reports aimed at resolving the conceptual basis of metastasis have suggested that although the metastatic ability is a central characteristic of cancer cells *per se*, the manifestation of metastasis is the culmination of a series of events that demands molecular and cellular "pliability" from the tumor cells undergoing

this process. Hence, while the tumor intrinsic properties govern the initial dissemination, the progression of metastasis is an evolutionary process that requires adaptation by the tumor cells at each step. Therefore, in-depth understanding of the molecular basis of metastasis is inevitable to devise novel therapeutic strategies for the treatment of recurrent metastatic cancers.

1.1.1 Molecular principles of metastatic traits

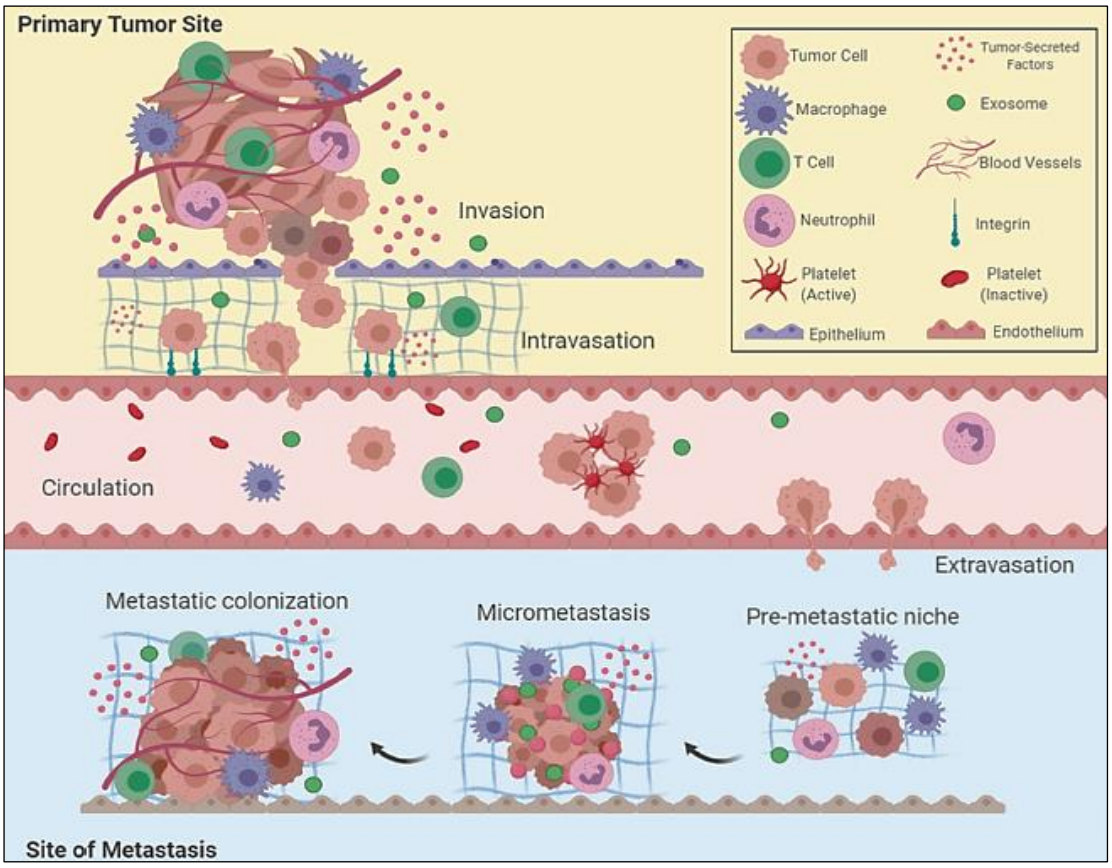

Figure 1. Overview of the metastatic cascade. The major steps of metastasis include local invasion, intravasation into blood or lymphatic circulation, extravasation at a distant site, and colonization through pre-metastatic niche formation.

Figure adapted from Fares, Jawad, et al. "Molecular principles of metastasis: a hallmark of cancer revisited." Signal transduction and targeted therapy 5.1 (2020): 1-17.

Although a large number of tumor cells is exfoliated from the site of origin, only few cells can emerge as metastases and even fewer can be detected and are clinically meaningful. While a previous study demonstrated that as many as 4 x 10^6 tumor cells are disseminated per gram of gross tumor mass, it is estimated that only 0.1% of the cells that gain entry into the circulation remain viable after 24 hours, of which only 0.01% successfully propagate at the secondary site [12, 13]. These findings suggest that, biologically, metastasis is a highly inefficient process and that dissemination from the primary tumor is relatively easier than the establishment of a secondary growth at foreign sites. The understanding of the metastatic principles requires careful considerations of genetic, cellular, and molecular bases owing to the dynamic acclimation of the tumor cells at each step of the metastatic cascade.

A) The genetic landscape: The first trigger

Neoplastic transformation sets in as a result of genetic mutation/s in driver gene/s, after which the tumor cells tend to accumulate genetic aberrations with each cycle of multiplication [14]. Using a genetic model of colorectal carcinoma (CRC), Fearon and Vogelstein (1990) demonstrated that the full-blown stage of CRC is preceded by the development of adenomas, which are benign precursor lesions [15]. This observation intrigued scientists and led to the hypothesis that there may be some specific alterations that confer these cells with the metastatic phenotype. Later, the large scale analysis of high-throughput sequencing of primary and metastatic tumors from CRC patients conducted by Jones et al (2008) revealed that while the transformation of CRC inevitably requires inactivation of the *APC* (Adenomatous polyposis coli) gene, the progression from the benign to malignant stage is achieved through the successive accumulation of other mutations in genes such as *TGF- β, KRAS, SMAD, TP53,* and *PIK3CA* [16]. However, this study revealed no genetic alterations that could be

specifically associated with matched metastases. Interestingly, this study also showed that the duration of progression required for tumor initiation (from the normal cell to precancerous lesion to carcinoma) is as much as 17 years, which is much longer than that required to acquire the ability to metastasize, which is as small as <2 years. This might justify why the original driver mutations are retained in the metastatic tumors. Moreover, comparative analyses of genetic signatures of the primary and metastatic tumors have revealed similar observations for pancreatic, renal, and breast cancers. Metastatic clones in pancreatic cancer may harbor amplifications of *CCNE1*, *KRAS*, and *MYC* genes, which is a common genetic signature of primary tumors of pancreatic cancer [17]. In renal carcinoma metastasis, mutant alleles of the *KDM5C, SETD2*, and *TP53* genes, which are commonly inactivated tumor suppressor genes in the primary tumor, have been identified in their metastatic counterparts [18]. Shah et al., (2009), demonstrated that a ER+ve breast cancer patient who developed metastasis after 9 years of primary tumor debulking harbored the same *ERBB2* driver mutation in the metastases that was originally present in the primary tumor [19]. Another study conducted on breast cancer revealed differential allelic frequencies between the primary and metastatic tumors. However, the affected genes were more or less similar with very few exceptions [20]. Till date, several genes that are associated with the invasiveness of the tumor cells have been identified, which qualifies them as pro-metastatic [21-23]. However, integrative analysis of the genomics data of primary vs. metastatic tumors has revealed some of the genes exhibiting somatic alterations while undergoing the course of metastasis across different cancers, including *TP53, CDKN2A, PTEN, PIK3CA,* and *RB1* [24, 25]. In case of epithelial ovarian cancer, comparative genomic hybridization, microarray, and genomics studies involving high resolution analysis of SNPs (single nucleotide polymorphisms) revealed no discernible differences in the

genetic profiles of primary tumors and their corresponding metastatic counterparts [26-29]. Although these reports suggest that the primary tumors are themselves genetically tuned to acquire metastatic traits, the clonal population of metastases has been seen to harbor only a part of the entire primary tumor genome [30]. Moreover, the individual metastatic tumors in different organs may or may not be identical. For example, while for pancreatic cancer, the lung and peritoneal metastases descended from different clonal populations of the primary tumor, for prostate cancer, the metastatic counterparts in different secondary organs were shown to arise from a single metastatic clone [31, 32]. Altogether these reports suggest that the primary tumors themselves harbor the genetic landscape that governs their metastatic trait.

Although no recurring driver mutations of metastases have been specifically discerned so far, high-throughput transcriptomics and phospho-proteomics studies have identified aberrant overexpression and overactivation of several receptor tyrosine kinase receptors including the epidermal growth factor family receptors (EGFR, HER1, HER2, neu), hepatocyte growth factor receptor (HGFR/c-Met), fibroblast growth factor 1/2 receptor (FGFR1/2), vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR)α, and insulin-like growth factor receptor (IGF1R) in metastatic tumors [33-36]. These receptor tyrosine kinases regulate principle physiological traits like proliferation, metabolism, differentiation, and morphogenesis, under normal conditions and therefore are often exploited by cancer cells to relay sustained oncogenic cues for continuous proliferation and subsequent metastatic progression [37, 38]. Several clinical, pre-clinical, and *in vitro* studies implicate their functions at various stages of metastasis from stromal invasion and migration to angiogenesis and organ-specific colonization. LaTonia et al., (2015) showed that IGF1R signaling promotes epithelial to mesenchymal transition, invasion, and motility

of triple negative breast cancer cells (TNBC) *via* activation of focal adhesion kinases [39]. On the other hand, VEGFR signaling in tumor cells enhances the process of lymph-angiogenesis, thereby promoting their systemic dissemination through newly formed blood or lymphatic vessels [40]. Moreover, tyrosine kinase receptors also play an important role in tumor–stroma interaction. For example, Wu et., (2018) demonstrated that co-culturing of cancer-associated fibroblasts with breast tumor cells leads to reciprocal activation of FGFR1 and EGFR, which mediates their crosstalk that is necessary for shaping a conducive microenvironment for tumor growth [41]. Owing to their role in the tumor–stroma crosstalk, receptor tyrosine kinases also direct the organotropic nature of tumor cells. EGFR signaling has been implicated in increased invasiveness and migration of non-small cell lung cancer and breast cancer and metastasis of breast tumor cells to brain [42-44]. Clinically, while overexpression of IGF1R was observed in the leptomeningeal metastasis of medulloblastoma, that of FGFR1 has been correlated with liver metastasis in colorectal cancer patients [45, 46]. Similarly, overexpression of PDGFR-α has been implicated in the colonization of prostate tumor cells to bones [47]. Taken together, deregulation of receptor tyrosine kinases on account of their aberrant expression or overactivation may impart differential metastatic properties to tumor cells, thereby dictating their course of dissemination.

B) Epithelial to mesenchymal transition (EMT): Preparation for escape

Epithelial cells are tightly cemented with adjacent cells through the extracellular matrix (ECM) in order to maintain tissue integrity and cellular architecture [48]. However, this compact arrangement and the epithelial nature of tumor cells render them immotile. Epithelial tumors, therefore, undergo a dynamic epithelial to mesenchymal transition (EMT), a trans-differentiation process, by virtue of which the tumor cells disengage

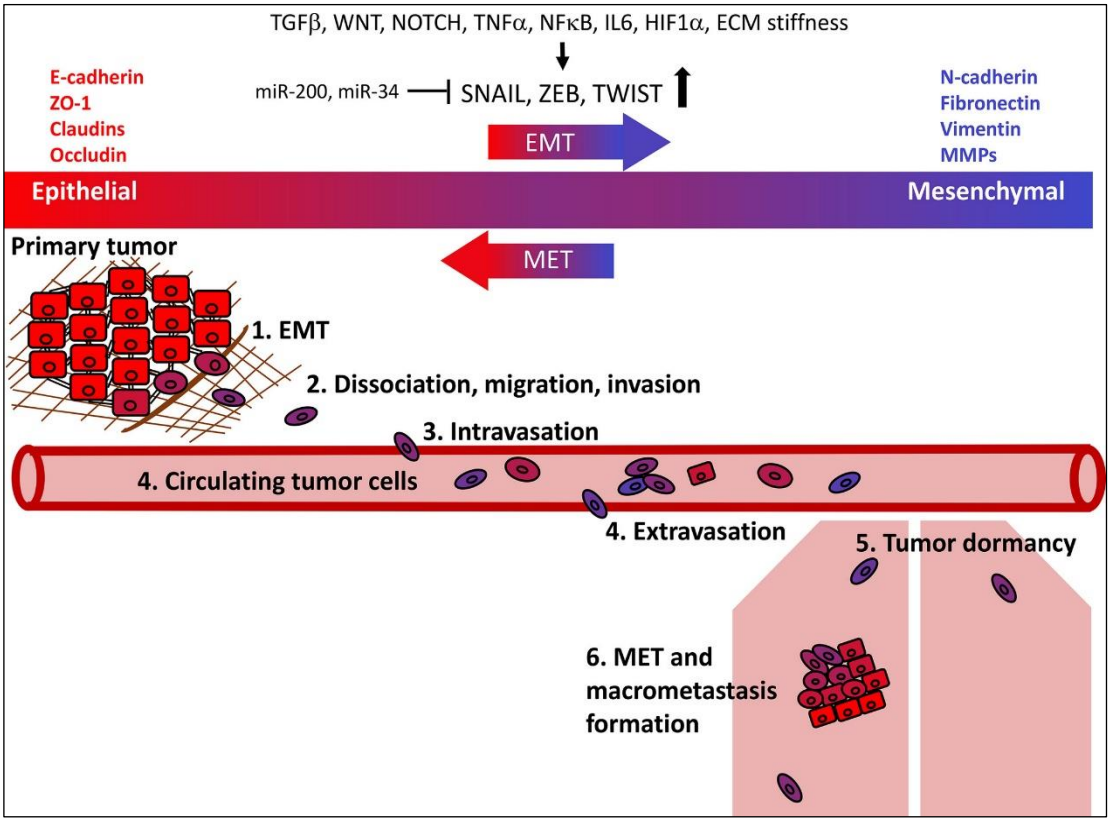

Figure 2. Molecular and cellular processes underlying epithelial to mesenchymal transition (EMT). At the primary site, tumor cells undergo EMT to escape the primary site and migrate into the neighbouring stroma. On encountering the blood capillaries, these cells enter into circulation and get systemically dispersed and thereafter arrested at distant sites, where they undergo MET to resume proliferation and form micro-metastases.

Figure adapted from Yeung, Kay T., and Jing Yang. "Epithelial–mesenchymal transition in tumor metastasis." Molecular oncology 11.1 (2017): 28-39.

themselves from the primary tumor (Figure 2) [49, 50]. Classically, the biochemical and biophysical alterations that manifest because of this transition confer reversible epithelial to mesenchymal plasticity on the tumor cells, which allows their systemic dissemination [51]. This switch is genetically controlled by a complex network of

transcription factors including SNAI1/2, Twist, ZEB1/2, and E12/E47 and is defined on the basis of the conversion of prototypical markers from E- and P-cadherin (marking the epithelial cells) to N-cadherin and vimentin (marking the mesenchymal cells) [52-54] (Figure 2). On reaching the secondary site, EMT reversal is triggered as the tumor cells are now required to anchor the foreign tissue and therefore need to be more stationary, this process is called mesenchymal to epithelial transition (MET) [55] (Figure 2). Recent studies have shown that the EMT process has a spectrum of intermediate stages, thereby refuting the probability of it being a binary transition [56]. Since spotting these highly dynamic transit phases *in vivo* is quite challenging, plasticity during the transit can be easily conceptualized using mathematical models, which are being validated for the biological existence of putative transit stages [57, 58]. Recent studies indicate the correlation of specific stages of the EMT with the specific stages of metastasis, thus locating their existence. The circulating tumor cells (CTCs) with pronounced mesenchymal characteristics show higher entrapment at the secondary site mediated by increased interaction with the resident endothelial and inflammatory cells, which in turn stimulate their proliferation [59]. After the period of initial growth, these early migrants secrete large quantities of cytokines, triggering inflammation and angiogenesis [59]. Experimental evidence suggests that the tumor cells that successfully retain the characteristics of both natures (neither completely epithelial nor completely mesenchymal) are more invasive and display better efficacy in combating the sheer stress during circulation and thus have a better chance to develop metastasis [59]. Moreover, the physical, metabolic, and molecular composition and cellular milieu of the microenvironment at the secondary site can also affect the EMT stages of the disseminated tumor cells (DTCs). While the cancer-associated fibroblasts have been shown to facilitate the migration of tumor cells through alignment of ECM components

like fibronectin, the matrix stiffness, hypoxia, and metabolic stress inducers have been demonstrated to promote EMT by transcriptionally reprogramming the tumor cells (suppression of epithelial genes and activation of mesenchymal genes at the secondary site) [60-62]. During colonization to bone, the tumor cells entrapped in the bone capillaries display the activation of the Wnt signaling pathway that induces MET transition and drives secondary growth [63]. Importantly, lung and pancreatic tumor cells undergoing EMT showed increased tolerance to cytotoxic drugs, suggesting that the genetic reprogramming occurring during metastasis might equip the tumor cells to combat systemic therapy, increasing the chances of recurrent metastasis [64-66].

C) Intravasation and circulation: A perilous journey on systemic highways

After undergoing EMT, the detached tumor cells invade through the basement membrane or adjacent ECM, radiating away from the primary site and entering into the lumen of blood capillaries or the lymphatic channels, a process known as intravasation (Figure 1) [67, 68]. The sophisticated experiments conducted using live cell fluorescence microscopy and artificial tumor microvessel platforms indicated that the tumor cells in the process of intravasation line the blood vessel and disrupt the endothelial layer using the mechanical force generated due to continuous proliferation of tumor cells (an active process) [69]. Further, tumor cells gain entry into circulation by squeezing through small vessel fenestrae (a passive process). However, this process takes a toll on the tumor cells as well. The physical pressure built during this process impacts the nuclear integrity of these cells, causing chromosomal rearrangements, which further augments their metastatic potential [70]. As soon as the tumor cells enter the circulation current (the tumor cells at this point are referred to as circulating tumor cells or CTCs) either as single cells or in clusters, the immediate challenge is to survive the sheer forces generated due to the blood flow. The successful interaction of CTCs

with the endothelial cells of the capillaries eventually decide their viability and, therefore, extravasation capacity at distant sites [71, 72]. The CTC clusters are heterogeneous in nature and may contain non-cancerous cells such as stromal or immune cells that enhance their chances of survival; for example, neutrophils shed along with the CTC clusters prevent activation of leukocytes during circulation [73-77]. Similarly, CTCs having a coating of platelets possess the necessary structural support to counteract the physical stress of circulation [78, 79].

D) Extravasation and colonization: Impinging new territory

The fine capillary beds of distant organs like lungs, liver, bone, and brain entrap these CTCs leading to extravasation, a diapedesis mimicking process facilitating entry of CTCs into the parenchyma of the secondary site (the tumor cells at this point are referred to as disseminated tumor cells or DTCs) (Figure 1) [80-83]. Herein, integrins play a vital role in mediating the anchorage dependent colonization of CTCs [84]. The entrapment of tumor cells and the following extravasation are influenced by the permeability of vasculatures, which greatly vary according to the organ. Organs such as bone and liver that are common metastatic sites for several cancers display high frequency of tumor cell colonization, owing to the vasculature having highly permeable sinusoids as compared to other organs, which have stringent basement membranes and tight barriers requiring biochemical forces for permeation [67]. Moreover, the pharmacological or genetic inhibition of RIP threonine-protein kinase (RIPK)-1, a mediator of necroptosis, significantly abrogated endothelial death, thus attenuating the extravasation of circulating melanoma and Lewis lung carcinoma cells *in vivo*, suggesting that the extravasation process is promoted by the CTC driven necrosis of endothelial cells [85].

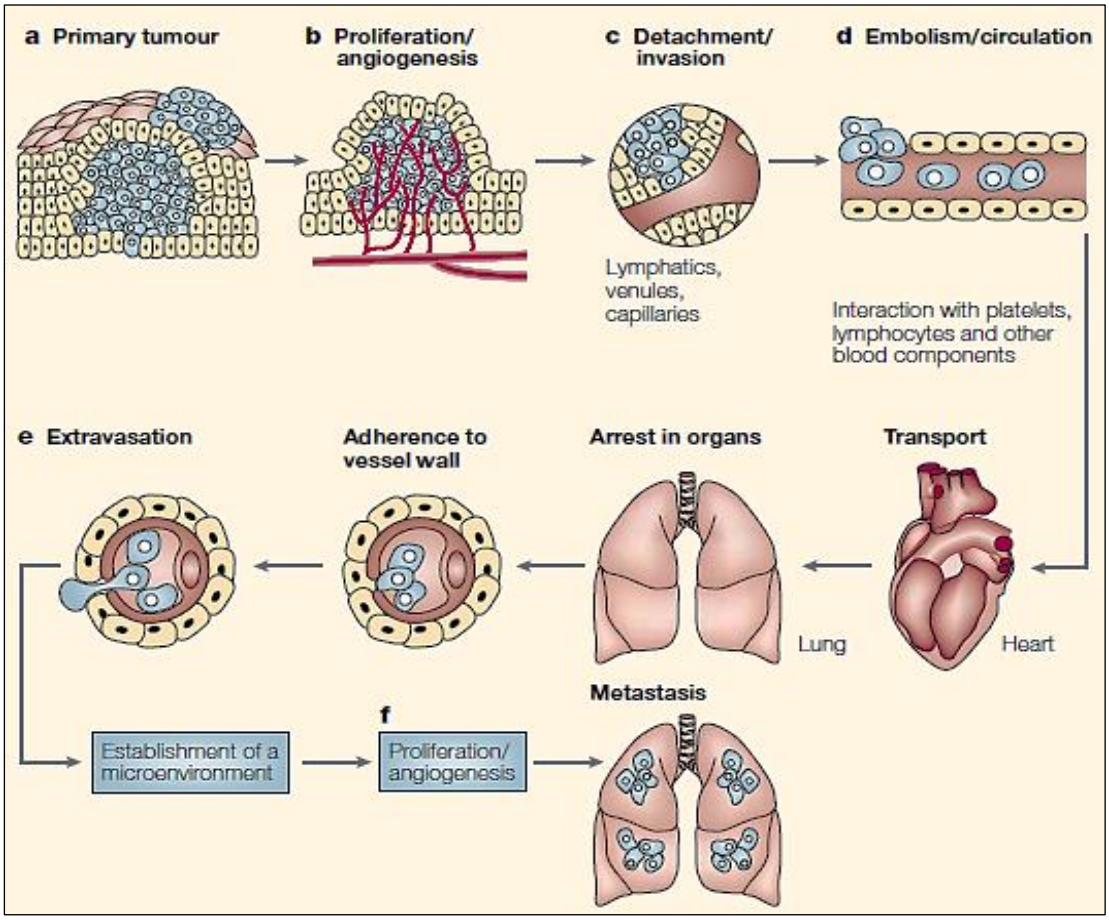

Figure 3. Schematic representation of hematogenous route of metastasis.

Figure adapted from Fidler, Isaiah J. "The pathogenesis of cancer metastasis: the seed and soil hypothesis revisited." Nature reviews cancer 3.6 (2003): 453-458.

1.1.2 Pathogenesis and the routes of metastasis

The commencement of the metastatic cascade precedes unrestrained growth of the neoplastic cells at the primary site after the initial transformation events have occurred (Figure 3a). As the tumor continues to form a sizable mass of approximately 2–3 mm^3 volume, angiogenesis, the extensive development of a new capillary network, is initiated (Figure 3b) [86, 87]. Subsequently, the tumor cells undergo EMT and invade the adjacent stroma, which can be easily detected in the histopathological sections of biopsied primary tumors and is an indicator of the aggressiveness of the cancer type

(Figure 3c) [88]. During this passage, tumor cells/aggregates get embolized into the blood through intravasation as soon as they encounter the blood capillaries that act as a vehicle for systemic dispersal of tumor cells (Figure 3d). The infiltration at the secondary organ is caused either by the physical entrapment of tumor cells due to smaller capillaries or due to their adherence to endothelial cells of the capillaries or sub-endothelial basement membranes (Figure 3). At this stage, the entrapped tumor cells can either proliferate within the capillaries or extravasate into the surrounding tissue parenchyma, forming a discontiguous mass also known as micro-metastases, thus completing the process of metastasis (Figure 3e). However, in order to maintain the equilibrium for continued growth at the secondary site, newly formed micro-metastases have to develop their own vasculature to draw nutrient supply and devise strategies to evade the immune system. This series of events is collectively referred to as hematogenous metastasis, which is a preferred route of dissemination for most epithelial cancers.

Although tumor cells can follow the hematogenous route of dissemination, they can also infiltrate into the lymphatic channels, contingent on their proximity to the primary sites, causing metastasis via lymph. Empirically, clinical evidence over the years has implied that carcinomas (tumors of epithelial origin) predominantly disseminate via the lymphatic route and the tumors having a mesenchymal origin, for instance melanoma, follow the hematogenous route for propagation [89]. This notion, however, is experimentally difficult to prove as the lymphatic and vascular systems are linked through extensive interconnections, making them virtually inseparable [90, 91]. The apparent preference of tumor cells for lymph nodes might be explained on the basis of their structural composition in reference to the blood capillaries. Although tumor vasculature is supposed to be overtly permeable owing to its “leaky” nature, lymphatic

channels are even leakier as they lack the intra-endothelial tight junctions of the capillaries [92, 93]. Further, the absence of the pericytes and smooth muscle layers, which line the endothelial layer in blood vessels, makes lymphatics easier to intrude [93]. Moreover, irrespective of whether they enter a blood vessel or a lymphatic channel, the tumor cells will ultimately enter into venous circulation as the lymph is drained into blood through left or right lymphatic ducts. Sentinel lymph nodes are the most common sites of metastatic dissemination, and the infiltration of tumor cells at these sites is a critical determinant of TNM (T; Primary tumor size; N: Lymph node positivity for tumor cell infiltration and M: Metastatic coverage) staging of the tumor [94], which is an important prognostic marker. For example, around 75% of the patients with prostate cancer showing lymph node metastasis at the time of diagnosis will eventually develop bone metastasis within 5 years, irrespective of the treatment regimen [95]. Although the proclivity of tumor cells for either route may be largely dependent on relative distribution of these vasculatures and their proximity to the primary tumors, active mechanisms have recently been proposed that underlie the choice of preferred route by the tumor cells. Genetic perturbation studies demonstrated that tumor cells with overexpressing vascular endothelial growth factor (VEGF)-C and VEGF-D show increased propensity to drain into lymph nodes through increased interaction with the endothelial cells lining the lymphatic channels [96-101]. Conversely, antibody mediated blockade of VEGF receptor 3 attenuated the lymphatic dissemination of tumor cells in breast and gastric orthotopic mouse models [102-104]. Similarly, when the phosphorylation of PDGF receptor was inhibited in an orthotopic prostate tumor model through administration of imatinib, a significant reduction was observed in the lymphatic spread of tumor cells highlighting the active involvement of PDGFR therein [105, 106].

Besides the overlapping routes of hematogenous and lymphatic dissemination, there exists another equally important route of dissemination called the transcoelomic or the peritoneal route of dissemination, wherein the tumor cells directly sloughed off into the peritoneal cavity are passively carried via the peritoneal fluid. While the dispersal of tumor cells via the first two routes largely depends on the hemato-lymphatic vascularity of the organ and the net supply of blood or lymph that the tumor bearing organ receives, peritoneal dissemination is independent of these factors. The most striking feature of this route is that the tumor cells are carried openly and not through any concealed vesicles, and the metastatic spread is usually confined to the abdomino-pelvic cavity. Although uncommon, this route is a primary mode of metastasis for gastrointestinal and genitourinary epithelial cancers such as pancreatic, gastric, colorectal, and ovarian cancers [107].

1.1.3 Ovarian cancer metastasis: An amalgamation of all three routes

The anatomical position of the ovaries makes them more vulnerable to transcoelomic dissemination as they are surrounded by abdominal organs bearing no physical boundaries (Figure 4). The emerging primary tumor causes rupture of the fibrous ovarian capsule exposing the tumor cells to the peritoneal cavity and circulating peritoneal fluid. The peritoneal fluid present in the abdomen bathes all the abdominal organs, facilitating exchange of various factors, and displays an inherent upward current due to respiratory forces from the pelvis to para-colic gutters to intestinal mesentery to the hemi-diaphragm [108-110]. This circulatory movement (Figure 4) of the fluid causes disposition of tumor cells leading to exfoliation in the form of single cell or multicellular aggregates at various peritoneal organs up the abdomen. While the primary neoplasm actively grows, the continuous shedding of the tumor cells into the peritoneal fluid results in the formation of a reservoir of actively metastasizing tumor

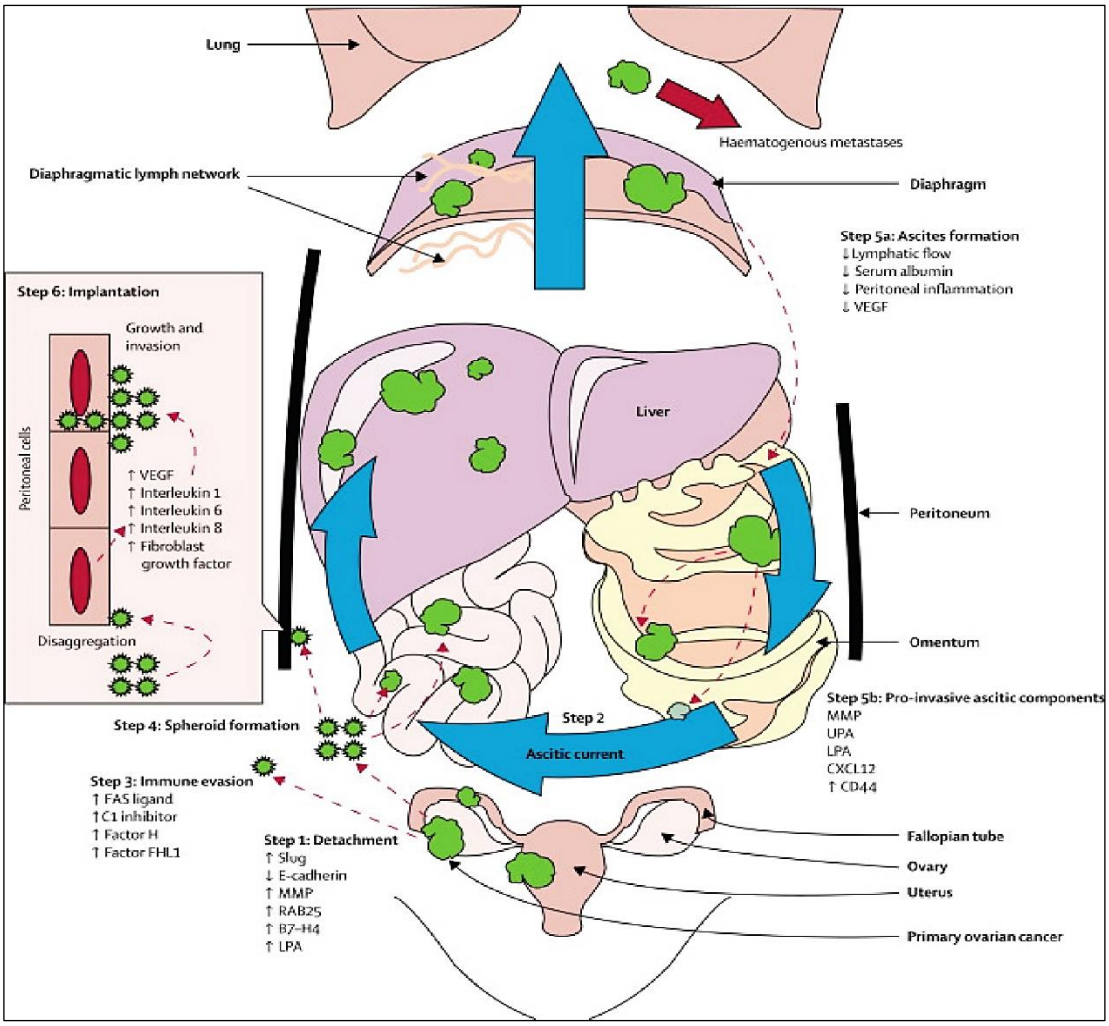

Figure 4. Schematic representation of transcoelomic (peritoneal) route of ovarian cancer metastasis.

Figure adapted from Tan, David SP, Roshan Agarwal, and Stanley B. Kaye. "Mechanisms of transcoelomic metastasis in ovarian cancer." The lancet oncology 7.11 (2006): 925-934.

cells, generating ascites and further worsening the situation. Although the exact etiology of ascites formation is not well established, accumulation of ascites is attributed to the blockade of lymphatic vessel fenestra caused through binding of suspended cells, which prevents re-circulation of the drained lymph [111]. Furthermore, VEGF secreted in the circulation and malignant effusion also draws more and more

peritoneal ascites [112, 113]. MMP2 and MMP9, type IV collagenases released by the tumor stroma, have been shown to stimulate VEGF secretion from the tumor cells, in turn inducing ascites formation [114]. Ascites fluid mainly comprises immune cells, mesenchymal cells, like mesothelial cells and fibroblast cells, and high levels of various secretory factors such as lactate dehydrogenase, VEGF, lysophosphatidic acid (LPA), urokinase-type plasminogen activator, lactate dehydrogenase, MMPs, chemokine ligand 12 (CXCL12), and a broad range of cytokines and chemokines, where the tumor cells float as multicellular spheroids [112, 115-120]. Epithelial cells require anchorage for growth, in the absence of which the cells undergo apoptosis, a phenomenon called anoikis. The spheroids observed in the ascites show extreme resistance to anoikis (anchorage independent growth), which is driven through the overexpression of B7-H4, and RAB25 small GTPase proteins [121, 122]. Experimental evidence suggests that the chemotactic signals relayed through hepatocyte growth factor (HGF) binding and subsequent overexpression of the interacting partners fibronectin and α5β1 integrin results in the homotypic adhesion of suspended cells, thus forming spheroids [123]. These spheroids inherently lack the membrane-bound FAS ligand and therefore can easily escape FAS dependent immune recognition [124]. Another important aspect of these spheroids is their increased chemotolerance, which is achieved at two levels. First, the spherical morphology provides physical protection to the tumor cells residing in the core, whereas overexpression of BCL-xL, an anti-apoptotic protein, prevents the initiation of apoptotic pathways upon exposure to drugs [125]. These spheroids latch onto the peritoneal organs by adhering to the mesothelium that covers all the abdominal organs including the omentum, diaphragm, and bowel serosa [126, 127]. These early adhesion events are mediated by the interaction of the cancer cells with the mesothelial cells lining the mesothelium and the basal membrane underneath composed of type I

and IV collagen, laminin, and fibronectin [128-130]. At this step, the function of matrix metalloproteinases (MMPs) comes into play [131]. MMP2, secreted in response to the initial adhesion, cleaves the fibronectin and vitronectin polymers into smaller fragments engaging integrin receptors (α5β1 and αvβ3), which further strengthens the adhesion [129]. The colonization of DTCs at the peritoneum is successively achieved through proliferation and invasion, wherein the ascites components play a very important role. LPA present in the ascites induces secretion of MMPs and urokinase plasminogen activator, thereby promoting the invasion and motility of tumor cells [132-134]. Moreover, LPA also mediates recruitment of the focal adhesion kinase to the focal adhesion points via ras-MEK kinase 1 pathway, which facilitates tumor cell migration [135]. Similarly, stromal cell-derived factor 1 or CXCL12 chemokine from ascites binds to its receptor CXCR4 present on the ovarian cancer cells, upregulates β1 integrin expression, and promotes motility of these cells [136]. These malignant ascites also stimulate the expression of CD44, an immunoglobulin receptor for hyaluronic acid. Since the peritoneal lining is rich in hyaluronic acid, this induction results in higher adhesion of tumor cells to the peritoneum [137-139]. The implantation of tumor cells on the peritoneum fosters inflammation, causing activation of the resident stromal and immune cells to secrete various cytokines like interlukin-1,-6, and -8, which further induce VEGF secretion from cancer cells, thereby causing more ascites formation, and thus a cycle of unfavorable events commences, creating favorable conditions for the propagation of tumor cells [140, 141]. Moreover, the omentum and subdiaphragmatic surface houses small, white colored, distinct patches called the milky spots, which are nothing but small regions of lymphoid tissues of aggregated mesenchymal and immune cells (macrophages and lymphocytes). These are hotspots for cancer cell attachment as they provide gateways for tumor cells to leach into the lymphatic system [109, 117,

142, 143]. After adhering to the omentum, the physical interaction with adipocytes results in the upregulation of fatty-acid-binding protein 4 (FABP4) and a fatty-acid receptor CD36 in the tumor cells, which facilitates the transfer of lipids from the adipocytes to tumor cells expediting lipolysis in the adipose tissue and oxidation in tumor cells [144, 145]. Thus, the omentum acts as a site that constantly fuels the propagation of tumor cells.

Over the years, the transcoelomic route of metastasis has been traditionally considered as the most prevalent route of ovarian cancer metastasis and therefore is widely accepted [119, 146]. This belief has mainly set in because first, in nearly 85% of the ovarian cancer patients, the disease is confined to the abdominal cavity encompassing the peritoneum, omentum, diaphragm, and pelvic organs, and the incidences of extra-abdominal metastasis are relatively less frequent [147]. Second, when the patients are equipped with perito-venous shunts to alleviate the discomfort caused due to ascites accumulation, the drainage of ascites along with the malignant cells into the circulation does not lead to distant metastasis, and the disease remains confined to the abdomen [148]. However, investigators have also been encouraged to explore the hematogenous route for these patients as a few clinical observations pointed in this direction. First, although spread through surface contact, ovarian cancer cells have been observed to consistently show a predilection for the omentum in clinical as well as pre-clinical models, indicating that this is not entirely a passive process [117]. Second, the disease is found to be disseminated to retroperitoneal and sub-mesothelial regions and also to distant sites at the time of diagnosis indicating distant metastasis [149-151]. Finally, the blood samples of these patients demonstrate the presence of CTCs [152, 153]. In a pioneering study, Pradeep et al. used an elegant parabiosis model to demonstrate that omental metastasis can occur through a blood-borne route in the absence of ascites

[154]. In this study, two female mice were laterally anastomosed to develop shared vasculature allowing the exchange of venous flow. While the intraperitoneal implantation of SKOV3 ovarian cancer cells developed peritoneal metastasis in the host mice with ascites accumulation, the guest mice exhibited omental metastasis in the absence of ascites. Since the assessment of the shared skin section between the host and the guest mice through CD31 (blood vessel marker) and LYVE1 (lymphatic vessel marker) revealed no signs of shared lymphatic networks, the omental dissemination of the tumor cells in the guest mice ought to have happened from the host mouse via a blood-borne route. Further, comparative gene expression profiles of the parental SKOV3 cells and the omental metastatic counterpart revealed the ErbB3-neuregulin1 (NRG1) pathway as a driver for omental metastasis through a blood-borne root. In another study, the hematogenous dissemination of ovarian cancer cells was studied using a xenograft mouse model, wherein the primary tumor cells were subcutaneously co-implanted with infantile hemangioma stem cells to promote angiogenesis at the site, which resulted in overt metastasis to distant sites like lungs [155]. Further, genetic silencing of chemokine receptor CXCR4 led to significant attenuation in the CTCs indicating CXCL12-CXCR4 signaling driven mechanism underlying the hematogenous spread of ovarian cancer cells [156].

Taken together, all these reports clearly indicate that ovarian cancer can exploit all three routes for its dissemination and that the hematogenous route might actually govern the organotropic fate of ovarian cancer metastasis.

1.2 Metastatic organotropism

Despite dissimilarities in the pathogenesis and underlying physical and biological mechanisms, all three routes (hematogenous, lymphatic, and transcoelomic) are

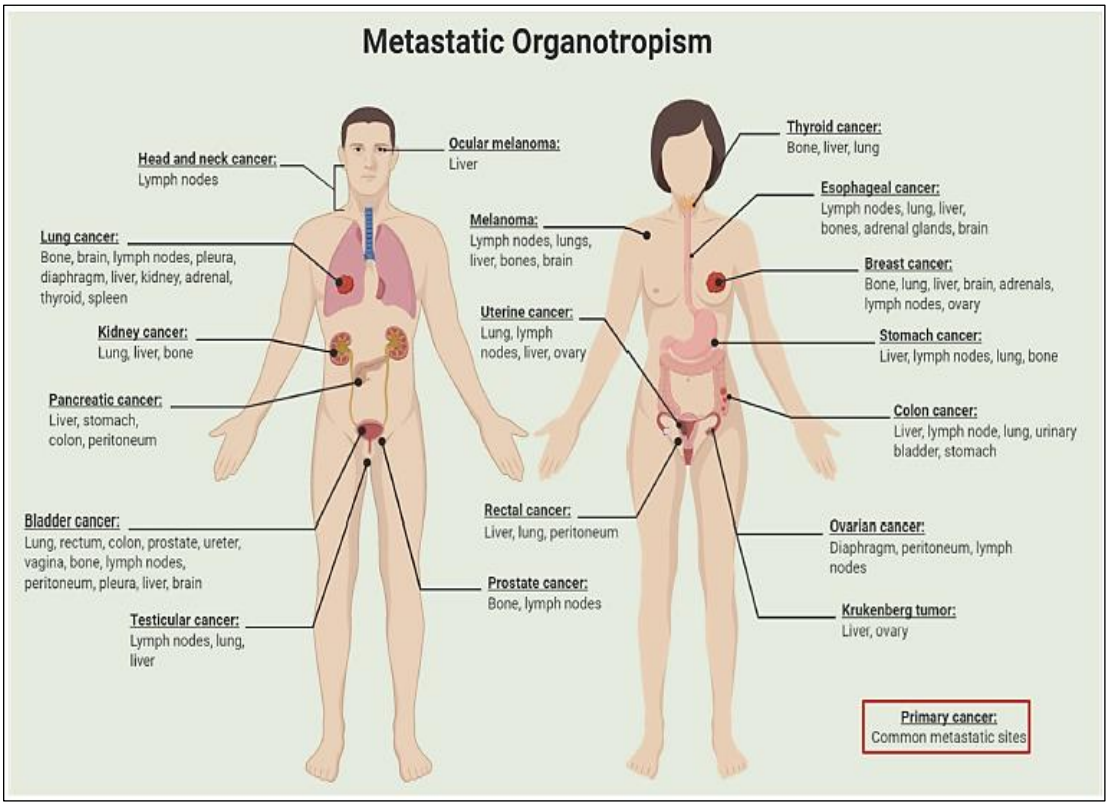

Figure 5. Clinically observed metastatic predilection for different cancers.

Figure adapted from Fares, Jawad, et al. "Molecular principles of metastasis: a hallmark of cancer revisited." Signal transduction and targeted therapy 5.1 (2020): 1-17.

unbiased, in the sense that they mediate random distribution of tumor cells across different body parts that are within their reach. However, clinically, not all types of tumors bear the potential to colonize all the organs. For example, while the preferred metastatic sites for breast cancer are bone, lungs, liver and brain, melanoma shows preferential colonization to lungs, brain, skin, and liver, whereas bone is the primary site for prostate cancer metastasis. Figure 5 and Table 1 enlist organ-selective metastatic sites for different cancers. In case of epithelial ovarian cancer (EOC), the therapy induced chemoresistance is clinically observed to modify the organo-tropic

dissemination of these cells. At the time of diagnosis, in nearly 85% of the patients, the disease is spread within the peritoneal cavity encompassing the peritoneum, omentum, and pelvic organs, whereas, at relapse, tumor cells have been observed to colonize liver parenchyma, spleen, lungs, breast, and even brain, which equates to dismal survival of these patients [147, 157-168]. Therefore, even though the different tumor types harbor specialized capacities to colonize specific organs, the intrinsic ability may be modified by external factors, for instance, therapy in case of ovarian cancer, that modulates their propensity of colonization. Stephan Paget, an English surgeon, documented similar observation way back in the 19th century. After careful observation of more than 900 different primary tumor samples, he observed a discrepancy in the relative blood supply of certain organs and the frequency of metastasis therein indicating that it is a selective process and not a random event (prevailing idea at that time). His extensive analysis led him to propose the *Seed and Soil hypothesis* (1889) which stated that the specific type of tumor cells (equated to seeds) have definite predilection for a milieu of specific secondary sites (equated to soil) [169]. In 1929, this

Cancer Primary Site	Most Common	Second Most Common	Third Most Common
Breast	Lungs	Liver	Bones
Colon	Liver	Peritoneum	Lungs
Kidney	Lungs	Liver	Bones
Lung	Adrenal	Liver	Lungs
Melanoma	Lungs	Skin/muscle	Liver
Ovary	Peritoneum	Liver	Lungs
Pancreas	Liver	Lungs	Peritoneum
Prostate	Bones	Lungs	Liver
Rectum	Liver	Lungs	Adrenal
Stomach	Liver	Peritoneum	Lungs
Thyroid	Lungs	Liver	Bones
Uterus	Liver	Lungs	Peritoneum

Table 1. Most common secondary metastatic sites for common cancer types.

Table adapted from Chapman, Jeremy R., and Stephen V. Lynch. "Donor-transmitted, donor-derived, and de novo cancer after liver transplant." Exp Clin Transplant 12.Suppl 1 (2014): 50-54.

theory was opposed by James Ewing, who proposed that the course of metastasis is dictated by the mechanical factors determined by the anatomy and the vasculature of the organ, a notion that prevailed for another four decades [170]. Finally, in the 1970s, the selective nature of metastasis was accepted. An experimental syngeneic mouse model of metastasis revealed that indeed the arrest of the metastatic cells at distant organs is mediated through the mechanical arrest by the capillary bed, whereas the proliferative cues and successive development of the secondary metastases is greatly influenced by the resident stromal cells [171].

Factors influencing the organotropic nature of the "seed" and congenial nature of the "soil'

A) Lineage

The capacity of tumor cells to colonize a particular organ could be a function of the lineage it belongs to. The migratory and invasive characteristics shown by the tumor cells are not necessarily malignant attributes as many non-cancerous cells perform these functions during development and growth and therefore the normal mechanisms may be manipulated to an advantage by the cancer cells in specific organs. For examples, the mechanism that controls invasion and migration of the mammary epithelial cells during morphogenesis also regulates the hyperplastic growth of these cells [172]. This mechanism can be utilized by luminal progenitor cells to invade and migrate into the lung stroma in the early stages of breast cancer [173]. Tail vein injection of untransformed murine mammary cells has been shown to cause infiltration in the lungs suggesting that the intrinsic invasiveness of the neoplastic cells is reflected by the lineage that they come from [174].

B) Vasculature

Although vascular systems act as pipelines facilitating dissemination of tumor cells to far off sites, the intrinsic properties of the tumor cells might cause differential interaction with the vasculature lining the distant organs, thereby increasing the chances of successful colonization. CD82 or KAil is a membrane glycoprotein with metastatic suppressor functions. Tumor cells having high expression of CD82 adhere to the endothelial cells via Duffy antigen chemokine receptor (DARC) causing senescence of the bound endothelial cells [175].Conversely, the loss of CD82 results in the increased dissemination of tumor cells [175]. Similarly, interaction with platelets can induce the formation of tumor cell aggregates, which shields the tumor cells from host immune surveillance and also assists in their extravasation [176-179]. Further, studies assessing the pattern of tumor infiltration in the brain, lungs, liver, and bone marrow, which are the most common sites of metastatic relapse, show that the hemodynamics of the secondary organs also greatly influences tumor cell infiltration. For example, in colorectal cancer, the permeabilization of the capillary sinusoids and the blood flow entering through the mesenteric circulation favor liver metastasis [180, 181]. In addition, colorectal carcinoma cells have been shown to specifically adhere to the endothelia of the lung and liver, suggesting an involvement of unique molecular interactions that engage tumor cells at these sites [182]. Such specific interactions have been identified for the pre-clinical breast cancer model. Overexpression of metadherin increases propensity of breast cancer cell lines to adhere to pulmonary vasculature, increasing lung metastasis [183]. Similarly, the organ-specific capillary structure can in turn promote infiltration. For instance, bone marrow sinusoids which have highly permissive fenestrae for the passage of hematopoietic cells tend to receive higher load of CTCs than secondary organs [184]. A similar phenomenon is observed in the liver, where the tumor cells can readily transverse the blood capillaries of the hepatic

vasculature [180, 181]. On the contrary, the lung vasculature is more restricted owing to the lining basement membrane and the alveolar cells that surround the endothelia. Therefore, the CTCs have to bypass these protective layers by specialized expression of the mediators like ANGpTl4 that cause trans-endothelial migration [185-188]. Further, the infiltration process may be guided by the specific interaction of α3β1 integrin expressed by the breast cancer cells with laminin 5 present in the basement membrane of the lung capillaries [189]. Recently, several mediators of extravasation have been found to be overexpressed in the primary breast cancer tumor that showed pulmonary recurrence such as epiregulin, pTGS2, and MMP1/2, which also mediate ECM remodeling [186, 190]. Cancer cells have to overcome similar challenges in order to invade the brain parenchyma as they need to surpass the blood–brain barrier formed through tight association of endothelial cells and the foot processes of the astrocytes [191]. As a result, the metastatic tumor cells require highly specialized processes to achieve this task, many of which remain to be elucidated.

C) Metastatic latency

The metastatic latency is determined as the time between the diagnosis of the primary tumor and the first presentation of the metastatic relapse. The extent of metastatic latency and the organ in which the dormant “seeds” are residing have implications in the development of organ-selective metastasis. Some cancers like pancreatic and lung show aggressive growth of micro-metastases as soon as the infiltration of the tumor cells occurs at the secondary site. Therefore, the rate of metastatic relapse for such cancers is overwhelming even if diagnosed at an initial stage. While the patients diagnosed with stage I lung adenocarcinoma exhibit a 5-year recurrence free survival rate of 60%–70%, that observed for breast cancer is as high as 98% [192-194]. Some of the cancers therefore exhibit distant metastasis even if the primary tumor is localized

like small cell lung carcinoma and show concomitant metastatic recurrence at distant sites to bone and brain like in case of lung adenocarcinoma [195, 196]. Melanoma is also another rapidly disseminating cancer that recurs in lungs, bone, liver, and even brain within 2 years of initial diagnosis [197]. A similar trend is observed within different subtypes of tumors. For breast cancer, the basal subtypes show a faster relapse than the luminal ones [198]. This short-lived latency is a consequence of the rapidly acquired metastatic competency which could be an inherent characteristic of the tumor cells or could arise during the early events of transformation, which makes the job easier for the disseminated cells. For instance, the transformation process of melanoma involves genetic alteration of genes that play specific role in the development of the melanocytic lineage [199, 200], and genetic regulators like polycomb chromatin remodeling complexes and microRNA-10b have been associated with metastatic processes [201-205].

On the contrary, some cancer types despite systemic dispersal of the tumor cells remain dormant for quite a long period of time delaying the clinical manifestation of metastasis. For breast cancer the metastatic latency of DTCs has been documented [206, 207]. Patients with the diagnosis of breast cancer without any clinical signs of overt metastases have shown infiltrated cells in their bone marrow [208, 209]. These findings clearly indicate that either these cells lack the capacity to form metastases or the metastatic process is somehow halted. Studies show that the tumor cells exhibit delayed dormancy by maintaining very low doubling time by suppressing Wnt signaling [210]. Additionally, these cells exhibit upregulation of stem cell genes such as SOX2 and SOX9, thereby granting them self-renewal capacity. Further, to minimize encounters with immune surveillance, immune recognition is avoided by downregulating the surface receptors [210]. Similarly, the genetic composition and unfavorable

microenvironment both can contribute to the latency. Studies performed with the pyMT mouse model of polyoma demonstrated that the tumor cells that lack β1 integrin expression fail to sense fibronectin as an environmental cue, exhibiting growth arrest [211]. Moreover, exposure to the foreign environment leads to the mounting of the stress response in the newly settled tumor cells causing an imbalance in the ratio of ERK and p38 MAPK pathways, which intricately control the proliferation (high ERK to p38 ratio) and dormancy state (low ERK to p38 ratio) of the tumor cells [212, 213]. However, the dormant state of the tumor cells can be dramatically flipped to the proliferative state on account of changes in the microenvironment. When DTCs harvested from transgenic BALBNeuT mice, which provided latency to the DTCs infiltrated in the bone marrow, with constitutive activation of the HER2/neu gene, were translated into the bone marrow of the wild type counterpart BALB/c mice, the proliferative state of the tumor cells was reinstated [214]. Most of the metastatic suppressor genes also perform their function by contributing to the metastatic latency such as KISS 1 which maintains the dormancy of the DTCs cells in distant organs by inhibiting the re-initiation of their proliferation [215]. Another example is the GpR56, an orphan G protein-coupled receptor which is hypothesized to mediate cell-cell and cell-matrix adhesion and also interacts with transglutaminase which is a widespread component of the ECM. Overexpression of GpR56 increases this interaction and suppresses the metastatic growth of melanoma cells [216]. The metastatic latency can also prevail if the DTCs fail to trigger the angiogenic processes owing to the presence of anti-angiogenic factors on arrival, like thrombospondin, which starves them by limiting the nutrient supply [217, 218].

D) Organ-selective speciation at the secondary site

For cancers like breast or prostate where the emergence of metastatic relapse is followed by a longer latency period, it is a fairly logical extrapolation to think that the infiltrated cells likely evolve to gain competence to flourish at the secondary site. Since the principle sites like lung, liver, brain, and bone marrow are absolutely heterogeneous in terms of their tensile strength and the cellular and molecular milieu, the DTCs have to acquire specialized functions to combat differential selection pressures offered at these sites. This can also be supported by the fact that these cancers (with light latent period) patients usually show emergence of distant metastasis at a particular site rather than concomitant metastatic development at multiple sites e.g. the metastatic site for prostate cancer at first relapse is usually bone, indicating that not all sites can be metastasized at a time with equal propensity or aggressiveness.

Further, not all CTCs are exfoliated from the primary sites, but the circulatory tumor burden is also contributed by the tumor cells disseminated from the metastatic sites as seen in patients with advanced stage metastatic disease, which has been demonstrated through an experimental *in vivo* mouse model. The tumor cells/cell lines harvested from the pleural effusion of the breast cancer patients give rise to different subpopulations that have differential organotropic capabilities [219]. Strikingly, the metastatic ability of these cells remains unaltered even in the *ex vivo* setting, suggesting that their phenotype has been modulated at the chromosomal level. Owing to the site-specific speciation, the genetic reprogramming of the DTCs at a particular site makes then unfit for colonization at another site. Metastatic breast cancer cells, which gain an ability to colonize bone, are required to produce several osteoclast activating factors such as tumor necrosis factor-α (TNFα), PTHrP, interleukin 6 and 11, and granulocyte macrophage-colony stimulating factor (GM-CSF) in order to create conducible environment for homing [220-223]. However, this expertise is futile at other sites like

lungs or brain where there are no osteoclasts present. Specific mediators for brain or liver colonization largely remain unknown. For brain, it is hypothesized that more than the molecular mediator, it is the cell to cell communication in the tissue parenchyma that governs the development of metastasis owing to several different cell types present in the brain. Consistent with this notion, the metastatic brain samples show overt gliosis (reactive changes in glial cells in response to an insult) and the interaction of tumor cells with the glial cells has been observed to be pro-metastatic *in vitro* [224]. Moreover, neuronal pathways can also be exploited for colonization in the central nervous system exemplified as the breast tumors proximal to the neuronal synapses facilitate brain metastasis via the N-methyl,-D-aspartate receptor pathway [225]. Metastatic cancer cells also communicate with the astrocytes through cGAMP by disrupting the protocadherin 7 mediated assembly of tumor cell–astrocyte gap junctions to activate interferon mediated signaling. These events lead to the activation of STAT1 and NF-κB signaling, which potentiate metastatic tumor development and chemoresistance [226].

E) Therapeutic perturbations

Metastatic recurrence is commonly observed across several epithelial cancer types including head and neck, breast, lung, prostate, and ovarian cancer as a consequence of intrinsic or acquired resistance to chemo/radiotherapies [227-231]. In literature, it is observed that the perturbations that confer drug resistance may also augment the metastatic features of tumor cells making them more competent for organ-selective homing. A subset of EGFR mutant lung tumors that acquire resistance to the targeted drugs erlotinib and gefitinib show amplification of the *MET* gene encoding hepatocyte growth factor receptor (HGFR) which is a tyrosine kinase receptor. The EGFR and HGFR pathways show a crosstalk at the receptor level following redundant signaling

pathways, thereby making the function and therefore dependency on EGFR dispensable [232, 233]. The pro-metastatic gene metadherin, which has been shown to play a key role in lung-tropism of breast cancer cells, also regulates the expression of *MET* gene [183, 234] Therefore, tumor cells having overactivation of MET pathway driven by metadherin may developed chemoresistant lung metastases. Alternatively, systemic therapies can also delay the latency period of tumor cells in specific organs thus favoring the evolution of tumor cells and increasing the chance of recurrence as seen in case of HER2 positive breast cancer patients where there is a rising incidence of brain metastasis post administration of Herceptin which is an anti-HER2 targeted therapy [191, 235]. Moreover, the organ-specific niche can also dilute the cytotoxic effect of therapeutic drugs. It is estimated that nearly 15-20% of breast cancer patients retain DTCc in the bone marrow even after completion of adjuvant or endocrine therapies [236, 237]. Although these studies exemplify the selection of metastatic trait as one of the outcome of systemic therapy, the possibility of the independent emergence aggressive metastasis as feature of tumor intrinsic reprogramming cannot be denied which might be more relevant in terms of rapidly progressing cancers like lung, melanoma and ovary for which the treatment efficacy drastically drops with successive recurrence.

Taken together, there is plethora of variables that influence both seed as well as the soil and it is the cumulative effect that is observed in the form of organ-selective metastasis.

1.3 Ovarian cancer

1.3.1 Epidemiology

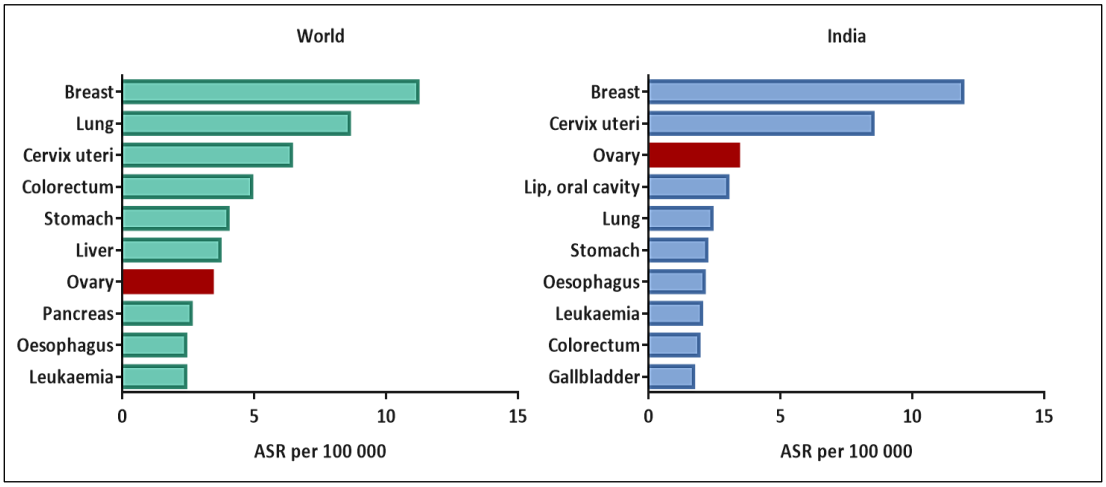

Figure 6. Mortality rates for cancers commonly seen in females worldwide and in India.

Figures adapted from Global Cancer Observatory (https://gco.iarc.fr).

Ovarian cancer (OC) is the third most prevalent cancer among women in India and eighth worldwide; in terms of mortality, it is the third leading cause of cancer-related deaths in India and seventh worldwide (Figure 6) [238-240]. The high mortality rate of ovarian cancer, relative to its incidence rate, makes it the deadliest among the other gynecologic malignancies.

1.3.2 Etiology and origin

The etiology and origin of ovarian cancer have been a long-standing problem and are least understood among the other major cancer types. Over the years, investigators put forth etiology in terms of genetic predisposition, like *BRCA* mutation. However, the exact etiology of this cancer remains largely unknown [241]. Although there exists an ambiguity about the origin of ovarian cancer, there is a consensus about the origin also

known as the tubal paradigm which suggests that the serous ovarian cancer, the major subtype of EOC cancer, originates from the percussion lesions of the epithelium lining the fallopian tubes rather than the surface epithelium of the ovary [242]. This widely accepted paradigm is based on the clinical observations of the fallopian tube showing dysplastic growth from women with *BRCA1* and *BRCA2* germline mutations [243-245]. The immediate precursor lesion is called serous tubal intraepithelial carcinoma (STIC) and is characterized by the continuous lining of non-ciliated tubal epithelial cells exhibiting nuclear atypia, high mitotic and KI-67 indices, and loss of cellular polarity and apoptotic bodies. These cells also show atypical p53 staining indicating genetic alterations (missense or deletion) [245-249]. This paradigm predicts that pre-malignant STIC eventually invades the underlying mucosa and subsequently detaches and spreads to the surfaces of ovaries, peritoneum, bowels, and omentum. Further, the evolution of migrated STIC cells through natural selection equips them to survive and proliferate, which eventually causes tumor formation.

1.3.3 Classification

According to the anatomical site of origin, ovarian cancer can be broadly categorized as epithelial, germ cell, and sex-chord stromal cell cancer [250]. Of these, the epithelial tumors account for around 60% of the total ovarian tumors, whereas almost 90% of the malignant tumors are epithelial in nature [250]. Epithelial tumors are further classified as serous (high grade and low grade) (80-85%), endometrioid (10%), clear cell (5%), mucinous (~3%), and transitional cell carcinoma (extremely rare) [250, 251]. On the basis of genetic, molecular, and histopathological features, EOC cancer has also been characterized into dualistic subtypes as type I and type II tumors (Figure 7) [252]. While the type I tumors have shared lineage with that of the benign neoplasms with borderline features, their presentation usually signifies low grade of the disease [253].

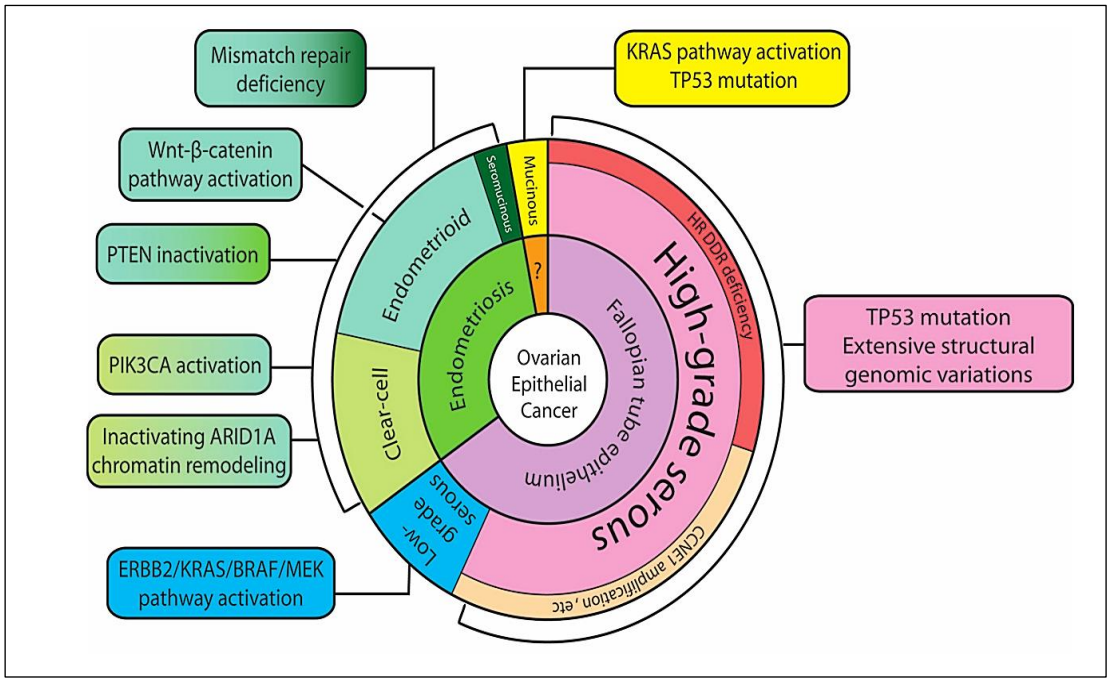

Figure 7. Classification of epithelial ovarian cancer (EOC). There are 5 major histological subtypes: high grade serous, low grade serous, endometrioid, clear cell, and mucinous.

Figure adapted from Shih, Ie-Ming, Yeh Wang, and Tian-Li Wang. "The origin of ovarian cancer species and precancerous landscape." The American Journal of Pathology (2020).

However, these tumors are quite indolent to the standard therapeutic regimen, as observed in the clinical setting [253]. These include low grade serous, low grade endometrioid, mucinous, and clear carcinomas [253]. Type II tumors include high grade serous, high grade endometrioid, and undifferentiated carcinomas accounting for approximately 75% of epithelial carcinomas [253]. Unlike type I tumors, type II tumors are highly aggressive in nature and usually present themselves at an advanced stage; however, they initially respond very well to the standardized treatment [253]. Type I tumors harbor mutations in specific genes like *KRAS, ERBB2, BRAF, CTNNB,* and

PTEN, with a relatively stable genome [254-256]. Further, while activating mutations in PIK3CA are common in them, *TP53* is rarely found to be mutated [257, 258]. On the other hand, the characteristic features of type II tumors is a mutant *TP53* signature, which is observed in more than 90% of the cases [253]. In addition, these tumors also show high chromosomal aberrations and translocation, exhibiting a highly unstable genome. Except for *CCNE1* (cyclin E1) gene, that shows amplification, specific mutations are rarely seen in this category [253].

1.3.4 Diagnosis and staging

The diagnosis of ovarian cancer is made on the basis of radiological findings obtained through transvaginal ultrasound and CT scan and the levels of carbohydrate antigen 125 (CA125) in the serum. Although other serum markers such as human epididymis protein 4 (HE4), carbohydrate antigen 19-9, and carcinoembryonic antigen (CEA) have been proposed, none of them have been able to surpass the credibility of CA125, which therefore remains a gold standard for diagnosis and assessment of the treatment response. These finding are confirmed through cytological examination of the ascites derived tumor cells, as primary tumor biopsy is rarely done in these patients at the time of diagnosis [259].

1.3.5 Front-line treatment

Ovarian cancer is often called the whispering disease as the disease remains unnoticed till the very advanced stage in a majority of the patients. Lack of early detection markers and vague symptoms such as blotting, pain in the lower abdomen, changes in bowel motion, nausea, and weight loss, which can be often mistaken as routine gastrointestinal or gynecological problems, results in the ignorance of early warnings [260]. As a result, >70% of ovarian cancer patients display an immense accumulation of ascites, harboring

actively metastasizing cells, encompassing the entirety of the peritoneal cavity at the time of diagnosis [128]. Surgery and chemotherapy are the cornerstone treatment modalities used in the treatment of ovarian cancer. Owing to the vast spread of the disease at diagnosis, the administration of neo-adjuvant chemotherapy (NACT) followed by radical debulking surgery has been a standard of care in the first-line treatment of EOC [261]. After primary tumor resection, a few cycles of chemotherapy are repeated to achieve minimal residual disease. For NACT, the combination of cisplatin and paclitaxel have remained the choice of drugs since the end of 1990s, as it provides the highest efficacy in limiting the disease [262, 263]. Later, cisplatin was replaced with carboplatin to achieve minimal toxicity [264]. Despite this standardized approach, the median progression-free survival is a mere 12–18 months, with a 5-year survival rate of <35% [261].

1.3.6 Emergence of chemoresistance and challenges in the management

Clinically, it is observed that a majority (~70%) of EOC patients who respond very well to the standard front-line therapy successively acquire chemoresistance to platinum-based drugs, thus developing the recurrent disease which is often metastatic in nature [265]. Based on the platinum free interval (PFI) which is defined as the time from the last cycle of platinum-based chemotherapy to the evidence of disease progression, the patients are categorized as platinum refractory (initial non-responders) (PFI= 0 months), platinum resistant (PFI= <6 months), partially sensitive (PFI = between 6–12 months), and sensitive (PFI = <12 months) (Figure 8) [266]. In case of recurrence, the probability of response to the platinum-based second-line therapy is depended on this

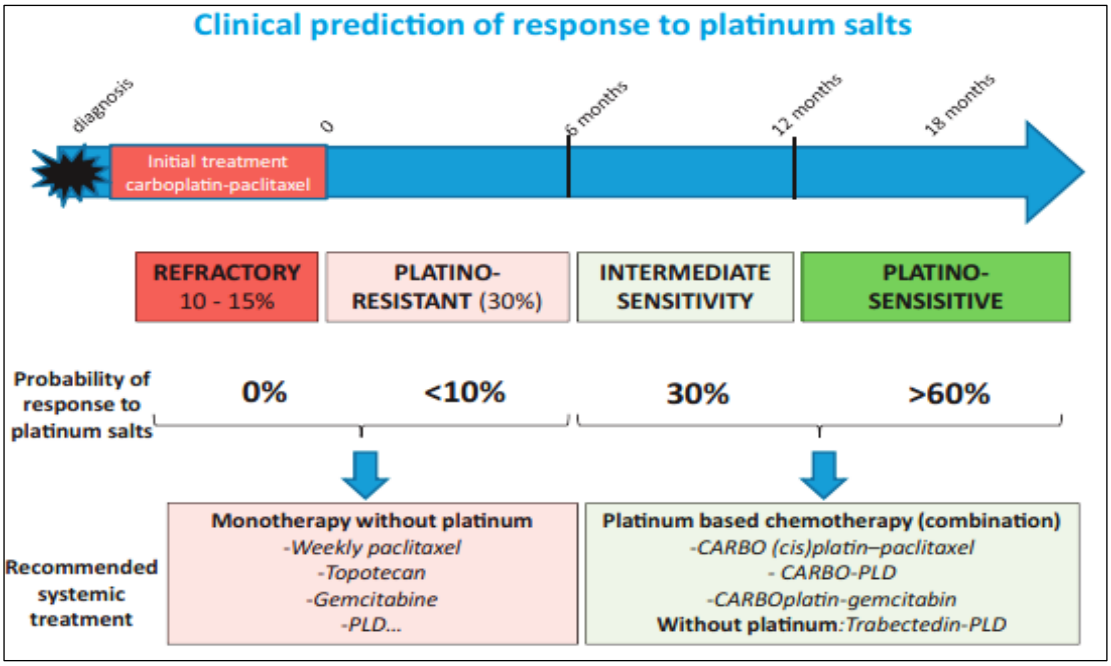

Figure 8. Assessment of platinum sensitivity in EOC patients. The recurrent cases of EOC are categorised as refractory, resistant, and sensitive to platinum drugs based on the platinum free interval (PFI) shown by these patients after the first-line treatment. PFI also helps decide the probable response to platinum-based drugs in the second-line treatment.

Figure adapted from Colombo, Pierre-Emmanuel, et al. "Sensitivity and resistance to treatment in the primary management of epithelial ovarian cancer." Critical reviews in oncology/hematology 89.2 (2014): 207-216.

categorization. While the sensitive relapse patients show >60% response to the platinum-based chemotherapy in the second line, the partially sensitive and resistant patients, at relapse, exhibit 30% and <10% response to similar treatment, respectively (Figure 8) [267]. Unfortunately, the PFI decreases with subsequent rounds of relapse and chemotherapy and the choice of other drugs like doxorubicin or gemcitabine is made on a case by case basis [267]. Currently, anti-VEGF antibody (bevacizumab) and the PARP inhibitor olaparib are the only FDA approved targeted therapies available for

the treatment of advanced stage EOC [268, 269]. However, while anti-VEGF antibody treatment caters to the patients who have symptomatic complications like ascites accumulation, PARP inhibitors are generally prescribed for patients with *BRCA* mutations, who constitute only about 15% of the total EOC patients [269, 270]. Therefore, development of therapeutic intervention strategies for the optimum management of metastatic recurrent EOC is the need of the hour.

In order to develop metastasis directed therapies for recurrent EOC, it is important to identify targetable molecular switches, which primarily regulate the metastatic behavior and organotropic nature of chemoresistant EOC cells. One approach to unveil such potential targets is to investigate the pro-metastatic signaling pathways and underlying molecular networks, which are perturbed owing to chemoresistance, thus imparting differential metastatic properties to chemoresistant EOC cells, regulating their organotropic behavior. As described previously (section 1.1.1 A), EGFR, VEGFR, PDGFR, c-Met/HGFR, and IGF1R are some of the signaling pathways that are commonly implicated in the metastatic progression of tumor cells across several cancers. A growing body of evidence suggests that the IGF1R signaling pathway also plays a role in the regulation of the chemoresistant phenotype of cancer cells [271]. Therefore, it is plausible that the IGF1R signaling axis, perturbed due to acquirement of chemoresistance, may contribute toward the differential metastatic phenotypes of chemoresistant EOC cells and their organotropic behavior.

1.4. IGF1R signaling

IGF1R is a homodimeric tyrosine kinase receptor that plays a critical role in the growth, development, and homeostatic functions in almost every organ of the body, controlling serval functions like proliferation, metabolism, protein synthesis, and inhibition of

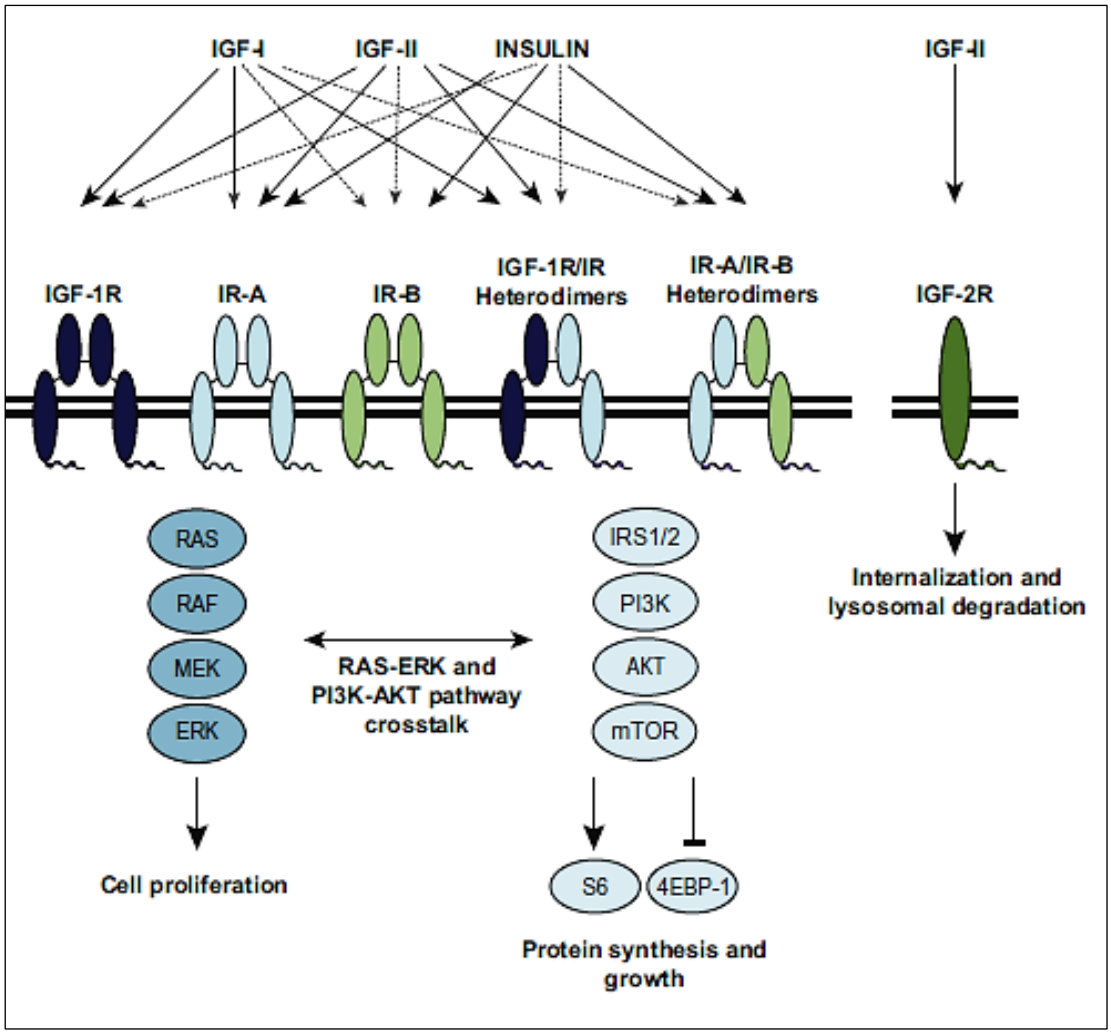

Figure 9. The IGF1R signaling system.

Figure adapted from Liefers-Visser, J. A. L., et al. "IGF system targeted therapy: Therapeutic opportunities for ovarian cancer." Cancer treatment reviews 60 (2017): 90-99.

apoptosis [271]. The IGF1R signaling system is composed of the following ligands: IGF1, IGF2 and insulin, which function by binding to IGF1R (primarily), insulin-like growth factor-2 receptor (IGF2R), and insulin receptor (IR), all belonging to the family of tyrosine kinase receptor and IGF ligand binding proteins (IGFBPs) that control the bioavailability of the ligands [272]. IGF1 binding to IGF1R leads to auto-phosphorylation of IGF1R which in turn phosphorylates insulin receptor substrate 1

(IRS1) thus activating the principle arms of signaling: PI3K/AKT and MEPK/ERK pathways that mediate the above-mentioned functions [272]. Owing to the receptor homology, there exists a great crosstalk between IGF1R and IR signaling (Figure 9). IGF1R can form a functional heterodimer with IR. Similarly, other ligands like IGF2 and insulin can also mediate its activation of IGF1R with much lesser affinity (IGF2 with 20% of the IGF1 affinity; Insulin with <1% affinity of IGF1). In addition, this system also employs IGF2R as a decoy receptor that acts as a sink, thus negatively regulating the function of IGF2 [272].

Implications of IGF1R signaling in chemoresistance and metastasis

Overexpression and hyper activation of IGF1R signaling has been implicated in chemoresistance across various cancers including, ovary, gastric, colon, and prostate cancers among others [271]. A study conducted on a gastric cancer cohort revealed that the patients bearing primary tumors with high IGF1R expression show poorer survival outcomes than those with low expression of IGF1R [273]. In another study of gastric cancer, the co-expression of IGF1R with multi-drug resistance-associated protein 1 (MRP1) prognosticated suboptimal response to adjuvant FOLFOX-4 chemotherapy [274]. Similarly, in HER2 negative breast cancer patients, the decreased IGF1R levels in the tumor tissue were correlated with better response to chemotherapy [275]. Moreover, inhibition of IGF1R signaling facilitated chemo-sensitization of bladder cancer cells that are insensitive to chemotherapy [276]. A similar chemo-sensitizing effect upon IGF1R silencing was observed for prostate cancer as well [277]. In addition to its role in the regulation of chemoresistance, IGF1R signaling has also been implicated in almost all steps of metastasis including adhesion, invasion, motility, EMT, and angiogenesis [278-280]. Notably, IGF1R function has also been reported to drive the colonization of tumor cells at secondary sites. For example, the colonization

of colon cancer cells to liver, osteosarcoma cells to lung or liver, and breast cancer cells to lungs *in vivo* have been observed to be mediated by active functioning of IGF1R [281-283].

Previously, we developed a clinically relevant, acquired chemoresistance (against cisplatin-paclitaxel) model in A2780 (EOC) cells, which showed oscillatory expression of insulin-like growth factor 1 receptor (IGF1R) at different stages of resistance [284]. While the resistant cells at the initiation phase of resistance (early resistant) possess high expressions of IGF1R and MAPK/ERK pathways, cells with highly resistant phenotype (late resistant) bear lower IGF1R expression but highly active PIK3CA-AKT signaling. Moreover, our own group has previously reported that the overexpression at the onset of chemoresistance assists EOC cells in the acquirement of chemoresistance and that the inhibition of IGF1R results in sensitization of chemoresistant EOC cells to cisplatin and paclitaxel [284]. Since all the above-mentioned studies underline the involvement of IGF1R signaling in the establishment of chemoresistance as well as organ-specific metastasis independently, in-depth understanding of how IGF1R signaling regulates differential metastatic properties of EOC cells during acquirement of chemoresistance may provide a foundation for the development of targeted therapies for recurrent EOC.

Rationale

Despite the development of targeted therapies and multidisciplinary approaches over the last decade, neither a significant improvement in the rate of relapse nor a cure for recurrent metastasis have emerged for epithelial ovarian cancer (EOC). A primary reason for this is the inadequate understanding of the cellular and molecular processes governing the distant or organotropic metastasis of chemoresistant ovarian cancer cells. In order to target the metastatic tumor population that is indolent to chemotherapeutic treatment, it is important to decipher the mechanism that imparts differential metastatic properties to chemoresistant EOC cells. Since IGF1R signaling is known to regulate both the chemoresistant and metastatic phenotypes, in-depth understanding of how IGF1R controls organ-specific colonization of chemoresistant EOC cells might help discern the molecular network controlling the metastatic organotropism of chemoresistant EOC cells, thus revealing potential targets for the treatment of metastatic recurrent EOC.

Hypothesis

We hypothesized that IGF1R signaling, perturbed owing to chemoresistance, imparts differential metastatic properties to chemoresistant ovarian cancer cells, leading to metastatic organotropism. The study has been designed with the following key questions:

Key questions

1. Does acquirement of chemoresistance influence the metastatic properties of ovarian cancer cells? If so, which steps of peritoneal metastasis (adhesion, invasion, and organ homing potential) are perturbed due to resistance?
2. Does IGF1R regulate metastatic properties of chemoresistant ovarian cancer cells?

3. What is the status of IGF1R expression in primary and metastatic tumor specimens from advanced stage ovarian carcinoma patients?

Aim

The aim of this study was to investigate the role of IGF1R in the regulation of differential metastatic properties and organotropism of EOC cells during acquirement of chemoresistance.

Based on this aim, the following specific objectives were designed:

Objectives

Objective 1: Monitor the metastatic potential of sensitive vs. early and late resistant cells *in vitro* and *in vivo* using optical imaging

Objective 2: Monitor the effect of IGF1R knock-down on metastatic potential of sensitive v/s early and late resistant cells

Objective 3: Investigate the role of differentially expressed adhesion molecules in driving the events of metastasis

Objective 4: Analyze the IGF1R expression in matched paired primary and omental metastatic tumor specimens obtained from patients with advanced stage ovarian carcinoma

Chapter 2

Non-invasive optical imaging guided monitoring of metastatic potential of sensitive, early and late resistant cells *in vitro* and *in vivo*

2.1 Introduction

Although existing anticancer chemotherapies successfully kill majority of tumor cells on initial administration, acquired resistance is often an inevitable outcome owing to the evolutionary dynamics that govern tumor progression [285]. Thus, therapy resistance and the subsequent emergence of recurrent disease, which is widely observed across several cancers, are the primary confounding factors that foster failure of cancer management strategies [286, 287]. For example, around 30% to 55% of patients with non-small cell lung carcinoma (NSCLC) relapse and eventually succumb to the disease after the initial front-line therapy [288]. Patients suffering from recurrent glioblastoma show a 5-year survival rate of <10%, with a mortality rate close to 100% [289]. Similarly, about 50%-70% of the patients diagnosed with advanced-stage epithelial ovarian carcinoma (EOC) are estimated to relapse within 1 year of the first-line therapy, with a probability of <30% to survive beyond 5 years [290, 291]. To date, an entire array of factors, ranging from genomic alterations at cellular level to perturbations at tumor level involving elements of the tumor microenvironment, have been considered as drivers governing the canonical acquisition of therapy resistance [292, 293]. However, drug tolerance is still believed to be essentially a cellular attribute, because it can be explained through the nature of established underlying molecular mechanisms, such as decreased drug retention achieved though the modulation of drug transporters, drug target alterations, detoxification through enhanced drug metabolism, increased DNA damage repair, and effective manipulation of pro-survival pathways to evade apoptosis [294-297].

Irrespective of the mechanism/s involved, the emergence of therapy resistance stems from the fact that cancer is a heterogeneous disease comprising a pool of subpopulations possessing distinct phenotypic and genotypic features, which also happens to be one of

the major reasons why tumor cells exhibit resistance in the first place [298-303]. This can also be exemplified in case of intrinsic resistance, which is displayed by a relatively more homogenous population and observed to be associated with specific cancer subtypes exhibiting enrichment of distinct driver mutations; for instance, gastric cancer with *HER2* overexpression, triple-negative breast (TNBC) cancer, and clear cell subtype of EOC, are all associated with intrinsic chemoresistance and dismal therapy response [304-306]. Most chemotherapies target tumor cells belonging to a specific proliferation stage; for example, DNA damaging drugs like cisplatin and doxorubicin cause chromosomal damage particularly in the S- and G2/M-phases of the replication cycle, taxol-based drugs like paclitaxel arrest proliferation of tumor cells undergoing mitosis, and topoisomerase inhibitors like topotecan and irinotecan cause cell death during the DNA replication phase [307-311]. Accumulating data suggest that this non-specific targeting approach eventually leads to the selection of a drug tolerant population exhibiting perturbed metastatic characters [312]. For instance, the conventional treatment using letrozole or doxorubicin has been shown to promote the mesenchymal characteristics in the residual population of tumor specimens of breast cancer patients indicating increased epithelial to mesenchymal transition (EMT) [313]. Moreover, pre-clinical studies have also demonstrated that chemotherapy can cause additional genetic modifications leading to altered intracellular signaling pathways in primary tumors accompanied by systemic changes that can promote tumor cell dissemination at distant sites [314, 315]. For example, cisplatin treatment mediated selection of CXCR4+ and CD133+ resistant population of lung cancer has been shown have increased metastatic potential to evade distant sites [316]. On the other hand, distant metastases also exhibit resistance to chemotherapeutic challenge at relapse [312]. Consequently, recurrent metastases arising from chemoresistant cancer cells are

often associated with dismal survival outcomes for epithelial cancers including head and neck, breast, lung, prostate, and ovarian cancer [227-231].

Epithelial ovarian cancer (EOC) is a malignancy that exhibits overwhelming incidence of relapse (~70%), due to acquirement of chemoresistance. At diagnosis, in ~85% of the patients, EOC is confined to the peritoneal cavity [147]. However, at relapse, tumor cells may colonize extra peritoneal organs such as liver parenchyma, spleen, lungs, breast, and brain, which show poor prognosis [147, 157-168]. Although this is a well-observed manifestation of recurrent EOC, whether chemoresistance perturbs the organ-selective dissemination of EOC cells is not clearly established. Uunderstanding the mechanism by which acquired chemoresistance modulates the tumor intrinsic metastatic properties of EOC cells and differentially attunes the tumor cells to the pro-metastatic cues of the microenvironment, thus manoeuvring their organ-selective nature, could be an important step toward determining the molecular orchestration that governs the manifestation of the recurrent metastatic disease. However, this probable association between the acquired chemoresistance and metastatic dissemination of EOC cannot be satisfactorily investigated in the clinical setting, owing to certain limitations. Once relapse is confirmed, the patients are immediately treated with second/third-line therapy with non-platinum agents. Biopsy of the metastatic tissues located in organs like liver, lung, and brain is extremely difficult. Moreover, for patients with multiple recurrences and/ distant metastases, long-term follow up is challenging due to extremely poor prognosis. Therefore, there is a dire need to develop animal models in which the unusual progression of chemoresistant EOC cells can be studied at the cellular and molecular levels. This chapter describes the effect of acquirement of chemoresistance on the metastatic potential and organotropic dissemination of EOC

cells at various stages of resistance, as studied using cellular and mouse models (orthotopic and intraperitoneal) of EOC.

2.2 Methodology

2.2.1 Development of cisplatin-paclitaxel dual chemoresistant model of A2780 EOC cells

The A2780 dual chemoresistant model was established previously in our lab using the modified pulse method. Briefly, the A2780 sensitive cell line (stably expressing Firefly luciferase 2-tdT florescence bifusion reporter) was treated with the IC50 of the cisplatin (50 µg/ml) and paclitaxel (8.5 µg/ml) at 37℃ for 2 hours, after which the cells were allowed to propagate in drug-free medium at 37℃ till they reached optimum confluency. After the first round of treatment, half of the fraction was cryopreserved, while the other half was subsequently treated with the same concentration for another two rounds to achieve stable resistance at that concentration. Next, to escalate resistance, the combination dose was successively increased after every three cycles of treatment, and the cells were treated as described above (the cells were cryopreserved at the end of each cycle). The resistant sublines thus obtained were characterized as early (A2780-dual^{ER}) and late resistant cells (A2780-dual^{LR}) based on their resistance indices (ratio of IC50 of the resistant cells to IC50 of sensitive cells) and viability at the IC50 concentration of the parental cell line, as determined using MTT assay.

Table 1. Characterization of A2780 chemoresistance model.

Sublines	Resistance index	Viability at IC50 concentration of cisplatin and paclitaxel
A2780 (Sensitive)	1	50%
A2780-dual^{ER} (Early resistant)	5	60-70%
A2780-dual^{LR} (Late resistant)	10	>90%

2.2.2 Luciferase-based live cell adhesion assay

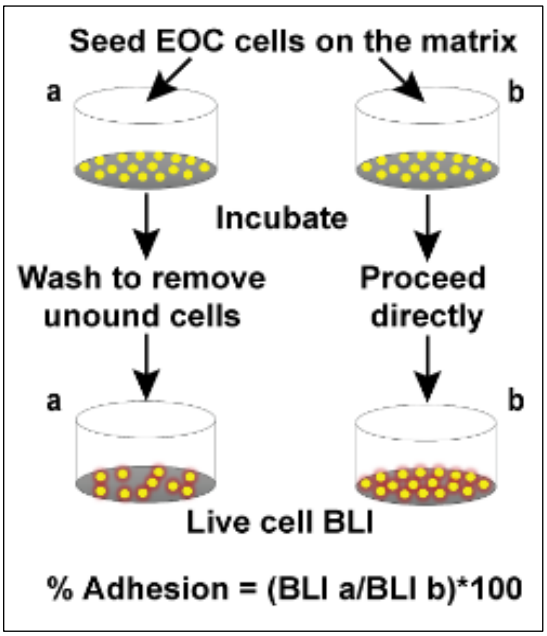

Figure 1. **Adhesion assay.** Schematic representation of indigenously developed luciferase-based adhesion assay with a formula for calculating percentage adhesion (Set a measures the adherent fraction whereas set b: measures the total fraction). BLI stands for bioluminescence imaging.

EOC cells (80,000) were seeded in quadruplicates in two sets (a and b) in 24-well plates coated with Matrigel or a monolayer of NIH3T3 fibroblast cells or immortalized ovarian surface epithelial (IOSE) cells and incubated at 37ºC for 2–16 hours. Next, the total fraction in set (b) was directly imaged without changing the medium, but only the adhered fraction was measured in the set (a) plate after replenishment with fresh medium to remove unbound/loosely bound EOC cells (Figure 1). For imaging, D-luciferin at a final concentration of 1 mg/ml was added to each well and the bioluminescence was measured. The percent adhesion was calculated as follows: [Average radiance of Set (a)/Average radiance of Set (b)] × 100 (Figure 1).

2.2.3 *In vitro* co-culture

Incomplete fibroblast conditioned medium (FCM): NIH3T3 or primary mouse lung fibroblast cells were grown to 70%–80% monolayer confluency, washed twice with PBS, and incubated with incomplete DMEM at 37^0 C for 16 hours. This medium, after

filtering through 0.45-µm filters, was used for co-culture experiments and referred to as FCM throughout the study.

2.2.4 Boyden chamber assay

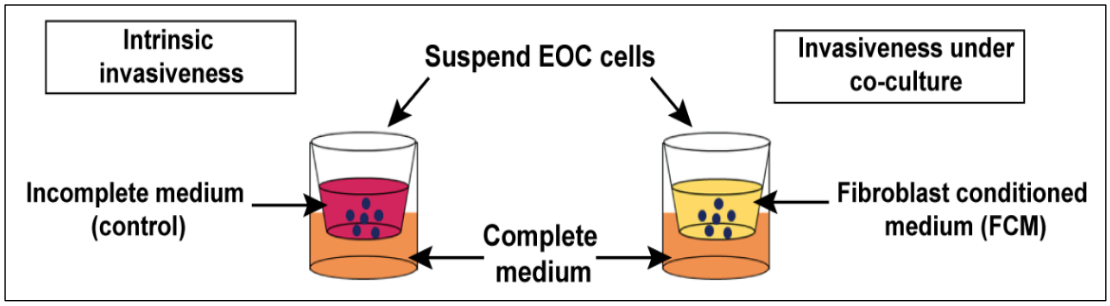

Figure 2. **Boyden chamber assay.** Schematic representation of the classical and modified Boyden Chamber assay devised to evaluate the invasive potential of EOC cells under co-culture condition with fibroblast cells.

EOC cells (50,000) suspended either in incomplete medium (to assess intrinsic invasiveness) or in FCM (to assess invasiveness under co-culture conditions) were seeded in Matrigel-coated Transwell inserts, which were placed in a compatible 24-well plate containing 750 µl of complete medium and incubated at 37^{o}C for 24 hours (Figure 2). After incubation, the inserts were washed with PBS, fixed using 4% paraformaldehyde (37^{o}C for 20 min), and stained with 0.5% crystal violet (room temperature for 5 min). The stained membrane was mounted on a glass slide using DPX and images were acquired for quantification.

2.2.5 Gelatin zymography

Gelatin zymography was performed as described previously with some modifications [317]. Briefly, FCM/co-culture supernatants of EOC cells were concentrated using SpeedVac, dissolved in laemmli buffer, and resolved using 8% polyacrylamide gel containing 10% gelatin. The gel was washed with renaturation buffer and incubated in

reaction buffer until the zone of clearance was clearly visible. The zymogram was developed by staining the gel with coommassie brilliant blue and images were acquired. When compared between co-culture medium and FCM alone, the relative activities of MMPs were calculated as follows: (Intensity of proteolytic zone of co-culture medium/ Intensity of proteolytic zone of FCM alone) × 100.

2.2.6 *In vivo* metastatic mouse models

An intra peritoneal (IP) xenograft mouse model was established by injecting 1 million EOC cells through the intra peritoneal route. On the last day of imaging, mice were injected with D-luciferin, sacrificed, and the organs were examined for metastatic nodules. The animals were followed up for tumor growth using non-invasive bioluminescence imaging for 6 weeks. An orthotopic xenograft mouse model was established by injecting 0.5 million EOC cells suspended in 9 µl of PBS in the ovarian bursa of NOD/SCID mice (4-6 weeks old), and tumor growth (primary and metastatic) was monitored using non-invasive bioluminescence imaging for 30 days as mentioned for the IP model. For orthotopic cohort analysis, only the mice with primary ovarian tumors along with metastasis were included in the study. Metastatic organs that showed high bioluminescence were fixed in paraformaldehyde and embedded in paraffin for sectioning. The presence of primary and metastatic tumors was confirmed on the basis of H&E sections of organs by an experienced pathologist.

2.2.7 Statistical analysis

Data presented in this study represent the mean ± standard error mean (SEM) of at least three independent biological replicates. The statistical significance was analyzed using unpaired Student's *t*-test, and *P*-values < 0.05 were considered significant. The *in vivo* metastatic trend was determined using the chi-square test.

2.3 Results

2.3.1 Chemoresistant EOC cells exhibit expansive metastatic spread with distant colonization

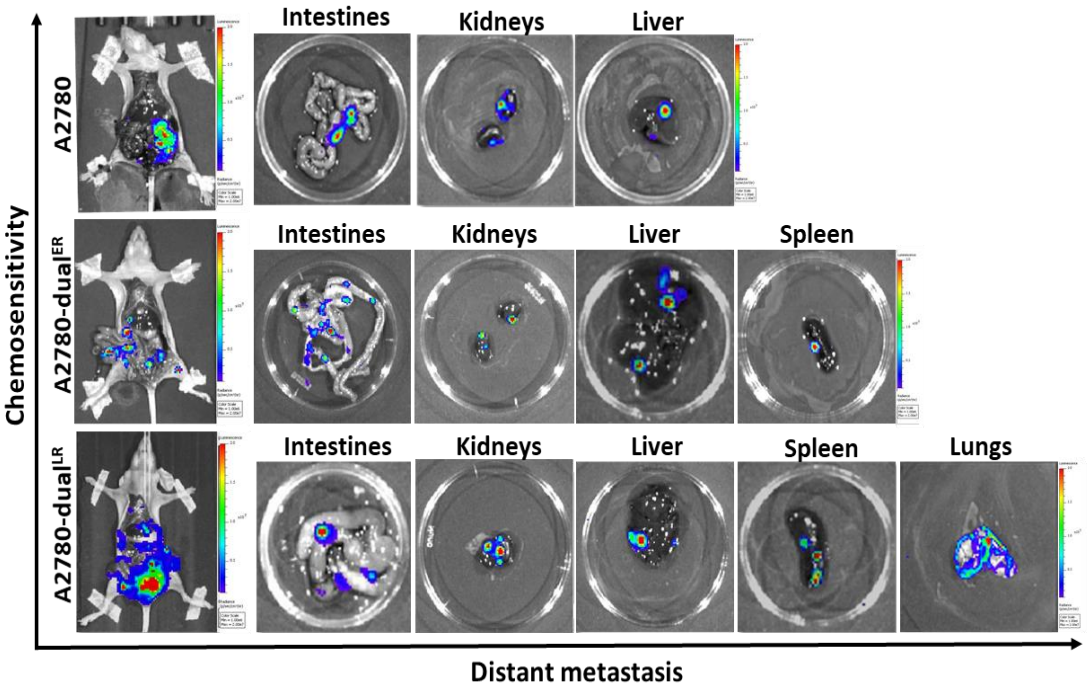

Figure 3. Chemoresistant cells exhibit increased tendency to colonize distant organs. Representative *post-mortem* bioluminescence images of the whole body and individual organs of A2780, A2780-dual^{ER} and A2780-dual^{LR} intra-peritoneal xenograft mouse models at the end of 6 weeks post implantation of tumor cells. An advancing trend of distant colonization is observed from sensitive (A2780) to early (A2780-dual^{ER}) to late stage chemoresistant cells (A2780-dual^{LR}), positively correlating with the degree of resistance (n=15 in each group). (The bioluminescence images are displayed at the same scale of 1.0e6 – 2.0e7).

Our group has previously developed a chemoresistant model of A2780 cells that mimics the dynamic course of acquired chemoresistance *in vitro* (as described in methodology 1.2.1). This cellular model represents the three distinct phases of chemoresistance,

namely sensitive (A2780), early (A2780-dual^{ER}), and late (A2780-dual^{LR}) stages [284]. While the early resistant cells (A2780-dual^{ER}) represent a population at the onset of resistance, the late stage resistant cells (A2780-dual^{LR}) represent a population that have attained extreme resistance. In order to understand the organ-selective colonization of sensitive vs. early and late chemoresistant EOC cells, an IP xenograft mouse model was employed, and the metastatic dissemination was observed using bioluminescence imaging, a highly sensitive technique used to identify micro-metastases. This model was developed primarily because it allows for the passive dissemination of tumor cells as observed in ovarian cancer. For intraperitoneal xenograft model, all the three groups (n=15) that were injected with equal number of A2780 /A2780-dual^{ER}/ A2780-dual^{LR} cells exhibited metastasis with similar prevalence (A2780 group: 40%, A2780-dual^{ER} group: 47% and A2780-dual^{LR} group: 40%) and colon, kidney, and liver were the common organs harboring metastases (Figure 3). In addition, while spleen metastasis was observed in the mice injected with A2780-dual^{ER} and A2780-dual^{LR} cells, lung metastasis was exclusively seen in the mice injected with A2780-dual^{LR} cells (Figure 3). Taken together, an intriguing pattern of enhanced colonization to distant organs was observed with an incremental resistant status of A2780 cells.

2.3.2 Chemoresistance imparts differential tumor intrinsic metastatic properties to EOC cells

To further delineate the intriguing pattern of organ-selective metastasis of chemoresistant cells, the initial key events in the metastatic cascade, such as adhesion and invasion of sensitive vs. resistant cells, were compared *in vitro*. In these experiments, the SKOV3 cell line (stably expressing Firefly luciferase 2-tdT fluorescence bi-fusion reporter), which is inherently resistant to cisplatin, was also

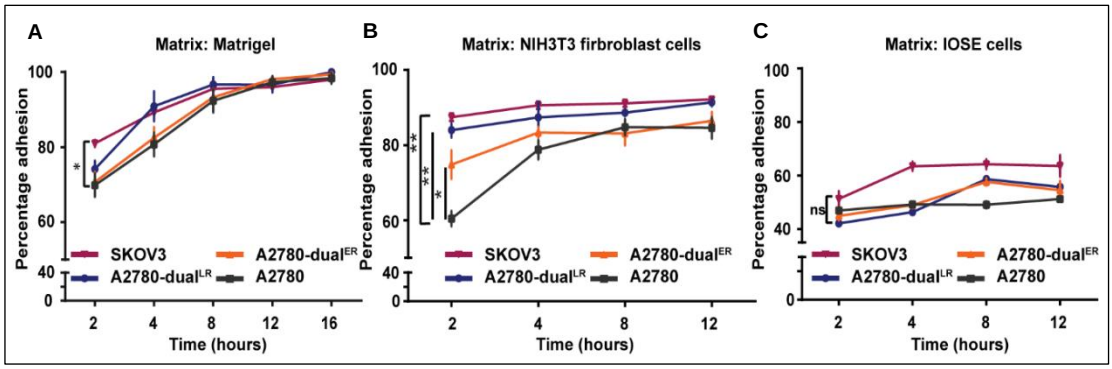

Figure 4. Chemoresistant cells exhibit escalated adhesion specifically toward NIH3T3 fibroblast cells. Adhesion properties, measured on Matrigel (A), NIH3T3 cells (B) and IOSE cells (C) as matrices, revealed greater and faster attachment of chemoresistant EOC cells (SKOV3>A2780-dual^{LR}>A2780-dual^{ER}) than sensitive cells (A2780) on the NIH3T3 matrix. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01.

incorporated to compare the metastatic phenotypes of intrinsic and acquired chemoresistant EOC cells. The adhesion potential was tested on Matrigel, NIH3T3 fibroblasts, and immortalized ovarian surface epithelial cells (IOSE) as matrices. On matrigel, both the sensitive and chemoresistant A2780 cells exhibited similar attachment kinetics, whereas SKOV3 cells showed a marginally higher (~11%) adhesion than A2780 cells (Figure 4A). While the attachment of sensitive and chemoresistant EOC cells was more or less similar on acellular Matrigel matrix, resistant cells displayed significantly higher adhesion on the NIH3T3 fibroblast matrix than the sensitive cells. At 2 hours, SKOV3, A2780-dual^{LR}, and A2780-dual^{ER} cells showed 27%, 23.6%, and 14.4% higher attachment than A2780 cells, respectively (Figure 4B). Similarly, the temporal kinetics of maximal attachment also varied with the degree of resistance. While 90% of SKOV3 and A2780-dual^{LR} cells attached on NIH3T3 cells within 2 hours, A2780-dual^{ER} and A2780 cells took 4 and 8 hours,

respectively, to achieve maximum attachment (around 80%) (Figure 4B). No difference in adhesion potential was found when immortalized ovarian surface epithelial cells were used as a matrix (Figure 4C).

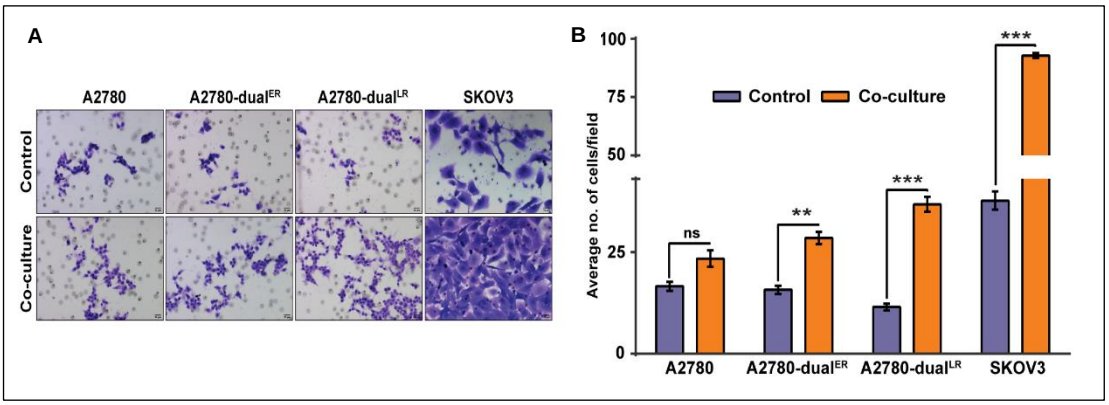

Figure 5. Chemoresistant EOC cells acquire increased invasive phenotype under co-culture conditions with NIH3T3 fibroblast cells. A-B) Incubation with fibroblast conditioned medium (co-culture) augmented the invasiveness of chemoresistant EOC cells (A2780-dual^{LR} > SKOV3 > A2780-dual^{ER} cells) whereas the intrinsic invasiveness of sensitive cells (A2780) remained unaltered under similar co-culture conditions. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

The intrinsic invasiveness of the A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells, as assessed using the Boyden chamber assay, revealed no significant differences (Figure 5A-B). On the contrary, SKOV3 cells were approximately 2.3-fold more invasive than A2780 cells (Figure 5A-B). Since resistant cells possessed heightened potential for adherence to fibroblast cells than A2780 cells, we tested whether NIH3T3 cells, in response, conditioned the invasive properties of these cells. To address this, EOC cells exhibiting different degrees of resistance were suspended in fibroblast conditioned medium (FCM) and the invaded fractions were compared with those of the control

(incomplete medium) using Boyden chamber assay. Incubation with FCM significantly increased the invasion potential of A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells by 1.8-, 3.2-, and 2.5-fold, respectively, compared to the corresponding controls (Figure 5A-B). For A2780 cells, however, a marginal increase (~1.4-fold) was observed in their invasiveness after incubation with FCM, which was also statistically insignificant (Figure 5A-B).

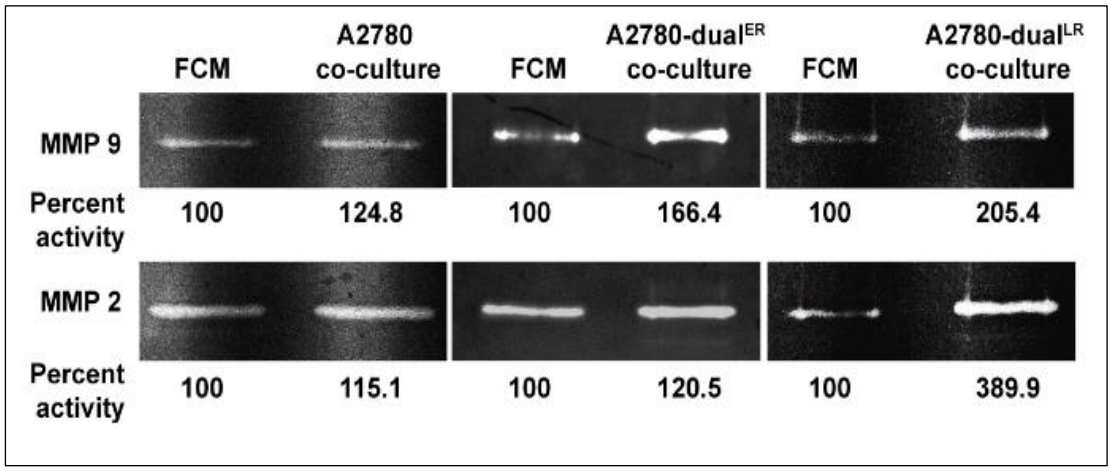

Figure 6. **Chemoresistant EOC cells exhibit increased proteolytic activity under co-culture conditions with NIH3T3 fibroblast cells.** The MMP9 and MMP2 activities observed in A2780-dual^{LR}- and A2780-dual^{ER}-NIH3T3 co-culture supernatants were higher than those in the fibroblast conditioned medium (FCM), with a modest increase seen in the A2780-NIH3T3 co-culture supernatant.

Further, the proteolytic activity of secretory MMPs from supernatants of the EOC cell-NIH3T3 co-culture, as measured using gelatin zymography, revealed their role in the enhanced trend of invasiveness, particularly for chemoresistant EOC cells. Since fibroblasts themselves secrete MMPs, the MMP activities observed in the cancer cell-fibroblast co-culture supernatants were calculated by normalizing them with the relative activities of MMPs present in FCM alone. In comparison to FCM alone, A2780-dual^{LR}

co-culture exhibited an overwhelming increase of 105.4% and 289.9% in the relative proteolytic activities of MMP9 and MMP2, respectively, while the same from A2780-dual^{ER} showed an increase of 66.4% and 20.5%, respectively (Figure 6). In contrast, A2780 cells showed only modest increase of 24.8% and 15.1% in the relative activities of MMP9 and MMP2, respectively (Figure 6). The *in vitro* metastatic properties of intrinsically resistant SKOV3 cells were found to closely resemble those of the A2780-dual^{LR} cells rather than the A2780-dual^{ER} cells.

2.3.3. Late stage chemoresistant cells exhibit remarkable propensity of lung colonization

Although EOC is thought to arise from tubal epithelial cells, it primarily develops in the ovary from where it further metastasizes through the peritoneal fluid, lymphatics, and blood stream [242]. Therefore, we developed orthotopic models using these sensitive and chemoresistant sublines (A2780-dual^{ER} and A2780-dual^{LR}) of A2780 along with SKOV3 cells to further investigate the effect of acquired chemoresistance on the metastatic organotropism of EOC cells through bioluminescence. Although both acquired and intrinsic chemoresistant models showed comparable growth of the primary tumors, the A2780-dual^{LR} model exhibited a wider metastatic spread, encompassing the intestines, kidneys, liver, spleen, and lungs (Figure 8A-B), than did the SKOV3 model, which showed colonization to intestines, kidney, liver, and spleen.

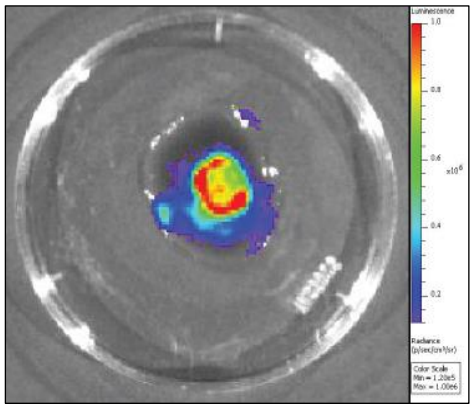

Figure 7. **Ascites from SKOV3 xenograft.** Exfoliated tumor cells present in the ascites collected from the mouse bearing SKOV3 orthotopic xenograft showing bioluminescence.

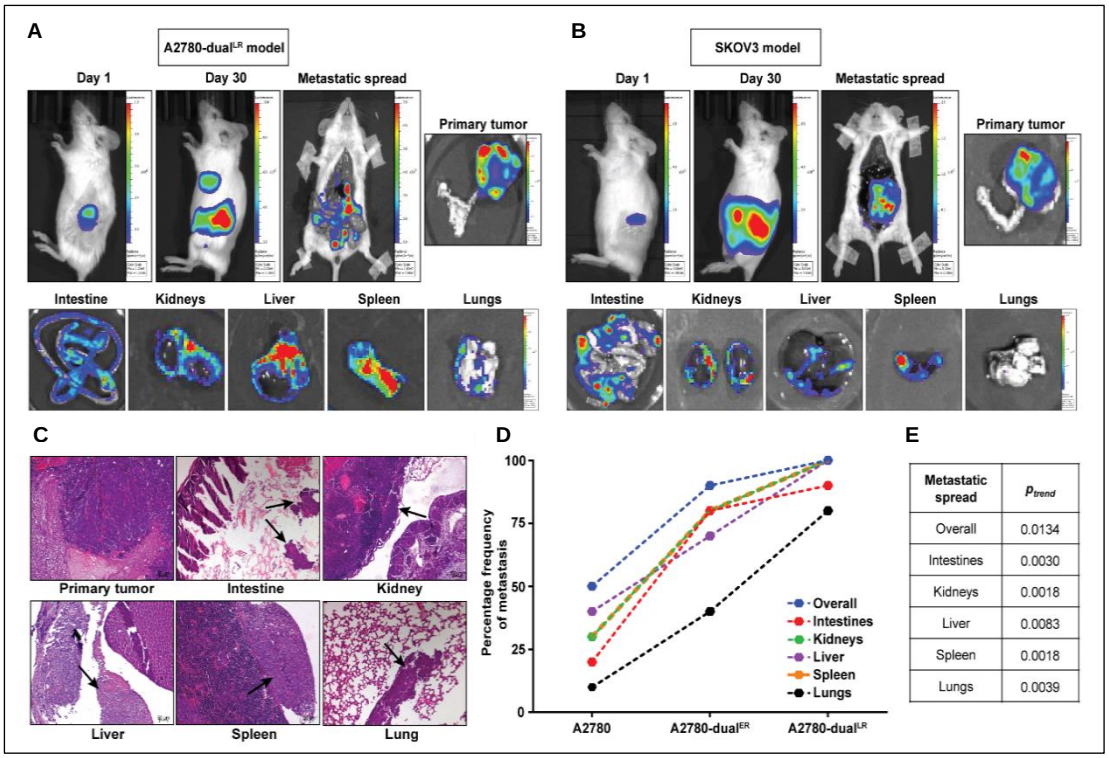

Figure 8. The propensity of lung homing progressively increases with the acquirement of chemoresistance. A-B) Representative bioluminescence images of A2780-dual^{LR} and SKOV3 orthotopic mouse models at day 1 and day 30 after implantation of tumor cells. *Post-mortem* bioluminescence imaging of the whole body and individual organs of mice revealed metastatic growth in intestines, kidneys, liver, and spleen along with the primary tumors. The A2780-dual^{LR} model additionally displayed lung colonization. C) Representative images of H&E stained sections of the primary tumor and metastatic organs studied. Arrows indicate metastatic tumor cells in respective organs. D) The metastatic dissemination of EOC cells was correlated with the degree of chemoresistance, (E) such that an increasing trend of distant metastases was observed with increasing degree of resistance as revealed using the chi square test. A2780-dual^{LR} cells showed a remarkably high incidence of lung colonization (n=10 in each group).

In addition, the SKOV3 model also exhibited ascites formation which was not observed in the A2780 model. However, formation of ascites masked the detection of organ specific nodules, owing to the presence of exfoliated tumor cells in the ascites that contributed to bioluminescence signal (Figure 7) and therefore was not considered for further studies.

A comprehensive analysis of the A2780 metastatic models revealed an advancing trend of dissemination with the progression of chemoresistance. The overall metastatic frequencies among the groups were found to be A2780-dual^{LR} (100%) > A2780-dual^{ER} (90%) > A2780 (50%) (p_{trend}=0.0134) (Figure 8D-E). Similarly, the degree of resistance also correlated with the frequency of colonization to intestines (p_{trend}: 0.003), kidneys (p_{trend}: 0.0018), liver (p_{trend}: 0.0083) and spleen (p_{trend}: 0.0018), confirmed using histopathological analysis (Figure 8C-E). Notably, A2780 cells in the late stage of chemoresistance (A2780-dual^{LR}) showed a remarkable incidence of lung metastasis (Figure 8D-E), indicating that chemoresistance augments the lung-tropic potential of EOC cells particularly in the extreme stage of resistance.

2.4 Discussion

Despite being a principle treatment modality for several cancers, chemotherapy has thus far demonstrated a limited potential in counteracting metastatic dissemination of tumor cells, even though the response is quite promising at the primary site. In addition, recent studies have clearly demonstrated the metastasis promoting effects of chemotherapy and that the residual drug tolerant population may follow an unusual course of organ-selective dissemination highlighting the paradoxical role of chemotherapy. Therefore, one of the major research priorities in the field of cancer therapeutics is to identify the metastatic behaviour of tumour cells that thrive in spite of the cytotoxic effects of chemotherapy, in order to assess the association between chemoresistance stage and the prospective metastatic complications at relapse. The data obtained from such studies may guide the path for the development of novel and precise therapeutic strategies for cancers exhibiting extensive incidence of metastatic recurrence, such as EOC. To address this need, we tracked the dissemination pattern of EOC cells belonging to various stages of chemoresistance, using intra peritoneal and orthotopic metastatic mouse models and established that chemoresistance augments the organotropic dissemination of EOC cells at distant organs, such as lungs, particularly in the late stage. Further, *in vitro* assessment of adhesion and invasion properties of sensitive vs. resistant EOC cells revealed an enhanced ability of chemoresistant EOC cells to initiate the early events of metastasis, which progressively increased with the resistant nature of these cells. Overall, these data indicate that chemoresistance aggravates the tumor intrinsic properties of EOC cells promoting the early events of dissemination, while advancing their organotropic nature of distant metastasis.

Intraperitoneal and orthotopic xenograft mouse models are popular models used to study metastatic dissemination of EOC cells, as the efforts to develop genetically

engineered mouse models have been hampered due to the leaky nature of the ovarian tissue-specific promoter mullerian inhibitory substance type II receptor (MISIIR) [318]. By far, SKOV3 orthotopic or intraperitoneal xenograft mouse models are some of the widely used *in vivo* models as they mimic most of the pathophysiological features of EOC [319]. However, the results of our study show that the SKOV3 orthotopic model may not be a suitable model to track the growth of organ-specific micro metastases owing to the enormous amount of ascites generated, which may also narrow the observation window due to ascites-related morbidity. The two striking characteristics of the A2780 orthotopic model developed in this study were the absence of ascites, which is a classical feature of EOC, and the manifestation of lung metastasis, which is a less frequent event for EOC. Although the absence of ascites did not limit the abdominal spread of A2780 cells (as it was comparable to that of the SKOV3 model), it did raise the possibility of an alternate route of dissemination. Previously, Pradeep et al., (2014) reported a hematogenous spread (excluding lymphatics) of EOC cells using a parabiosis model and also discovered the ErbB3-neuregulin1 (NRG1) pathway as a driver for omental metastasis through a blood-borne root [154]. This report and our own findings suggest that the metastatic organotropism of EOC cells to distant sites such as lungs may essentially be a phenomenon exploiting the hematogenous route of dissemination. Moreover, the orthotopic implantation of A2780 sensitive cells led to their colonization in the lungs, whereas the lung-tropic effect was not observed when these cells were injected via the intra peritoneal route. In agreement with this observation, the organ homing potential of A2780 cell line has been reported to be contradictory in nature. While lung metastasis was documented in subcutaneous and intraperitoneal implantation models of the A2780 cell line [320, 321], tail-vein injection of A2780 cells was shown to cause ascites formation and mild bowel obstruction with

no signs of lung metastasis [155]. Hu et al., (2008) demonstrated the metastatic colonization of A2780 cells to distant sites like liver and diaphragm using an orthotopic xenograft mouse model [322]. However, this study did not report the accumulation of ascites or any event of lung metastasis. In all the above-mentioned reports, only the chemosenitive A2780 cells were employed but not their resistant counterparts. We observed lung metastasis only in 10% of mice when chemosenitive A2780 cells were injected orthotopically, but the incidence of lung tropism enhanced significantly with the degree of resistance. Thus, the route of inoculation and drug tolerant nature of A2780 cells might influence the dissemination pattern and organ-selective colonization in the tumor xenograft mouse models.

The colonization event and subsequent growth of disseminated tumor cells at the secondary site precedes the initial critical steps like adhesion to organ parenchyma and localized invasion, ensuring anchoring of the tumor cells to the guest site [323] Our data showed that the chemoresistant EOC cells intrinsically bore increased adhesion potential toward fibroblast cells. Moreover, although chemoresistance was not found to alter the intrinsic invasiveness of A2780 EOC cells, the chemoresistant cells indeed acquired aggressive invasiveness with increased MMP2 and MMP9 activities when co-cultured with the fibroblast conditioned medium, which seems to have been mediated through the paracrine activity of the secretory milieu of NIH3T3 fibroblast cells. In literature, there are several reports that suggest the active involvement of the fibroblasts in aiding the early events of metastasis [324]. For example, co-culture of primary and metastatic tongue tumor cells (head and neck cancer) with fibroblasts were found to increase the invasiveness of the tumor cells through the induction of MMP2 and MMP9 secretion which was found to be regulated by the paracrine interaction of CXCL12 and CXCR4 [325]. Similarly, cancer associated fibroblasts were shown to produce

fibronectin rich extracellular matrix, thus promoting αv integrin mediated migration and invasion of prostate tumor cells [62]. Singer et al., (2020) demonstrated that the human derived breast cancer associated fibroblasts can activate MMP2 secretion in a paracrine model leading to increased invasion of breast tumor cells [326]. In another study, cancer associated fibroblasts were found promote the invasiveness of breast tumor cells through the secretion of osteopontin, which in turn activates integrin and CD44 signaling, thereby promoting the metastatic phenotype [327].

Taken together, our findings demonstrate that chemoresistance governs the metastatic phenotype of EOC cells in part by enhancing their tumor intrinsic metastatic properties and making them more receptive to the pro-metastatic stimulation provided by stromal cells, like fibroblasts, which may explain the increased probability of chemoresistant EOC cells to form micro-metastases at distant sites.

Chapter 3

Monitoring the effect of IGF1R silencing on the differential metastatic organotropism of sensitive, early, and late resistant cells *in vitro* and *in vivo*

3.1 Introduction

The pre-requisite for cancer growth and its progression into the metastatic phase is unrestrained proliferation of tumor cells at the primary site [328]. Research over the past decades has helped ascertain that tyrosine kinase receptors, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), transforming growth factor receptor (TGFR), and insulin-like growth factor receptor (IGF1R), provide the necessary oncogenic cues for sustained development of the neoplasms [329-332]. Of these, the IGF1R signalling pathway is found to be overexpressed and over-activated widely across major cancer types including lung, breast, melanoma, pancreas, colon, prostate, and ovarian cancer [271, 280, 333]. The first evidence demonstrating the involvement of IGF1R in neoplastic transformation was documented by Sell et al., (1994), who demonstrated that mouse-derived fibroblast cells that were deficient in IGF1R lacked the capacity to transform upon transduction with various viral genes such as Ha-ras, SV40 T antigen, and c-src [334]. Conversely, when ectopic expression of IGF1R was generated in IGF1R deficient cells through genetic perturbation, the transforming ability was restored [335, 336]. Subsequent studies established that besides its involvement in the early progression of cancer, IGF1R signalling is implicated in various steps of the transcoelomic and hematogenous metastatic cascades, like epithelial to mesenchymal transition (EMT), anoikis resistance, cellular motility, stromal invasion, angiogenesis, and colonization at the secondary site [337].

The metastatic programming of cancer cells is initiated as they undergo EMT. IGF1R is reported to induce this process via different mechanisms [338]. IGF1R-mediated activation of AKT can stabilize slug protein, which negatively regulates the epithelial marker E-cadherin, facilitating disengagement of the tumor cells from the primary site

[338]. In addition, IGF1R induced activity of focal adhesion kinase (FAK), STAT3, and NF-kB is also reported to trigger EMT [39, 339]. After undergoing EMT, the exuviated tumor cells (having an epithelial origin) need to acquire anoikis resistance in order to survive the anchorage-independent growth conditions during circulation through blood, lymph, or peritoneal fluid. IGF1R acclimates the tumor cells to these conditions by inhibiting the activation of p53 and p21 stress sensor proteins [340]. Further, studies conducted on ovarian and breast cancer cells demonstrated that IGF1R imparts anoikis resistance through RACK1-mediated activation of AKT and STAT3 or through AKT mediated expression of the LIP protein, a suppressor of anoikis as observed in ovarian and breast cancer cells [341-343]. During stromal invasion, which is the next phase of metastasis, IGF1R plays a pivotal role by controlling the motility of tumor cells though cytoskeletal rearrangement, thus driving the biophysical force, or by controlling the activity of proteolytic enzymes, thereby driving the biochemical force required for invasion by the tumor cells. Guvakova et al., (2002) showed that the activation of PI3K/AKT pathways through IGF1R stimulation cause rapid relocalization of fascin, a cytoplasmic protein involved in actin bundle formation, to the microspike formation in the migratory front of tumor cells, thus increasing their motility [344]. Moreover, IGF1R has been demonstrated as a promoter of the urokinase proteolysis system by acting as a positive transcriptional activator of urokinase-type plasminogen activator (uPA) and its receptor (uPAR) in many cell lines [337, 345]. While IGF1R stimulated activation of uPAR increased the motility of prostate cancer cells, its suppression through inhibition of IGF1R resulted in the attenuation of invasive properties in breast and colon cancer cells [346-348]. Additionally, inhibition of IGF1R has been shown to downregulate the angiogenic potential of multiple tumor cells through the suppression of vesicular endothelial growth factor (VEGF), thus curbing

their metastatic potential [281, 349]. In pancreatic cancer and endometrial cancers, the angiogenesis promoting function of IGF1R was identified through the upregulation of VEGF via nuclear translocation of hypoxia induced transcription factor-1 (HIF-1α) [350, 351]. Moreover, colonization of tumor cells at the secondary site entails specialised tumor intrinsic characteristics that help their settlement at the foreign site. The function of IGF1R has been implicated in the colonization events as well. Reinmuth et al., (2002) demonstrated that the replacement of functional IGF1R with the dominant negative mutant abrogates colonization of colon cancer cells to liver, which is a common site for metastasis in colon cancer [281]. A similar observation, where inhibition of IGF1R signalling resulted in the attenuation of preferential metastasis to liver, was also documented in the case of lung and breast cancer cells [352]. Similarly, for prostate cancer cells, which exhibit a high propensity to colonize bone parenchyma, it has been observed that these cells after colonizing to bone operate a self-amplifying autocrine loop of IGF1 in the stroma that prevents their apoptosis and provides the necessary growth signals [353]. All these reports clearly establish the pro-metastatic role of IGF1R that governs primary tumor cell dissemination, enables the circulating tumor cells to withstand harsh transit conditions, and finally aids in their settlement at the secondary site, thus promoting their colonization to distant organs.

Clinical studies show that distant metastases at relapse often have an indolent nature as far as the standard treatment, which efficaciously controls the corresponding primary tumor growth, is concerned [312]. Interestingly, overexpression and overactivation of IGF1R has been associated with the increased drug tolerance ability of several cancers including breast, prostate, bladder, gastric, and ovarian cancer cells [271, 354]. Experimental findings from previous studies show that IGF1R resists chemosensitization of cancer cells though various mechanisms including PI3K/AKT

mediated inhibition of apoptotic cues on exposure to drugs, manipulation of the expression of ABC family drug transporters to keep the intracellular drug levels down, and promotion of integrin mediated interaction with ECM components to induce pro-survival signals [355-361]. Taken together, all the above-mentioned studies provide overwhelming evidence for the implications of IGF1R in the regulation of the metastatic and chemoresistant phenotypes of cancer cells.

As reported earlier, the A2780 chemoresistant model utilized in this study exhibits an oscillatory expression pattern of IGF1R at different stages of resistance [284]. While the resistant cells at the initial phase of resistance (early resistant;A2780-dual^{ER}) exhibit high expression of IGF1R and MAPK/ERK pathways, cells with a highly resistant phenotype (late resistant;A2780-dual^{LR}) bear lower IGF1R expression but highly active PIK3CA-AKT signaling [362]. Similarly, our group has reported earlier that the primary tumor samples harvested from EOC patients with high grade serous subtype post neoadjuvant chemotherapy (NACT) exhibit significant elevation in the IGF1R expression compared to their ascites-derived chemonaive counterparts [284, 363]. Moreover, the post NACT analysis of the omental metastatic tumor specimens from these patients also revealed an increase in the IGF1R levels in residual viable tumor cells when compared to their chemonaive counterparts [363].

As described in chapter 2, the chemoresistant cells bearing a differential pattern of IGF1R signalling demonstrated differential metastatic properties and organ-selective propensities. Since IGF1R signaling has been implicated as a driver in chemoresistance as well as organ specific metastasis independently, it may serve as a common signaling axis regulating the differential organotropic nature of chemoresistant EOC cells. However, the mechanism through which IGF1R signaling controls the metastatic organotropism of chemoresistant EOC cells has not been understood yet. This chapter

describes the role of IGF1R in the regulation of differential metastatic properties and the organotropic behavior of chemoresistant EOC cells.

3.2 Methodology

3.2.1 Lentivirus mediated gene silencing using targeted shRNA

IGF1R gene was silenced using the following targeted sequence: 5'AGACCTGAAAGGAAGCGGAGA3'. The shRNA sequences against *IGF1R* was cloned into a pLL3.7 lentiviral vector containing an eGFP gene. The lentiviral particles, containing the shRNA cassettes, were transduced into the EOC cells, and stable clones were selected through FACS sorting using eGFP.

3.2.2 Luciferase-based live cell adhesion assay

As previously described in chapter 1 methodology section 2.2.2.

3.2.3 *In vitro* co-culture

As previously described in chapter 1 methodology section 2.2.3.

3.2.4 Boyden chamber assay

As previously described in chapter 1 methodology section 2.2.4.

3.2.5 Gelatin zymography

As previously described in chapter 1 methodology section 1.2.5.The relative MMP activities of wild type and IGF1R KD cells in co-culture were compared using the following formula: (Intensity of proteolytic zone of wild type in co-culture medium/ Intensity of proteolytic zone of corresponding IGF1R KD in co-culture) × 100.

3.2.6 Primary lung fibroblast culture

Primary mouse lung fibroblast cells were isolated as described earlier [364]. Briefly, the mouse was euthanized through cervical dislocation and the lungs were isolated after perfusion with PBS to avoid contamination with blood cells. The isolated tissue was

minced and sequentially digested with dispase (37ºC for 30 min) and trypsin (37ºC for 20 min) followed by neutralization using complete DMEM. Cells were pelleted down by centrifugation and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and characterized on the basis of morphological features (Figure 1A) and through the cytoplasmic expression of vimentin as assessed through immunofluorescence (Figure 1B).

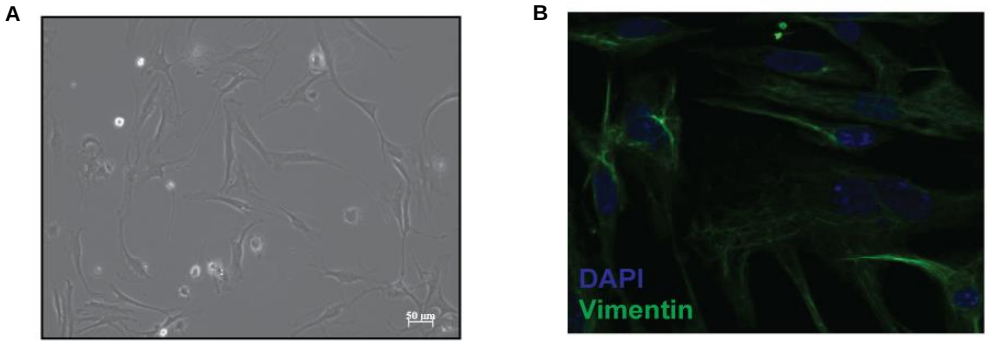

Figure 1. **Characterization of primary mouse lung fibroblast cells.** Representative image of primary mouse lung fibroblast cells characterized by (A) flat, spindle-shaped morphology and (B) homogenous cytoplasmic expression of vimentin determined using immunofluorescence.

3.2.7 *In vivo* orthotopic mouse model

As previously described in chapter 1 methodology section 2.2.6.

3.2.8 Statistical analysis

Data presented in this study represent the mean ± standard error mean (SEM) of at least three independent biological replicates. The statistical significance was analyzed using unpaired Student's *t*-test and *P*-values < 0.05 were reported as significant. *In vivo* metastatic frequencies were compared using Fisher's exact test.

3.3 Results

3.3.1. IGF1R silencing decapacitates the metastatic ability of chemoresistant cells

As previously described, the prominent trait associated with our chemoresistant model is the pulsatile nature of IGF1R expression. During onset of resistance (early stage), upregulated IGF1R expression is believed to assist in progressive acquirement of resistance, which on attainment of severe resistance (late stage) is dispensable for maintenance of resistance [284]. Moreover, the intrinsically resistant SKOV3 cells also exhibit higher IGF1R expression than A2780 sensitive cells. To understand whether IGF1R is engaged in the regulation of differential metastatic properties of chemoresistant cells, the gene was silenced (IGF1R knock down/KD) (Figure 2A-B) through lentivirus mediated approach, and the effect was monitored on the metastatic properties. After IGF1R KD, the relative expression of IGF1R was found to be downregulated by 2.1-, 8.6-, 2.3-, and 8.6- fold across A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, respectively (Figure 2A).

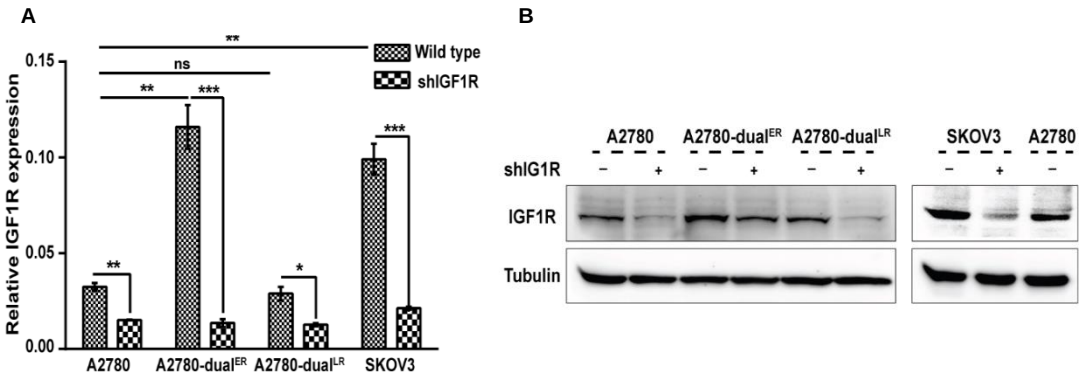

Figure 2. Validation of IGF1R knock-down across A2780 chemoresistant model and SKOV3 cells. A) Transcript and B) Protein levels of IGF1R across A2780, A2780-dual^{ER}, A2780-dual^{LR} and SKOV3 cells and their corresponding knock-down counterparts. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

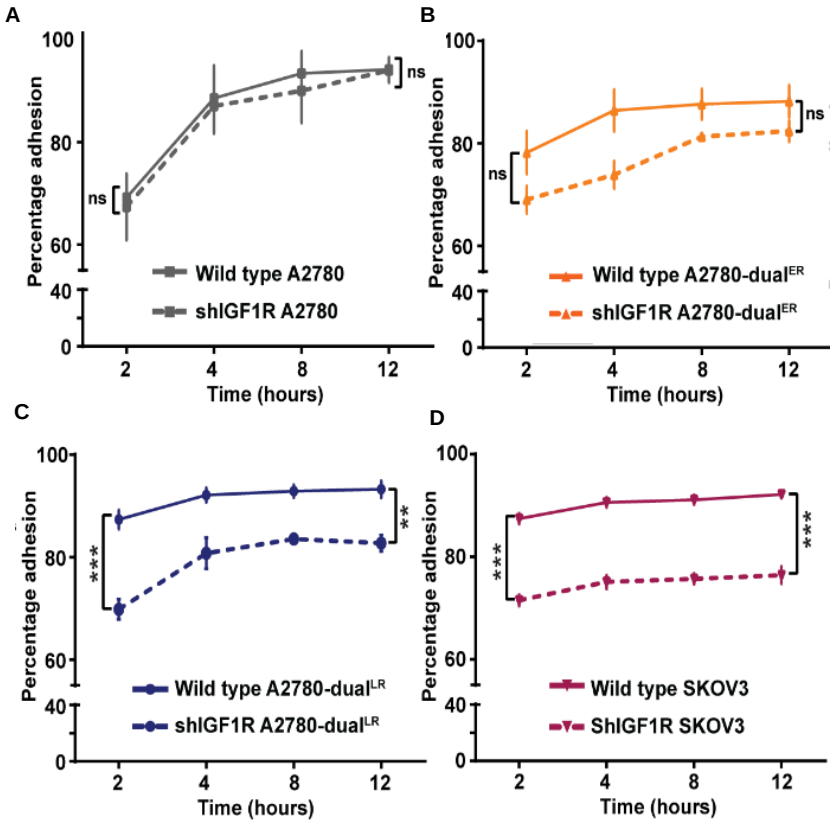

Figure 3. Effect of IGF1R silencing on the adhesion of sensitive and chemoresistant EOC cells toward NIH3T3 fibroblast cells. IGF1R silencing did not alter the adhesion potential of sensitive (A2780) (A) and early resistant (A2780-dual^{ER}) cells (B), whereas it attenuated the fibroblast specific adhesion potential of late stage (A2780-dual^{LR}) (C) and intrinsically resistant (SKOV3) (D) EOC cells. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

IGF1R silencing resulted in about 20% and 10% decrease in the initial (2 hours) and final (12 hours) adherence for A2780-dual^{LR} cells and SKOV3 (~16%) on fibroblast cells, respectively (Figure 3D-E). Such decrease in adherence for A2780-dual^{ER} cells was not statistically significant (Figure 3B), and no change was observed for A2780 cells (Figure 3A).

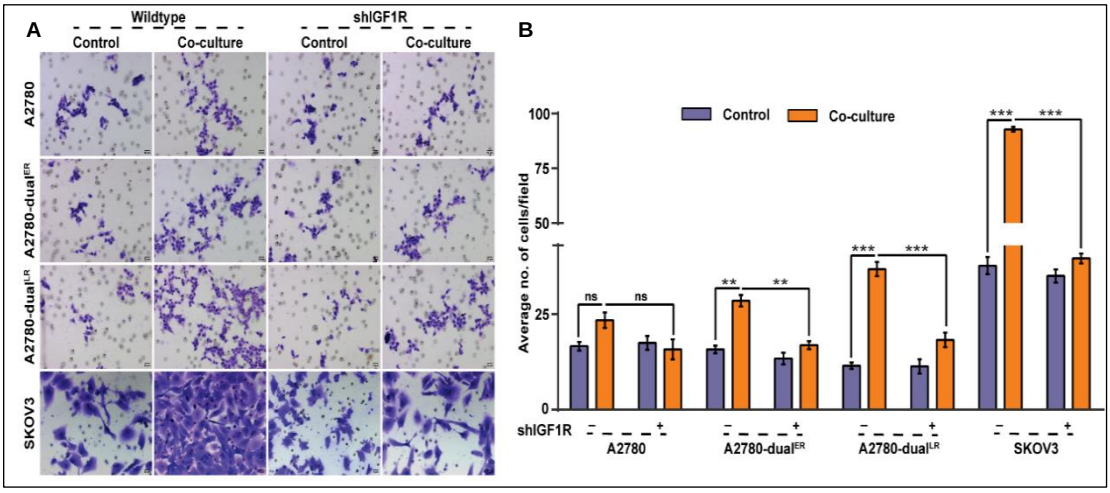

Figure 4. IGF1R silencing attenuates invasiveness EOC cells acquired under co-culture conditions with NIH3T3 fibroblast cells. A-B) IGF1R knock-down did not alter the intrinsic invasiveness of EOC cells, whereas it drastically abated the enhanced invasive phenotype of A2780-dual^{LR}, A2780-dual^{ER}, and SKOV3 cells, particularly observed under co-culture conditions, with no significant impact on the invasiveness of A2780 cells. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

IGF1R KD did not affect the intrinsic invasiveness of the sensitive or resistant cells (Figure 4A-B). Intriguingly, silencing of IGF1R caused a significant reduction in the invasiveness of resistant cells under co-culture conditions. After IGF1R KD, the invaded fractions of A2780-dual^{LR}, SKOV3, and A2780-dual^{ER} cells decreased by 2, 2.3, and 1.7 fold, respectively, compared to their corresponding wild type controls (Figure 4A-B). A marginal (1.5-fold) decrease in the invaded fraction for chemosenitive A2780 IGF1R KD cells compared to wild type was observed which did not meet statistical significance (Figure 4A-B).

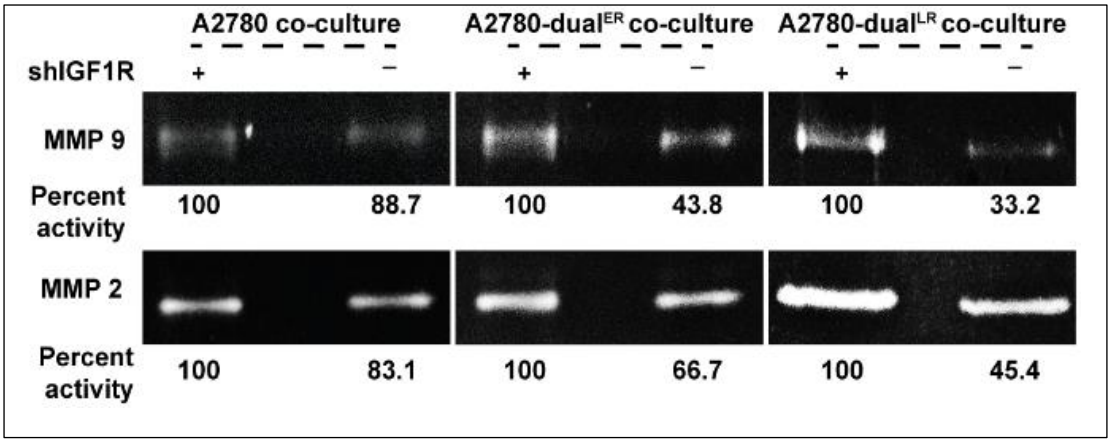

Figure 5. **IGF1R knock-down cells exhibit decreased proteolytic activity under co-culture conditions with NIH3T3 fibroblast cells.** As compared with the wild type condition, IGF1R knock-down severely reduced the secretory MMP9 and MMP2 activities in A2780-dual^{LR} and A2780-dual^{ER} cells when incubated with the fibroblast condition medium (FCM) with a mild decrease observed for sensitive cells (A2780).

Upon IGF1R silencing, MMP9 activities of A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells were decreased by 11.3%, 56.2%, and 66.8%, while MMP2 activities decreased by 16.9%, 33.3%, and 54.6%, respectively under co-culture conditions (Figure 5). These data indicate that IGF1R signaling plays a vital role in the regulation of tumor intrinsic properties required by the chemoresistant EOC cells to initiate key events of metastasis.

3.3.2 IGF1R inhibition attenuates metastatic spread of late stage chemoresistant cells to distant sites

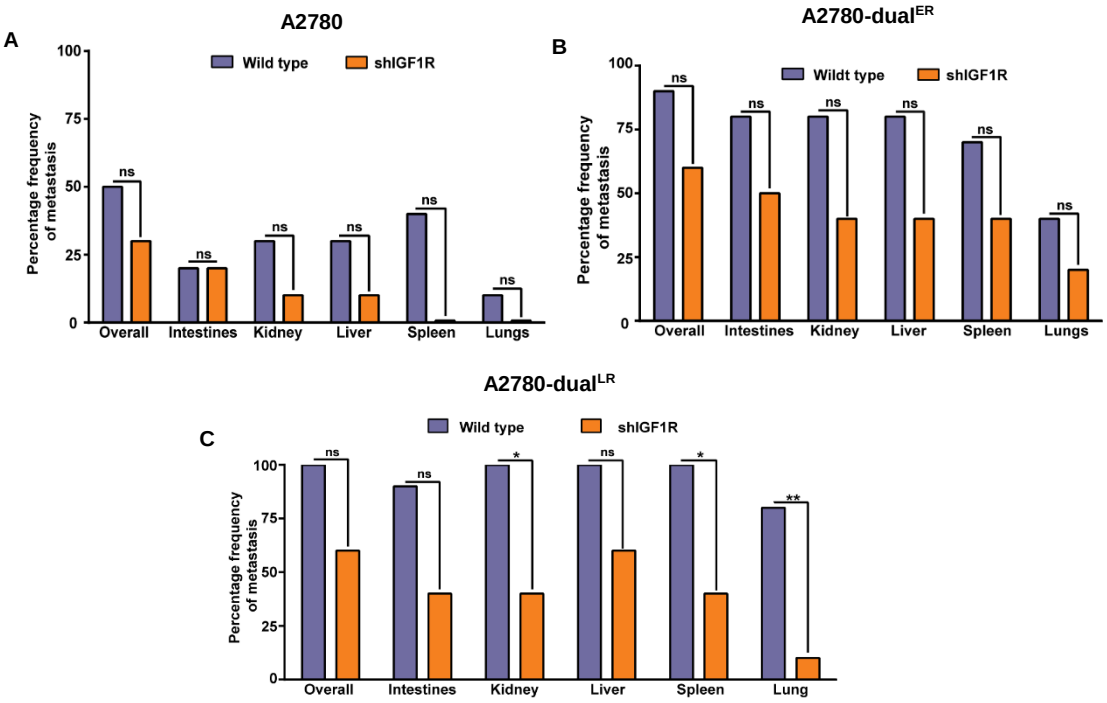

Figure 6. IGF1R knock-down arrests distant metastasis of late stage chemoresistant EOC cells. IGF1R silencing did not significantly affect the metastatic potential of sensitive (A2780) and early resistant (A2780-dual^{ER}) cells, whereas it significantly compromised the metastatic ability of late stage chemoresistant cells (A2780-dual^{LR}) to colonize kidneys, spleen, and lungs (n=10 in each group), as determined using Fischer's exact *t*-test.

When the development of metastasis after IGF1R silencing was assessed *in vivo*, the colonization of A2780-dual^{LR} cells was significantly reduced in lungs, kidney, and spleen, but this reduction was most prominent for the lungs (Figure 6) as determined using the Fisher's exact test. Though similar trends of reduced incidence of metastasis were observed for A2780 and A2780-dual^{ER} cells, they did not meet statistical

significance (Figure 6), indicating that the function of IGF1R is indispensable in the colonization of chemoresistant EOC cells bearing extreme resistant to distant sites.

3.3.3. IGF1R inhibition disrupts chemoresistant EOC cells-primary lung fibroblast interaction

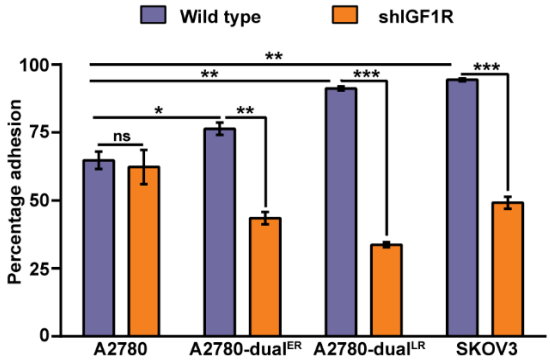

Figure 7. IGF1R silencing impairs adhesion of chemoresistant EOC cells toward lung fibroblast cells. Chemoresistance progressively increased the propensity of EOC cells to adhere to primary lung fibroblast cells, with A2780-dual^{LR} and SKOV3 cells exhibiting maximum adhesion. IGF1R knock-down significantly hindered the adhesion of chemoresistant EOC cells to lung fibroblasts but did not alter the adhesion potential of A2780 sensitive cells. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

The exceptional pattern of lung homing potential of EOC cells, that showed linear increment with the degree of resistance, raised the possibility of active involvement of lung tissue components and microenvironment. Indeed A2780-dual^{LR} cells showed about 26% and 11% higher adhesion on primary lung fibroblast cells than the A2780 and A2780-dual^{ER} cells, respectively (Figure 7) and SKOV3 cells showed approximately 30% higher attachment than the A2780 cells (Figure 7). Additionally, A2780-dual^{LR} cells exhibited 1.8 and 1.6 times higher invasion than A2780 and A2780-

dual^{ER} cells (Figure 8A-B), when incubated with the conditioned medium form primary lung fibroblast cells. SKOV3 cells also showed 3.2 times higher invasion than A2780 cells under similar condition (Figure 8A-B). To assess the direct influence of IGF1R signaling in driving lung metastasis by late resistant cells, similar experiments were performed in the IGF1R silenced background. IGF1R KD caused >50% and 33% decrease in the adhesion of A2780-dual^{LR} and A2780-dual^{ER} cells upon lung fibroblast cells but such effect on the adhesion potential of A2780 cells was negligible (Figure 7).

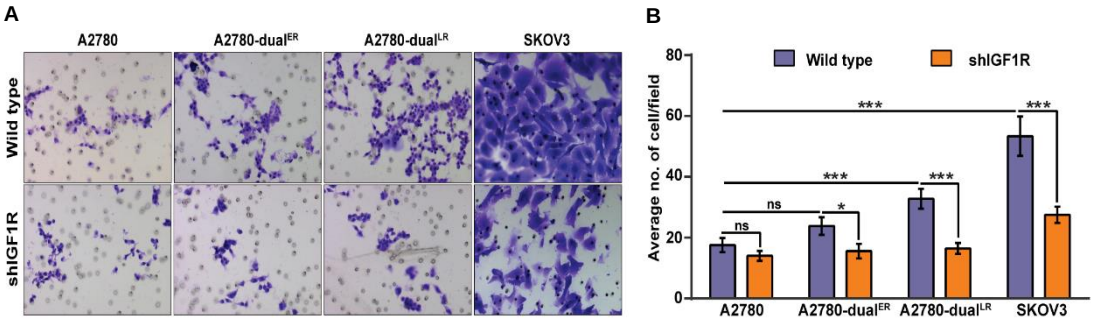

Figure 8. IGF1R silencing causes reduction in the invasiveness of chemoresistant EOC cells under co-culture conditions with lung fibroblast cells. A-B) The invasive phenotype of A2780-dual^{LR} and SKOV3 cells was significantly potentiated under co-culture conditions with the primary lung fibroblast cells, whereas that of A2780-dual^{ER} and A2780 sensitive cells did not exhibit any prominent effect under similar co-culture conditions. IGF1R knock-down diminished the invasive phenotype of A2780-dual^{LR} and SKOV3 cells acquired under similar co-culture conditions; however, it reduced the invasion potential of A2780-dual^{ER} cells only marginally and did not alter the invasiveness of A2780 sensitive cells. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

Moreover, while IGF1R silencing suppressed the invasiveness of A2780-dual^{LR} and A2780-dual^{ER} cells by almost 2- and 1.5-fold respectively, it had a negligible impact on the invasion of A2780 cells under co-culture conditions (Figure 8A-B). Similarly, SKOV3 cells exhibited about 45% decrease in adhesion (Figure 7) and almost 2-fold decrease in invasion (Figure 8A-B) after IGF1R silencing. Overall, these finding suggest that IGF1R signalling is crucial for chemoresistant EOC cells in mediating the initial key events like adhesion and invasion at the secondary site.

3.4 Discussion

The emergence of distant metastases at relapse and their tolerance to standard therapeutic regimen has been a long standing problem in the management of cancers [312, 365]. This observation becomes even more apparent in cancers like EOC, where the progressive acquirement of resistance can be tracked in terms of platinum free interval and can be correlated with the emergence of extra peritoneal metastases at distant sites [265]. The comprehension of the molecular triggers that perturb the metastatic behavior caused by chemoresistance at this stage is of prime importance in order to avoid confounding effects of chemotherapy. Moreover, in depth understanding of these triggers might unravel potential therapeutic targets which can be exploited for therapeutic purpose in the recurrent setting. We therefore sought to determine the implications of IGF1R signaling in the regulation of metastatic properties and organotropism of EOC cells during acquirement of chemoresistance. Secondary organs colonized by wild type and IGF1R-KD EOC cells at different stages of chemoresistance were analysed using an optical imaging-guided approach in corresponding orthotopic models. The findings revealed that IGF1R regulates the organ-homing ability of chemoresistant cells, particularly those in the late stage of chemoresistance, to colonize distant sites, such as kidneys, spleen, and lungs. Further, *in vitro* investigation of the key events of metastasis using primary lung fibroblast cells revealed that IGF1R signaling is central to the establishment of initial events of colonization of chemoresistant EOC cells, involving the stromal components of the microenvironment (in this case fibroblasts) at the distant sites, like lungs.

Herein, we observed that the suppression of IGF1R did not limit the overall metastatic dissemination of chemoresistant cells; however, it did alleviate their metastatic potential to colonize distant organs particularly at the late stage of chemoresistance,

suggesting that IGF1R signalling is more crucial in aiding in the colonization events at the secondary site than in the early phase of dissemination. In literature, there exist few reports that highlight the importance of IGF1R in the colonization event. In a study by Sachdev et al., (2004) the overexpression of truncated IGF1R, which harbors the ligand binding domain but lacks the auto-phosphorylation sites in the c-terminus, in a metastatic variant of breast cancer cell line completely abrogated their ability to colonize lungs. Similarly, Chen et al (2009) showed that the inhibition of IGF1R suppresses the colonization of hepatocellular carcinoma cells to lungs [366]. However, why tumor intrinsic IGF1R function is crucial for colonization to specific sites is not clearly understood. Moreover, the detailed mechanism by which IGF1R signalling mediates the pre-metastatic niche formation at the secondary site remain largely unknown. The upcoming findings suggest that the abundance of IGF1 levels at the secondary site might govern IGF1R mediated dissemination of tumor cells at these sites. For example, IGF ligands of bone stroma have been shown to promote colonization of breast tumor cells to bone by promoting their proliferation and survival through the activation of AKT and NF-kB pathways [367]. Interestingly, neutralizing antibody against IGF1R diminished these effects in spite of the availability of IGF1 ligand, indicating IGF1–IGF1R crosstalk in the tumor microenvironment [367]. Similarly, a recent study reported that cancer associated macrophages are the primary source of IGF1 in the primary and metastatic breast tumors and blocking IGF1 in combination with paclitaxel significantly reduced the incidence of lung metastasis in the pre-clinical model [368]. Therefore, besides the tumor intrinsic properties, IGF1R driven predisposition of tumor cells to colonize these sites could also be rationalized based on the receptive microenvironment these sites offer.

The initial physical and paracrine (through secretory components) interactions established between tumor cells and resident stromal components, like fibroblasts, mainly govern the viability of tumor cells at the secondary site. Our data demonstrated that the silencing of IGF1R attenuates the adhesion of chemoresistant cells toward resident lung fibroblast cells also abating their responsiveness to the pro-invasive secretory cues of the lung fibroblast cells. Previous studies have demonstrated the involvement of IGF1R in the reception of the pro-tumorigenic cues by the tumor cells from fibroblasts, which also help them maintain their resistant phenotype. For example, Vaquero et al., using co-culture techniques similar to ours, showed that fibroblasts provide the necessary pro-survival signals to erlotinib-resistant cholangiocarcinoma cells through IGF2 mediated activation of IGF1R signalling that sustains their viability [369]. Another study by Ireland et al., (2016) on pancreatic cancer showed that cancer associated fibroblasts can activate IGF1R signalling in the tumor cells by secreting IGF1 ligand thus promoting chemoresistance in them [370]. Therefore, besides pro-colonizing function, IGF1R mediated cross-talk between tumor cell and stroma can help cancer cells maintain a steady state of chemo-tolerance at the secondary site.

Overall, our findings demonstrate that IGF1R signaling governs the primary interactions between chemoresistant EOC cells and resident fibroblasts at distant sites such as lungs, which underlie the initial metastatic events, thereby promoting their colonization at these sites. Intriguingly, the late stage chemoresistant cells exhibit relatively lower expression of IGF1R than the early stage chemoresistant EOC cells; however, the effect of IGF1R inhibition was more pronounced in case of the late chemoresistant cells. Therefore, this observation suggested a possibility of involvement of another signalling pathway, which along with the IGF1R signaling, co-regulates the

metastatic phenotype of late stage chemoresistant cells. This possibility has been explored in the next chapter.

Chapter 4

Investigating the role of differentially expressed adhesion molecules and EOC–fibroblast secretome in driving the events of organotropic metastasis

4.1 Introduction

Once set in, metastasis is an ongoing process facilitating systemic dispersal of millions of cells every day [60, 371]. Despite it being a stochastic process, there is a non-random bias associated with it in terms of the colonization at the secondary organ. For example, epithelial ovarian cancer (EOC) cells almost always metastasize to the omentum, whereas colorectal cancer cells show a high propensity for liver metastasis at first instance [372, 373]. Similarly, breast cancer cells that possess inherent tendency to colonize liver, bone, lung, and brain exhibit differential preferences for secondary sites based on the subtype that they inherit. For instance, while the triple-negative breast cancer (TNBC) cells primarily disseminate to the visceral organs, the metastatic manifestation of the luminal subtype is commonly observed in the bones [374, 375]. This non-radon pattern of metastasis observed across different cancers or within subtypes of a specific cancer have long been identified as a phenomenon called metastatic organotropism [376, 377]. The seminal *seed and soil hypothesis* proposed by Stephan Paget provides a premise to this observation. According to this hypothesis, the metastatic tumor cells (referred to as "seeds") only colonize to a particular site that provides the necessary amenable microenvironment (referred to as "soil") for their growth [169, 378]. In accordance with this, both the intrinsic properties of the tumor cells and specific niches at the secondary organs govern the development of disseminated tumor cells into micro-metastases.

Several *in vitro* and *ex vivo* studies have revealed that aberrant cellular adhesion is causally involved in the progression and metastasis of cancer [379, 380].The tumor adhesion properties, in particular, correlate with the metastatic capacity of tumor cells to undergo extravasation at the secondary site, colonize at the foreign environment, and subsequently grow into an individual tumor mass [381]. Of the four major families of

adhesion molecules viz. cadherins, selectins, integrins, and immunoglobulins, integrins and integrin-dependent processes have been implicated in almost every step of cancer progression, including secondary metastasis at distant organs [382, 383]. Integrins are complex transmembrane adhesion molecules, existing as heterodimers formed out of non-covalent interactions between α and β subunits [384, 385]. Till date, 18 types of α integrins and 8 types of β integrins have been discovered in the mammalian system that assemble to form 24 unique functional pairs [386]. Integrin signaling is bi-directional and therefore, is unique in itself. The inside-out signaling induces conformational changes in the ligand-binding domain of the receptors and therefore changes the binding properties of ligands which are primarily extracellular matrix components (ECM) like fibronectin, vitronectin, collagen, and laminin, whereas the outside in signaling is relayed upon binding of integrins to these ligands through the activation of focal adhesion kinases (FAKs) and Src family kinases that regulate the intracellular processes like cell migration and invasion [387]. Although single integrins can bind to multiple ligands and same ligands can be shared by multiple integrins, the function of specific integrins has been attributed in different steps of the metastatic cascade and organ homing. For example, while the overexpression α6β4 integrins was correlated with the invasive capacity of colon cancer cells, α5β1 and α4β1 were found to govern the fibronectin-mediated migration and invasion of tumor cells [388]. Moreover, β1 integrin has been implicated in the adhesion of tumor cells to capillary endothelial cells, thus promoting their extravasation at the secondary site [389]. As far as homing of tumor cells to distant sites is concerned, a study conducted on a murine model showed that the loss of α4β1 integrin abrogates lung and spleen colonization potential of T-lymphoma cells [390]. Similarly, while α4β1 integrin was found to promote the peritoneal metastasis of ovarian cancer cells, αvβ3 integrin was implicated in the lymph

node metastasis of melanoma and pancreatic cancer cells [391-393]. Further, αvβ3 has been widely implicated in bone metastasis across breast and prostate cancers [394-398]. In addition to their role in distant organ colonization, integrin receptors are also known to be overexpressed in tumor cells when there is acquirement of chemoresistance and tumor recurrence. For example, administration of taxol based treatment to breast cancer cells results in an enrichment of tumor population expressing α6 integrin which remains indolent to the treatment [399]. A similar observation was also reported by Zheng et al., (2013) who used a spontaneous model of non-small cell lung carcinoma (NSCLC) to show that the residual tumor population after cisplatin treatment is enriched with tumor cells exhibiting overexpression of β4 integrin [400]. Moreover, β3 integrin is believed to drive acquired resistance toward EGFR inhibitors like lapatinib and erlotinib via activation of NF-kB signaling [401]. As a whole, integrin mediated cellular adhesion is one of the tumor intrinsic attributes that centrally govern the site specificity as well as drug resistance of metastatic tumor cells.

Although the function of integrins can independently control the metastatic phenotype of tumor cells, recent studies provide compelling evidence that integrin signaling also regulate the metastatic properties of tumor cells through synergistic association with the receptor tyrosine kinase signaling [402]. This crosstalk is facilitated by the ability of growth factor ligands to bind to the integrin receptor and the redundant nature of the downstream signaling pathways like PI3K/AKT and MAPK/ERK which both (integrin and receptor tyrosine kinase signaling) the classes of signaling pathways relay. For example, α6β4 shows cooperative binding to IGF1R and EGFR, whereas αvβ3 can also associate with IGF1R and c-Met receptor [402]. While α6β4–EGFR association has been observed to govern the mobility and invasiveness of breast cancer cells, αvβ3-c-Met co-operation has been shown to promote breast tumor cell colonization to bone

[403, 404]. Therefore, the relative abundance of integrin receptors may also contribute to the metastatic function of tumor cells driven by tyrosine kinase receptors like IGF1R.

Although tumor intrinsic properties help tumor cells enter the microenvironment of the foreign organ, a conducible niche for successive development is tuned only when there is a reciprocal crosstalk between tumor cells and the tissue parenchyma [405]. A wealth of experimental findings suggest that the pre-metastatic niche is shaped through the secretory milieu generated upon the primary interactions of disseminated tumor cells with the stromal components mediate this crosstalk [405]. For example, bone stromal cells like osteoblasts secrete various chemoattractants like CXCL12, OPN, BMPs, and RANKL that mediate recruitment of disseminated tumor cells to the bone marrow [406, 407]. Similarly, while tenascin-C secreted by lung stromal cells promotes the viability of infiltrated tumor cells and their subsequent colonization in the lungs, secretory claudin-2 has been demonstrated to mediate the tumor cell–stroma interaction, thereby promoting metastasis in the liver [408, 409]. Besides stromal secretory factors, secretory factors from tumor cells play a critical role as well. For instance, salivary cystatins, the plasminogen activators and collagen functionality proteins secreted by breast cancer cells, were found to promote bone metastasis in experimental as well as clinical settings [410]. Similarly, secretion of Nidogen1 by breast and melanoma cancer cells promoted their propagation in lung tissue *in vivo* [411]. Proteins like EFNA3, CD137L/TNFSF9, and SOSTDC1 secreted by colorectal cancer cells were found to promote colonization in liver [412]. In EOC, although the function of secretory molecules like CSF-1 and N-glycans has been reported to be pro-metastatic, their specificity for secondary sites remains unexplored [413, 414]. Further, whether chemoresistant EOC cells preferentially colonize specific organ/s assisted by specific secretory factor/s has not yet been evaluated.

Our previous findings described in chapter 2 and chapter 3 demonstrated that EOC cells, which showed a linear progression of lung-tropism with the degree of chemoresistance, also gained specialized adhesion potential toward the resident lung fibroblast cells which in turn elevated their invasiveness through the secretory milieu under co-culture conditions. Further, IGF1R silencing severely abrogated the adhesion of chemoresistant EOC cells to lung fibroblast cells while decreasing their invasiveness under co-culture conditions, also significantly altering the capacity of chemoresistant EOC cells to colonize lungs, particularly in the late stage. To understand the mechanistic basis of these findings we explored the profile of adhesion molecules in sensitive and chemoresistant EOC cells, assessed their alteration due to IGF1R silencing, and investigated their role in mediating the key events of organ-specific colonization. In addition, we also screened the co-culture secretome of sensitive and chemoresistant EOC cells to identify and assess the role of specific molecule/s governing the colonization events of late stage chemoresistant cells to distant sites such as lungs.

4.2 Methodology

4.2.1 Immunofluorescence

For co-localization studies, EOC cells were fixed on a coverslip using 4% paraformaldehyde, blocked with 3% BSA, and first stained with the primary α6 integrin antibody at room temperature for 2 hours followed by the secondary antibody treatment (both at room temperature for 2 hours). Before probing for IGF1R, the coverslips were blocked using 3% BSA (at room temperature for 1 hour). Next, the cells were probed with the primary IGF1R antibody followed by the appropriate secondary antibody (at room temperature for 2 hours), after which the cells were mounted using vectashield. The images were acquired using a Carl Zeiss, LSM 710 microscope and analyzed through ImageJ software.

4.2.2 Lentivirus mediated gene silencing using targeted shRNA

ITGA6 and *S100A4* genes were silenced using the following targeted sequences 5'CCATCACAGTAACTCCTAA3' and 5'GCTCAACAAGTCAGAACTAAA3' specifically designed against the respective genes. The shRNA sequences against *S100A4* was cloned into a pLL3.7 lentiviral vector containing an eGFP gene. For *ITGA6* silencing, the targeted shRNA sequence was cloned into doxycycline inducible pTRE2pur vector with a puromycin selection marker. The lentiviral particles, containing the shRNA cassettes, were separately transduced into the EOC cells, and the respective stable clones were selected through FACS sorting using eGFP (for *S100A4*) or puromycin selection (for *ITGA6*). For ITGA6 knock-down, the EOC cells were treated with 10 µg/ml concentration of doxycycline for 48 hours.

4.2.3 *In vitro* reverse co-culture

Co-culture medium: EOC cells were either suspended or grown in fibroblast conditioned medium (FCM; as described in chapter 2 methodology section 1.2.3) at 37°C for 24 hours. The medium harvested at the end of this incubation period was used for reverse co-culture experiments and referred to as the co-culture medium throughout the study.

4.2.4 nLC-MS and data analysis

The culture supernatants were concentrated using acetone precipitation. The concentrated protein samples were digested with trypsin (in-solution method), subjected to Triple TOF 5500+ nanoscale liquid chromatographic tandem mass spectrometry (nLC-MS/MS), and peptide spectra were acquired. Significant protein candidates from each MS/MS spectrum were identified using protein pilot software at a 0.01% false discovery rate and $p<0.05$ significance level.

4.2.5 Immunohistochemistry (IHC)

IHC was performed as described previously [363]. Briefly, 5-µm-thick sections of tumors were deparaffinized, hydrated, and treated with peroxide block for 14 min at room temperature (Abcam), which was followed by heat-induced epitope retrieval in sodium isocitrate buffer (pH 6) in a microwave at 640 V for 10 min and 320 volts for 5 min. Next, the sections were incubated with the protein block (Abcam) and sequentially treated with the primary antibody of S100A4 [HPA007973, Sigma (USA); (1:200 dilution)] and incubated at 4^{o}C overnight and secondary antibody (Abcam) at room temperature for 2 hours, after which they were developed using HRP conjugated DAB substrate (Abcam). Blinded grading was done based on the intensity and the extent of positivity as scored by an experienced pathologist. The immunoreactive score was calculated using the formula: intensity × extent of positivity.

4.2.6 *In vivo* metastatic mouse models

As previously described in chapter 1 methodology section 1.2.6. The tumor volume was calculated as follows: ½ x Length x $(Width)^2$.

4.2.7 *In vitro* and *in vivo* niclosamide treatment

For *in vitro* experiments, 1 mg/ml niclosamide (2′, 5-dichloro-4′-nitrosalicylanilide) stock was prepared using dimethylsulfoxide (DMSO) followed by 3-4 cycles (30 sec on/off) of sonication. All *in vitro* treatments were administered for 48 hours. For *in vivo* dosing, NOD/SCID mice with an average weight of 20 gm received 100 mg/kg body weight of niclosamide solution or vehicle (formulated in 0.5% w/v methylcellulose/0.1% v/v Tween 80), daily through gavage from day 10 post orthotopic implantation till the end point (30 days).

4.2.8 Statistical analysis

Data presented in this study represent the mean ± standard error mean (SEM) of at least three independent biological replicates. The statistical significance was analyzed using unpaired Student's *t*-test, and *P*-values < 0.05 were reported as significant. *In vivo* metastatic frequencies were compared using Fisher's exact test.

4.3 Results

4.3.1 IGF1R–α6 integrin interaction underlies the metastatic behavior of A2780-dual^{LR} cells

As described earlier in chapter 3, the superior metastatic characteristics of A2780-dual^{LR} cells even with lower IGF1R levels than A2780-dual^{ER} cells, as observed *in vitro* and *in vivo*, reflected the possibility of the involvement of additional signaling pathways. We, therefore, looked into the integrin profile across A2780 chemoresistant model, owing to their role in cellular adhesion and their cooperativity with tyrosine kinase receptors to regulate metastatic properties as described previously. Of all the integrins (α1, α2, α5, α6, β1, β4, and β5), whose expressions were profiled in the chemoresistant model, α6 and β4 integrins were overexpressed in A2780-dual^{LR} cells compared to in A2780 and A2780-dual^{ER} cells (Figure 1A). These integrins were also found to be downregulated across the model after IGF1R silencing (Figure 1A); however, maximum reduction was observed in A2780-dual^{LR} cells which was also confirmed at the protein level (Figure 1B-C). A similar downregulation α6 and β4 was also observed in IGF1R knock-down SKOV3 cells (Figure 1B-C). The α6 and β4 integrins together constitute a functional heterodimer and are known to form a tripartite complex with IGF1R after stimulation with the IGF1 ligand. As a consequence, IGF1R signaling can in part be activated by binding of IGF1 ligand to α6 and β4 integrins, thereby relaying the downstream ERK-Akt signaling [415]. Therefore, we evaluated the co-localization of α6 integrin and IGF1R in the presence and absence of the IGF1 ligand across the model using overlap coefficient as a measure of this complex formation.

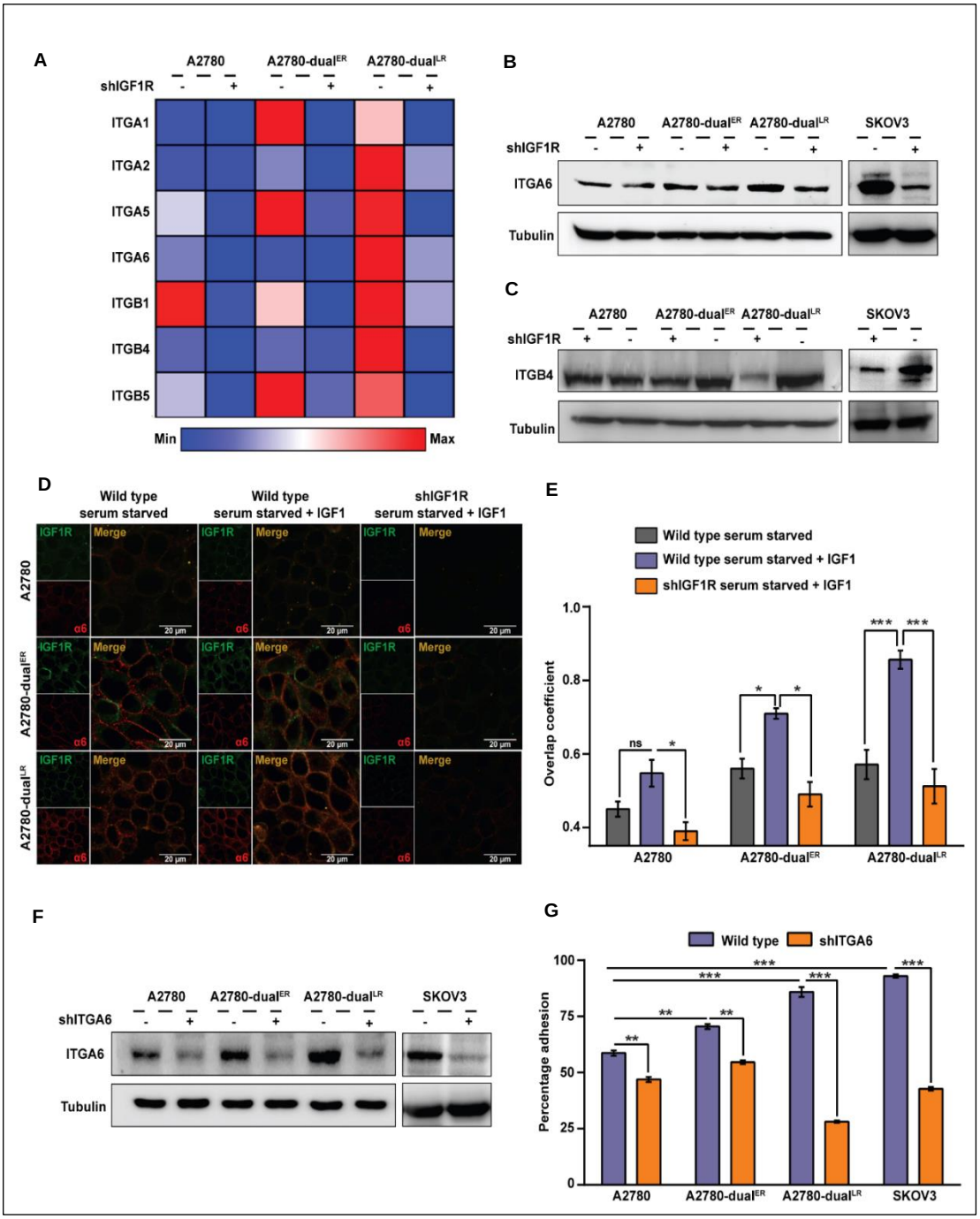

Figure 1. **IGF1R–α6 integrin coalition governs the metastatic phenotype of A2780-dual^{LR} cells.** A) Heat map showing the relative expression profiles (transcript level) of various integrins (α1, α2, α5, α6, β1, β4, and β5) mapped in the A2780 chemoresistant model along with the corresponding IGF1R knock-downs. B-C) After IGF1R knock-down, the expression of α6 and β4 integrins was downregulated across the A2780 chemoresistant model and SKOV3 cells, as

assessed using western blot analysis. D-E) The basal level of IGF1R-α6 integrin co-localization was enhanced after IGF1 (100 ng/ml) treatment in A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells by 1.2, 1.3, and 1.5-fold, respectively, as measured using the overlap coefficients. IGF1R knock-down caused a significant reduction in the overlap coefficients by 1.4-, 1.4-, and 1.7-fold, respectively, for A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells, in the presence of the IGF1 ligand. F) Representative western blot showing downregulation of α6 integrin across the A2780 chemoresistant model and SKOV3 cells and the corresponding inducible knock-downs after doxycycline treatment (10 μg/ml for 48 h). G) α6 integrin mediated the adhesion of EOC cells to lung fibroblasts; α6 integrin knock-down significantly reduced the adhesion potential of A2780-dual^{LR} and SKOV3 cells followed by A2780-dual^{ER} and A2780 sensitive cells toward lung fibroblasts. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells were found to have an overlap coefficient of 0.45, 0.56, and 0.57, respectively, at the basal level (serum starved conditions) (Figure 1D-E), which increased to 0.55, 0.71, and 0.86 (1.2, 1.3, and 1.5-fold higher), respectively after IGF1 treatment (100ng/ml) (Figure 1D-E). After IGF1R silencing, the overlap coefficient of A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells decreased to 0.39, 0.49, and 0.51 (1.4, 1.4, and 1.7-fold reduction), respectively, in the presence of the IGF1 ligand (Figure 1D-E). These findings highlighted the probability of a ratio-metric interaction between α6 integrin and IGF1R at different stages of resistance leading to the cooperative regulation of the metastatic phenotype of these cells.

To investigate the role of α6 integrin in the regulation of adhesion properties of EOC cells toward lung fibroblasts, a doxycycline inducible knock-down of *ITGA6* gene was carried out across all the stages of the A2780 cellular model and in SKOV3 cells (Figure 1F). As expected, *ITGA6* silencing resulted in a decrease in adhesion of both sensitive and resistant cells to lung fibroblast cells (Figure 1G). While SKOV3 and A2780-dual^{LR} cells exhibited 50% and 59% reduced adherence, respectively, A2780-dual^{ER} and A2780 cells showed a respective reduction of 16% and 12% in adhesion (Figure 1G), suggesting an important role of α6 integrin in conjugation with IGF1R for adequate adhesion of SKOV3 and A2780-dual^{LR} cells to lung fibroblast cells.

4.3.2 Primary lung fibroblast cells induce secretion of S100A4 by chemoresistant EOC cells in turn causing their activation

Our data indicate that the presence of two types of cellular components (cancer cell and fibroblasts) and the microenvironment of the co-culture governs the metastatic properties, involving the IGF1R-α6 pathway. In order to understand the prime factors responsible for increased lung colonization by chemoresistant cells, an extensive secretome profiling was undertaken as follows: set A: Lung fibroblast cell supernatant (fibroblast cell control); Set B: A2780/ A2780-dual^{ER} / A2780-dual^{LR} cell supernatant (cancer cell control); set C: A2780/ A2780-dual^{ER} / A2780-dual^{LR} IGF1R KD cell supernatant (KD control); set D: A2780/ A2780-dual^{ER} / A2780-dual^{LR} cell-lung fibroblast co-culture supernatant; and set E: A2780/ A2780-dual^{ER} / A2780-dual^{LR} IGF1R KD cell-lung fibroblast co-culture supernatant (Figure 2A).

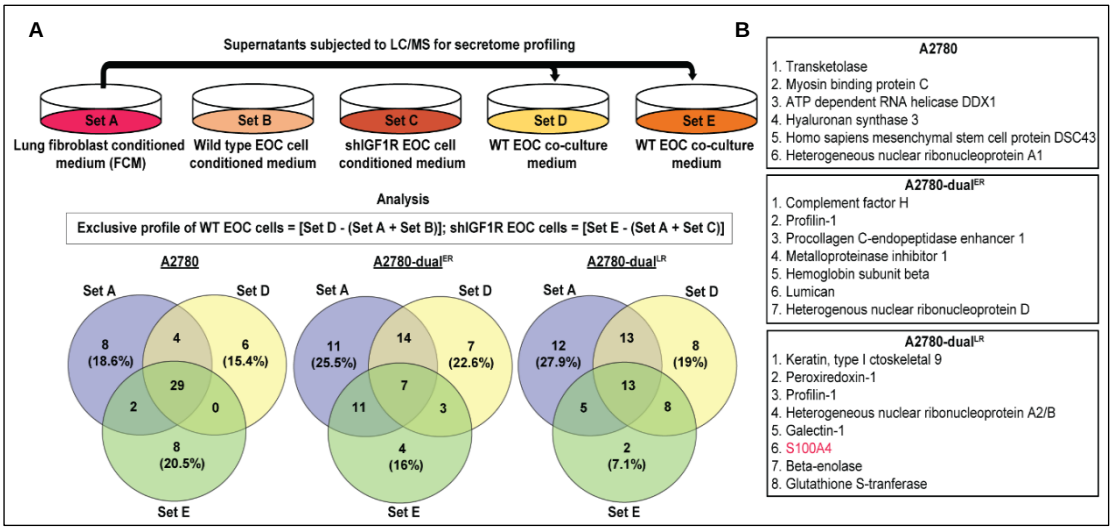

Figure 2. Secretome profiling of co-culture supernatants. Graphical representation of various conditioned media subjected to LC/MS for secretome profiling. The Venn diagram depicts the number/percentage of overlapping and exclusive candidates between lung fibroblast conditioned medium (FCM), wildtype EOC co-culture medium, and shIGF1R EOC-co-culture medium for A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells. B) List of exclusive proteins secreted by A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells under co-culture conditions after subtractive analysis of the co-culture secretome.

To identify the exclusive candidates secreted under co-culture conditions (upon induction with lung FCM) from A2780, A2780-dual^{ER} and A2780-dual^{LR} cells, whose secretion was abrogated after IGF1R knock-down, subtractive analysis was performed by normalizing their co-culture secretome profiles with the secretome profiles of fibroblast and cancer cells (wild type and IGF1R knock-down) (Figure 2A). The identified exclusive candidates secreted by A2780, A2780-dual^{ER}, A2780-dual^{LR} are listed in Figure 5B. A total of eight candidates were found to be exclusively secreted by A2780-dual^{LR} cells (Figure 2B), which includes S100A4 protein, known to be

involved in the metastatic processes such as invasion through MMP secretion, ECM remodeling, and angiogenesis. [416].

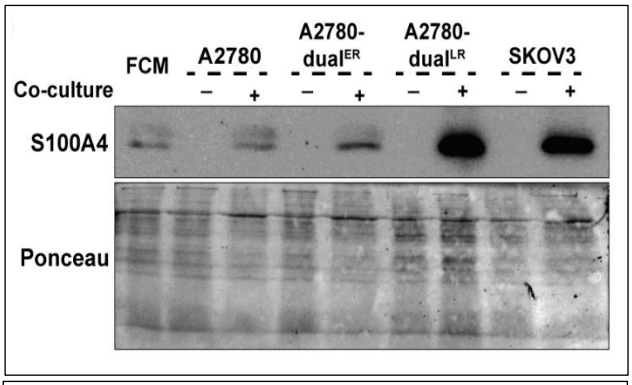

Figure 3. **Validation of secretory S100A4**. Western blot analysis validating abundance of S100A4 secreted from A2780-dual^{LR} and SKOV3 cells under co-culture conditions as compared to that in FCM.

Western blot analysis revealed that the abundance of secretory S100A4 in the co-culture supernatant of A2780-dual^{LR} cells was significantly higher than that of FCM (control) and supernatants of A2780-dual^{ER} and A2780 sensitive cells (Figure 3). Since the amount of secretory S100A4 for A2780 sensitive cells, A2780-dual^{ER} cells, and FCM were quite low (Figure 3), it might not have been detected in our LC/MS analysis. The abundance of secretory S100A4 in the co-culture supernatant of SKOV3 cells was also significantly higher than

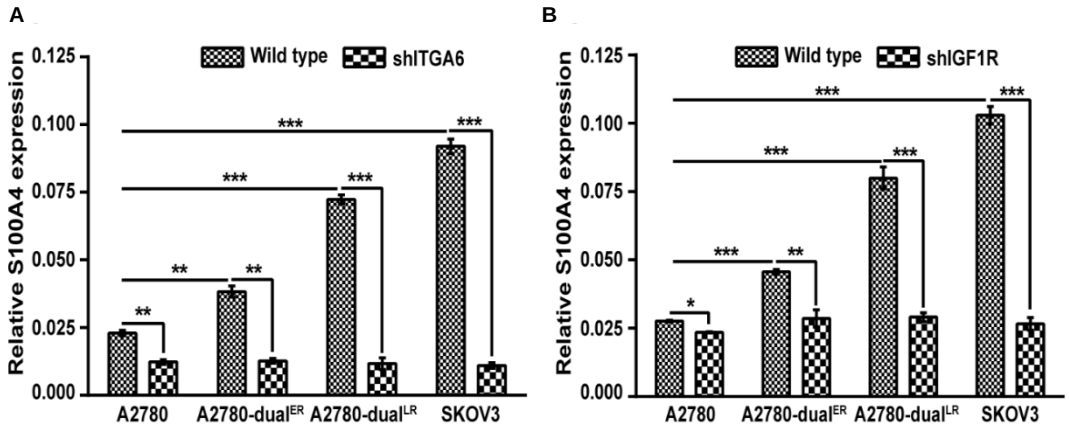

Figure 4. **Knock-down of ITGA6 or IGF1R causes downregulation of S100A4.** The effect of genetic silencing of α6 integrin (A) and IGF1R (B) on the relative expression of S100A4 across A2780 chemoresistant model and SKOV3 cells.

that in FCM, whereas it was almost comparable to that in A2780 dual^{LR} co-culture supernatant (Figure 3).

The α6β4 integrins, which were highly upregulated in chemoresistant cells, are known to regulate S100A4 expression through the NFAT5 transcription factor [417]. In agreement with this, while A2780-dual^{LR} cells showed around 3-fold higher expression, A2780-dual^{ER} exhibited around 1.7-fold higher expression of S100A4 than did the A2780 sensitive cells (Figure 4A-B). Similarly, SKOV3 cells were found to exhibit approximately 4-fold higher expression of S100A4 than the A2780 sensitive cells (Figure 4A-B). After α6 integrin knock-down, a decrease of 8.4-, 6.2-, 3.0- and 1.9-fold in the transcript levels of S100A4 across SKOV3, A2780-dual^{LR}, A2780-dual^{ER}, and A2780 cells, respectively, was observed (Figure 4A). Furthermore, IGF1R silencing, which caused a downregulation of α6 and β4 integrins in our model, also reduced S100A4 expression by 3.9-, 2.8-, 1.6-, and 1.2-fold across SKOV3, A2780-dual^{LR}, A2780-dual^{ER}, and A2780 cells, respectively (Figure 4B).

4.3.3 Genetic and pharmacological silencing of S100A4 suspend lung colonization of late stage chemoresistant EOC cells

To confirm the involvement of S100A4 in lung metastasis, IHC was performed on the metastatic lung tissue harvested from a mouse harboring A2780-dual^{LR} orthotopic xenograft, which showed intense nuclear-cytoplasmic staining of S100A4 specifically in the infiltrated tumor cells, thus confirming the abundance of S100A4 at the metastatic site (Figure 5). We assessed the role of secretory S100A4 in the observed lung metastasis by investigating several features of metastatic cells under genetically

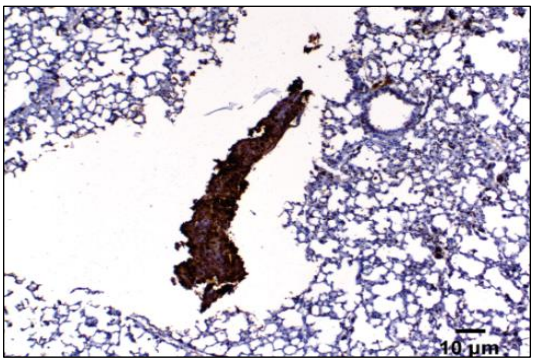

Figure 5. Lung metastatic tissue showing S100A4 staining. Representative IHC image showing nuclear-cytoplasmic staining of S100A4 in the tumor cells in the metastatic lung tissue.

perturbed S100A4 expression. Genetic silencing of S100A4 (Figure 6A) substantially reduced its secretion from A2780-dual^{LR} and SKOV3 cells when co-cultured with fibroblasts but barely affected the secretion from A2780-dual^{ER} and A2780 cells under similar conditions as compared to FCM (Figure 6B). At the secondary site, the interaction of resident fibroblasts with the disseminated tumor cells instruct the activation of fibroblasts which in turn instigates a hospitable environment for the formation of metastases [418]. Therefore, primary lung fibroblast cells were first incubated with the co-culture supernatants (reverse co-culture; Figure 7A) from wild type and S100A4 knock-down of A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells and the subsequent activation of fibroblasts was measured through α-SMA and vimentin expression. After incubation of fibroblast cells with co-culture supernatants of A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, α-SMA transcript levels were observed to be 1.6-, 2-, 2.6-, and 3.2-fold higher, respectively, than the basal levels (Figure 7B). Notably, when the fibroblast cells were incubated with supernatants of S100A4 KD A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, a decrease to the order of 1.3-, 1.5-, 2-, and 1.7-fold was observed in the α-SMA transcript levels (Figure 7B). Vimentin expression also demonstrated a similar trend. After incubation with the co-culture supernatants of wild type A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, the

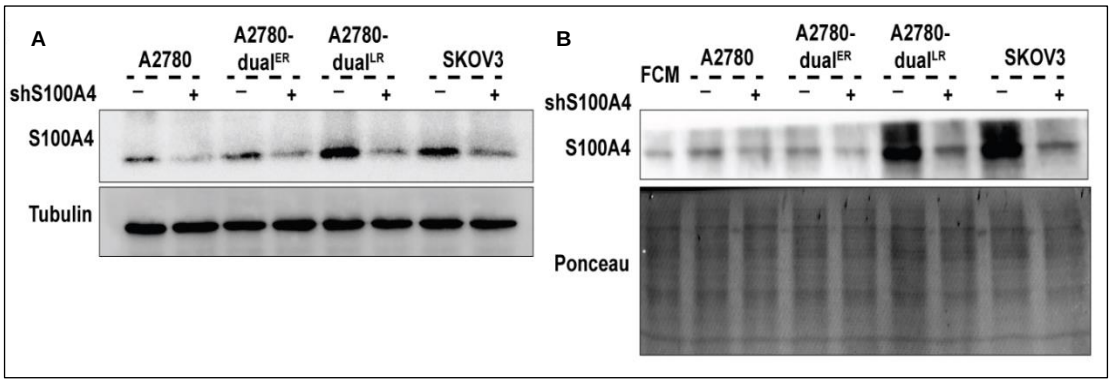

Figure 6. **S100A4 knock-down and its effect on its secretion under co-culture conditions.** A) Representative western blot showing the intracellular expression levels of S100A4 across the A2780 chemoresistant model and SKOV3 cells along with the respective S100A4 knock-downs. B) S100A4 knock-down in A2780-dual^{LR} and SKOV3 cells resulted in significant reduction in the secretion of S100A4 under co-culture with primary lung fibroblast cells.

vimentin expression in lung fibroblast cells was, respectively, 1.5-, 2.1-, 2.4-, and 2.8-fold higher than the basal levels (Figure 7C). On the other hand, upon incubation with the co-culture supernatants of S100A4-silenced A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, the vimentin expression in lung fibroblast cells showed a decrease of 1.2-, 1.8-, 1.8-, and 2.1-fold, respectively (Figure 7C). Activated fibroblasts are known to acquire an invasive phenotype which in turn causes ECM remodeling, an event that supports the growth of disseminated tumor cells. Therefore, the effects of secretory S100A4 on the invasiveness of primary lung fibroblast cells in culture supernatants of wild type and S100A4-KD A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells were compared. Lung fibroblast cells incubated with co-culture supernatants of A2780-dual^{LR} and SKOV3 cells showed 2-fold higher invasion than the control, while approximately 1.5- and 1.3-fold higher invasion rates were observed

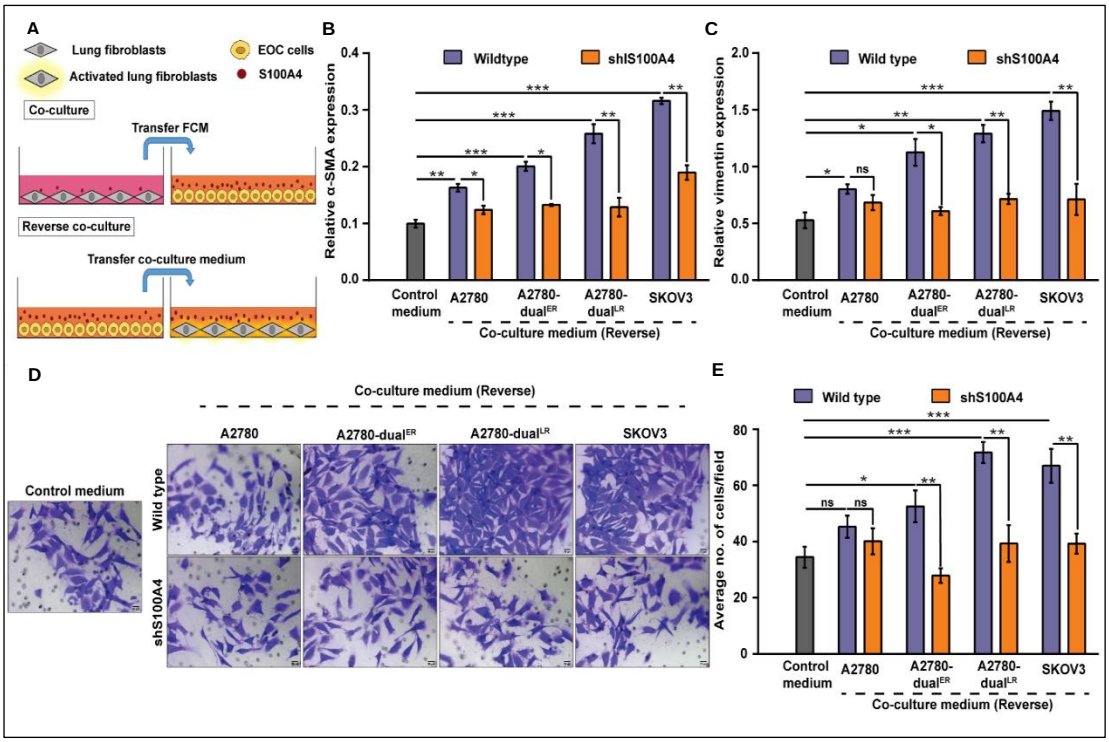

Figure 7. S100A4 secreted from chemoresistant EOC cells under co-culture conditions mediates activation of lung fibroblasts reciprocally. A) Schematic representation of the reverse co-culture strategy designed to evaluate the reciprocal effect of secretory S100A4 on primary lung fibroblast cells. B-C) Assessment of relative expression levels of α-SMA and vimentin in primary lung fibroblast cells under reverse co-culture conditions indicated S100A4-mediated activation of lung fibroblast cells that correlated with the S100A4 levels in the co-culture medium. D-E) Similarly, increasing S100A4 levels in the co-culture medium proportionally increased the invasiveness of lung fibroblasts under reverse co-culture conditions. Data are represented as mean ± SEM. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001.

when these cells were incubated with the co-culture supernatants of A2780-dualER and A2780 cells, respectively (Figure 7D-E). Intriguingly, silencing of S100A4 in A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells led to 1.5-, 1.8-, 1.8-, and 1.7-fold

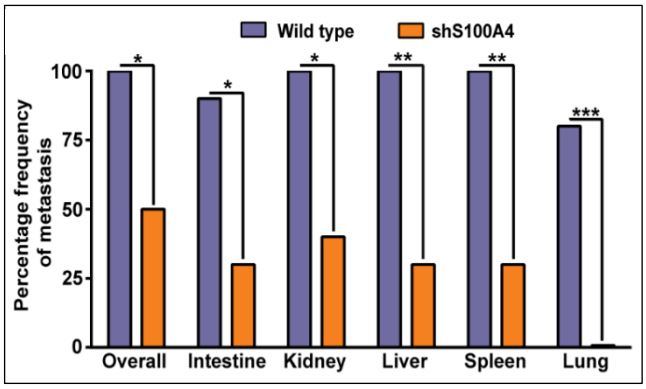

Figure 8. Effect of S100A4 knock-down on the metastatic spread of A2780-dual^{LR} cells. Knock-down of S100A4 reduced the metastatic spread of A2780-dual^{LR} cells with complete abrogation of lung homing. *p<0.05, **p<0.01, ***p<0.001.

lower invasion by fibroblast cells, respectively, in the above-mentioned setting (Figure 7D-E).

Since the inhibition of S100A4 in chemoresistant EOC cells significantly impacted the activation status of lung fibroblast cells *in vitro*, the effect of its inhibition was further evaluated on the organotropic conditioning by analyzing the metastatic colonization of S100A4-KD A2780-dual^{LR} cells *in vivo*. S100A4 KD suppressed the overall metastatic spread of A2780-dual^{LR} cells by 50% (p=0.0325), with complete abrogation of lung metastasis (p=0.0007) (Figure S8). Interestingly, S100A4 KD also caused a 70% reduction in spleen and liver metastasis (p=0.0031) and a 60% reduction in kidney (p=0.0108) and intestinal metastasis (p=0.0198) (Figure S8). Based on these findings, we further utilized a pharmacological approach of S100A4 inhibition and assessed its therapeutic potential. Niclosamide, a routinely used FDA-approved anti-helminthic drug, is a potent transcriptional inhibitor of S100A4 [419]. As compared to genetic silencing, niclosamide treatment was found to

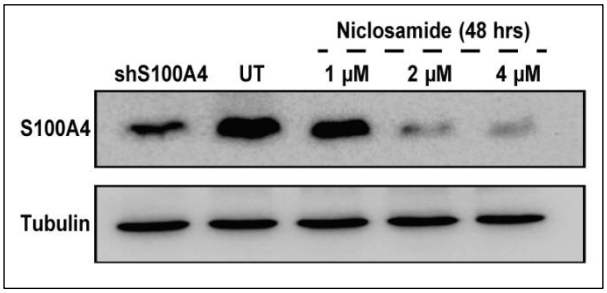

Figure 9. Niclosamide mediated inhibition of S100A4. Dose dependent efficacy of niclosamide in the downregulation of S100A4.

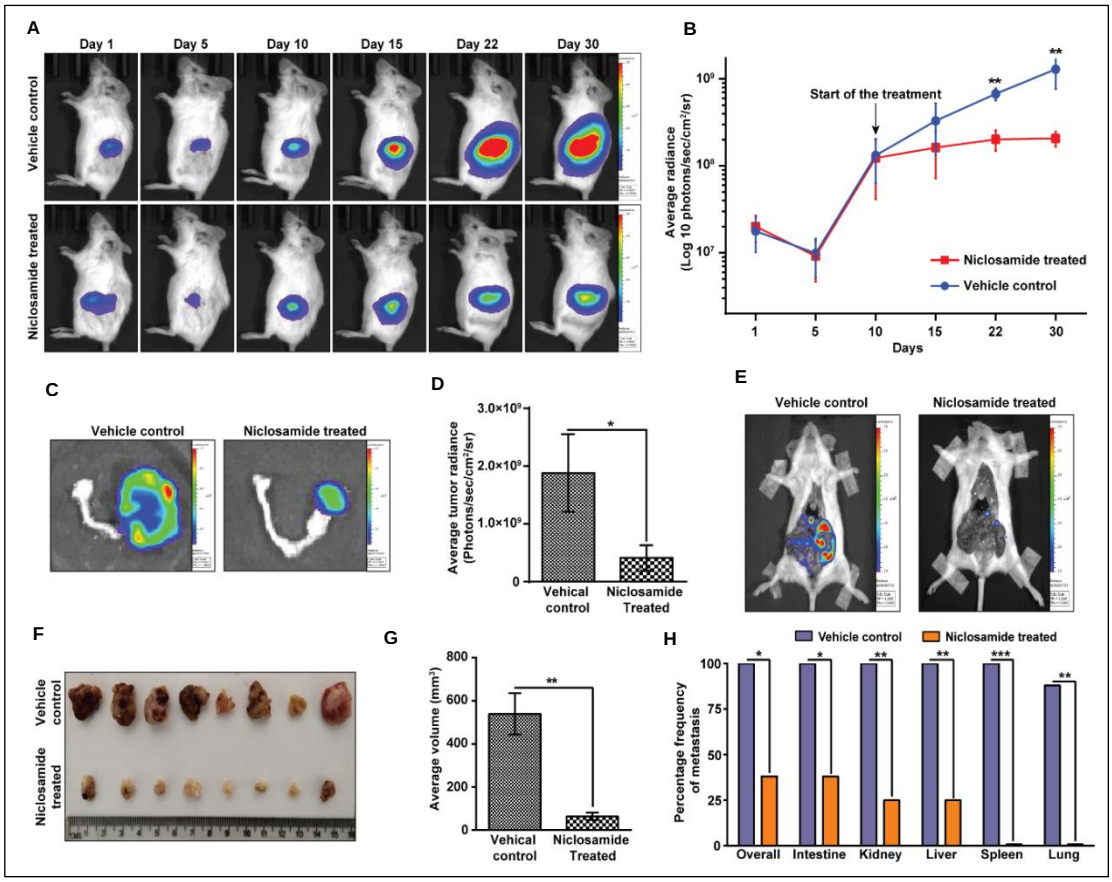

Figure 10. Pharmacologic inhibition of S100A4 abrogates organ-specific spread of A2780-dual^{LR} cells. A-B) As assessed through real-time monitoring using bioluminescence imaging, the A2780-dual^{LR} orthotopic xenograft displayed an exponential growth curve (day 5 onward) in the vehicle-treated group (n=8), whereas that in the niclosamide-administered group (n=8) displayed stunted growth after start of treatment (after day 10). C-D) Representative bioluminescence images along with the quantification of mean bioluminescence intensity showing around 4.5-fold decrease in the signal intensity in the primary tumors of niclosamide-administered group as compared to the vehicle treated group. E) *Post mortem* whole-body bioluminescence imaging of metastatic dissemination clearly showed significantly reduced tumor burden in the niclosamide-administered group as compared to the vehicle-treated control. F-G) Representative photographic image of the primary tumors along with the quantification of mean tumor volume exhibiting

about 9-fold decrease in the tumor mass in the primary tumors of niclosamide-administered group as compared to the vehicle-treated control. H) Pharmacologic inhibition of S100A4 effectively restricted the metastatic dispersal of A2780-dual^{LR} cells and completely abolished the colonization of A2780-dual^{LR} cells to spleen and lungs (n=8 in each group), as determined using Fischer's exact *t*-test. Data are represented as mean ± SEM. ns: non-significant, **p*<0.05, ***p*<0.01, ****p*<0.001.

be highly efficacious in suppressing S100A4 expression (Figure 9). The real time monitoring of the primary tumor using *post mortem* analysis of primary tumors from control vs. treatment group confirmed significant reduction in tumor volume after treatment. While the average bioluminescence intensity of primary tumor was 4.5 times lower in the treatment group than the vehicle control (Figure 10C-D), around 9-fold reduction in tumor volume was observed after niclosamide treatment (Figure 10F-G).

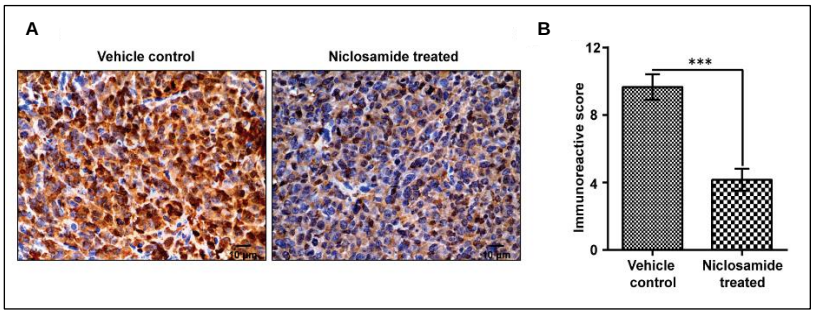

Figure 11. **Effect of niclosamide treatment on S100A4 expression in the primary tumor.** A) Representative IHC images and (B) comparative immunoreactive scores showing decreased nuclear-cytoplasmic S100A4 staining in the niclosamide-treated primary tumors as compared to the vehicle-treated controls.

The effect of downregulation of S100A4 expression after niclosamide treatment was also assessed in primary tumor tissues through IHC retrospectively. Compared to vehicle-treated primary tumors, niclosamide-treated tumors displayed about 2.3-fold reduction in the nuclear-cytoplasmic staining

intensity of S100A4 (Figure 11). The overall metastatic frequency was significantly reduced by 63% in the niclosamide treatment group as compared to the vehicle-treated group (p=0.0256) (Figure 10E,H). While niclosamide treatment resulted in 63% reduction in intestinal colonization (p=0.0256), it caused a 75% reduction in colonization to kidney (p=0.007) and liver (p=0.007) (Figure 10H). Remarkably, the niclosamide treatment caused complete loss of colonization in spleen (p=0.0002) and lung (p=0.0014) for late stage chemoresistant A2780 cells (Figure 10H).

4.4 Discussion

The management of metastatic recurrent EOC is challenging because treatment benefit, even with newer therapies, is seen in <20% of patients [420]. Owing to the decreasing response to platinum-based drugs with successive rounds of treatment and relapse, combinatorial chemotherapy involving the use of non-platinum-based drugs remains the mainstay for the management of metastatic recurrent EOC [421]. Recent studies demonstrating the inexorable contribution of the stromal microenvironment in the regulation of organotropic and chemoresistant traits of tumor cells suggest that targeting the heterotypic interactions between tumor cells and stroma could be a potential avenue that can be explored for the management of the recurrent disease. In this study, we explored this approach by identifying the key determinants (membrane bound and secretory) that govern the interaction of chemoresistant EOC cells with the resistant fibroblasts of distant sites, such as lungs. The heightened propensity of chemoresistant cells for distant organs like lungs was found to be primarily governed by IGF1R–α6 integrin interaction in the late stage of chemoresistance. Destabilization of this interaction through genetic silencing of either IGF1R or α6 integrin significantly reduced attachment of chemoresistant cells (particularly in the late stage) to primary lung fibroblast cells and decapacitated their invasiveness under co-culture. A comprehensive analysis of the secretome profile of EOC cell–primary lung fibroblast co-culture revealed S100A4 as a critical determinant for colonization of chemoresistant EOC cells to distant sites including lungs. Further, genetic and pharmacological inhibition of S100A4 resulted in the complete abrogation of lung metastasis of EOC cells bearing extreme resistance. Our study thus reveals IGF1R–α6 integrin–S100A4 signaling as a key network that intricately controls the metastatic organotropism of

EOC cells during acquirement of chemoresistance, thus revealing new avenues for the treatment of metastatic recurrent EOC through the targeting of S100A4.

In the pre-clinical setting, targeted therapy using IGF1R has proven to be efficacious, but a discouraging response has been observed in the clinical scenario when it is used as a single agent or an adjuvant therapy [422]. The counteractive effect of signaling pathways that overlap with the IGF1R pathway, including insulin receptor, EGFR, HER2, HER3, and integrins (αvβ3 and α6β4), is a primary reason for this response [423-427]. Herein, we observed that the late stage chemoresistant cells, despite having relatively low levels of IGF1R, exhibit maximum co-localization of IGF1R–α6 integrin upon IGF1 stimulation indicating maximal tripartite complex formation and possible activation of downstream signaling pathways. Therefore, more than the distribution of IGF1R alone, it is the relative distribution of these compensatory pathways that may govern the IGF1R driven fate of tumor cells.

To our knowledge, this is the first study that reports a direct interplay of IGF1R, α6 integrin, and S100A4 as a mechanism that governs lung-tropic ability of chemoresistant EOC cells. Hoshino et al. (2015) demonstrated that the tumor cells which metastasize to the lung tissues release exosomes specifically expressing α6β4 integrins. These exosomes upon fusion with the lung fibroblast cells trigger S100A4 upregulation, thus promoting lung colonization of tumor cells [428]. Herein, we observed that the destabilization of IGF1R–α6 integrin interaction in chemoresistant EOC cells through the genetic silencing of either IGF1R or α6 integrin results in the downregulation of S100A4. Since S100A4 is a bona fide target of α6β4 integrins, the downregulation was more severe in the α6 integrin compromised background than the IGF1R knock-down background, in which case the downregulation possibly occurred through the downregulation of α6 and β4 integrins.

Our study demonstrates that the function of S100A4 is indispensable in priming the "soil" at the secondary sites like lungs. The pre-metastatic niche is shaped through a complex interaction (physical as well as secretory) between the seeded tumor cells and the surrounding stroma. Fibroblast cells are an integral component of tumor stroma that play a vital role in proliferation, invasiveness, angiogenesis, and metastasis of cancer and also aid the cancer cells in the formation of a conducive microenvironment at the secondary site on arrival [429]. We showed that lung fibroblasts could induce chemoresistant EOC cells to secrete S100A4, leading to their activation and pre-metastatic niche formation, thereby increasing the metastatic colonization of late stage chemoresistant EOC cells. Previous studies have also highlighted the functional contribution of S100A4 to the formation of pre-metastatic niches by stromal cells. In a study by O'Connell et al. (2011), ablation of S100A4-expressing stromal cells, especially fibroblasts, attenuated the metastasis of breast cancer cells in lungs and that of colorectal cancer cells in liver. These S100A4^{-}positive stromal cells were found to support the development of metastases through the secretion of extracellular matrix proteins and secreted growth factors, such as VEGF-A and Tenascin-C [408]. Similarly, the secretion of S100A4 by T-lymphocytes was found to promote formation of a conducive microenvironment for tumor cells through the induction of cytokines in the lungs of an MMTV-PyMT transgenic breast cancer mouse model.

Clinically, association of S100A4 with distant metastasis has been reported in gastric and thyroid cancer patients [430, 431]. Studies have demonstrated that high levels of circulating S100A4 protein or transcripts correlate with an advanced FIGO stage and shorter progression-free and overall survival in EOC patients, but the underlying molecular mechanism and association with distant metastasis and chemoresistance were not investigated [432, 433]. In this study, we reveal a plausible IGF1R–α6β4

integrins–S100A4 axis steering the metastatic spread of chemoresistant cells in a pre-clinical model. Future studies assessing the correlation of upregulated S100A4 expression with distant metastases in EOC patients will prove helpful in validating the role of S100A4 in the clinical scenario. Currently, a pharmacologic approach for S100A4 inhibition using niclosamide is being explored under phase II clinical trial (the NIKOLO trial: NCT02519582) for the treatment of metastatic chemorefractory colorectal carcinoma [434]. Our findings may pave the way for the clinical translation of niclosamide in the treatment of recurrent metastatic EOC as well.

Chapter 5

Analyzing the IGF1R expression in matched paired primary and omental metastatic tumor specimens obtained from patients with advanced stage ovarian carcinoma

Chapter 5

5.1 Introduction

Among all the major subtypes of epithelial ovarian cancer (EOC), the high grade serous subtype has the highest prevalence, accounting for nearly 75%-80% of the total malignant ovarian neoplasms with an epithelial origin [435]. Due to lack of serum- or tissue-based biomarkers that can reliably detect early signs of tumor progression and the manifestation of atypical symptoms resembling common gastrointestinal or gynaecological dysfunction, the diagnosis of high grade serous epithelial ovarian cancer (HGSOC) is significantly delayed [436]. As a result, more than 75% of the patients are diagnosed at an advanced stage, by which time the disease has already spread to the peritoneal organs [437]. The first-line treatment, on account of the vast spread of the disease, consists of a cisplatin-platinum combination-based neoadjuvant chemotherapy (NACT), which is followed by radical tumor debulking surgery to achieve minimal residual disease [438]. Despite promising clinical response to the first-line therapy, almost 70%-80% of patients eventually relapse owing to acquired chemoresistance, and their response to the successive lines of treatment is sub-optimal at best [439, 440]. Consequently, high grade serous ovarian cancer (HGSOC) is the most lethal subtype of ovarian cancer with a 5-year survival rate that barely reaches 30% [435, 441]. Even with the considerable development in the field of chemotherapeutics and targeted therapies, the cure rate for HGSOC patients has unfortunately remained unimproved, which can be attributed in part to the unavailability of biomarkers that can prospectively predict therapy response and associated risks of disease recurrence in patients [442].

Currently, CA125 is considered as the most credible biomarker for EOC for diagnostic purposes and is also used to monitor the treatment response in these patients [443, 444]. However, the reliability of CA125 as a standalone marker is under question. Clinical studies have demonstrated that up to 20% of advanced stage EOC patients do not exhibit

increased serum CA125 levels [445]. Non-cancerous gynecologic pathologies such as follicular cysts, benign neoplasms, endometriosis, and cystadenomas can also contribute to the modulations in serum CA125 levels, especially in pre-menopausal patients [446, 447]. Moreover, CA125 levels have also been known to be affected by the nature of the treatment regimen administered. [448]. Despite it being the biomarker of choice for the diagnosis and prognosis of EOC for decades, the use of CA125 levels as a biomarker for therapy response has not contributed significantly toward the cure rate for EOC. Thus, the development of new biomarkers for the prediction of treatment outcomes in HGSOC patients is long due [449]. Till date, HE4, mesothelin, M-CSF, CEA, Bikunin, Osteopontin, β-hCG, α-fetoprotein, and LDH are some of the best-known, potential serum-based biomarkers for EOC [450-452]. In addition, a wide range of tissue-based biomarkers, including receptor tyrosine kinases like EGFR and VEGFR, and other additional markers including K-cadherin, KISS1, Ki-67, claudins IGF-2, Matriptase, PAX8, WT1, TROP2, PR, p21, SLPI, p53, MMPs, EpCam, F-Spondin, and ER have been evaluated for their prognostic value in HGSOC [450, 451]. Similarly, several drug influx (SLC family proteins like hCtr1) and efflux transporters (ABC and ATP family proteins) have also been investigated for their prognostic merit for HGOSC, on account of their modulation observed in the chemo-treated tumor samples exhibiting the multidrug resistant phenotype [453-457]. However, none of these serum- or tissue-based biomarkers have been able to surpass the validity of CA125 [363]. While the global mortality of HGSOC continues to rise, there is an urgent demand for the development of novel biomarkers that can prospectively predict survival outcomes for these patients in a given therapeutic setting. IGF1R is one of the emerging biomarkers being explored for its prognostic value across different types of cancer.

IGF1R signaling plays a central role in the regulation of common homeostatic functions in almost all tissues under normal physiological conditions [354, 458]. IGF1R overexpression is often observed in primary and metastatic tumors across several cancers, including colon, prostate, pancreatic, melanoma, medulloblastoma, and ovarian cancer [35, 271, 272, 459]. Although IGF1R overexpression is often observed in EOC, its prognostic merit for HGSOC remains to be investigated. In the previous chapters (2-4), we described the implications of IGF1R in the regulation of organotropic nature of chemoresistant EOC cells and underlying metastatic properties. This chapter describes the expression profile of IGF1R, as assessed using immunohistochemistry (IHC), in primary and paired omental metastatic tumor tissues that were harvested from 19 HGSOC patients and the prognostic merit of IGF1R therein.

5.2 Methodology

5.2.1 Patient cohort and tumor sample collection

In this study, 19 patients diagnosed with advanced stage HGSOC who were scheduled for NACT were recruited with informed consent between May 2013 and May 2017. After administration of NACT, matched primary and omental metastatic tumor samples were collected during the radical debulking surgery from all 19 patients. The patients in this cohort were followed-up for 3 years for clinical progression of the disease and survival status.

5.2.2 Immunohistochemistry (IHC)

IHC for IGF1R and hCtr1 was carried out as previously described in chapter 4 methodology section 1.2.4 with some modifications. For IGF1R, heat-induced epitope retrieval was carried out in sodium isocitrate buffer (pH 6) at high power for 20 min in a microwave, whereas for hCtr1, the tissue sections were boiled in sodium isocitrate buffer (pH 6) in a pressure cooker for 6 min. Next, the sections were incubated with the primary antibodies (dilution; incubation) of IGF1R (undiluted; at 37℃ for 60 min) and hCtr1 (1:200, at 4℃ for overnight), after which they were subjected to secondary antibody treatment and developed using HRP DAB as described previously. While for IGF1R IHC, A2780-dual^{ER} (possessing high IGF1R expression) tumor xenograft served as a positive control, human colon tissue was used as a positive control for hCtr1. In both the cases, secondary control was used as a negative control. Blinded grading was performed based on the intensity and the extent of positivity, as scored by an experienced pathologist. The immunoreactive score was calculated using the formula: intensity × extent of positivity (as scored by an experienced pathologist)) [460]. The

H-score was calculated using the IHC profiler with the formula: H-score= [(3 × % high positive cells) + (2 × % moderate positive cells) + (1 × % low positive cells) [461].

5.2.3 Statistical analysis

The tissue based IGF1R levels between the primary and omental metastatic tumors were compared using student's *t*-test. While the overall survival (OS) was considered as the duration between the time of registration and death or last follow-up, the disease-free survival (DFS) was considered as the duration between date of registration and occurrence of relapse or death. Kaplan–Meier and log rank test, respectively, were used to determine and compare the OS and DFS; in addition, bivariate analysis was conducted to study the correlation between IGF1R and hCtr1 immunoreactive scores using IBM SPSS statistics 21.0 software. *p*-value <0.05 was considered as statistically significant.

5.3 Results

5.3.1 Clinico-pathological features of the patient cohort and survival

Of the 19 advanced stage HGSOC patients that were recruited in this study, 8 patients were deceased, 8 patients showed relapse, and 3 patients were asymptomatic at the end of the follow-up period (Table 1). For this cohort having a mean age of 55.95 years, the median OS (Figure 1A) and DFS (Figure 1B) were observed to be 33.25 and 16.07 months, respectively, and the median follow-up was of 28.71 months. Table 1 summarizes the clinico-pathological features and survival outcomes for individual patients.

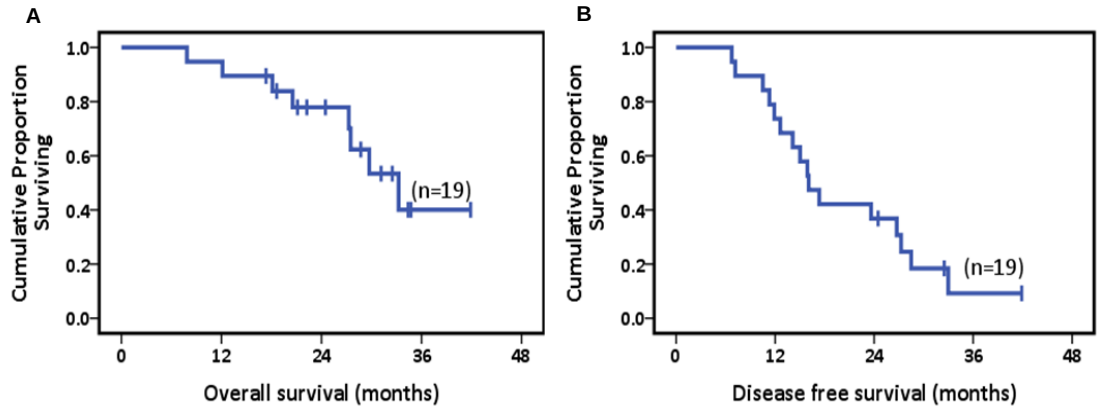

Figure 1. Survival analysis of patients. A) Kaplan–Meier overall survival (OS) curve of HGSOC patients with median OS of 33.25 months. B) Kaplan–Meier disease free survival (DFS) curve of HGSOC patients with median DFS of 16.07 months.

5.3.2 IGF1R expression in primary and metastatic tumor tissues and its prognostic merit

Although all 19 omental metastatic samples had viable tumor cells, in four cases (P1, P7, P10, P19), no viable primary tumor tissues were left. Therefore, IHC could only be-

Table 1. Clinico-pathological features and survival outcomes for HGSOC patients recruited in this study (n=19).

Patient No.	Age	Stage	Status	Overall survival (months)	Disease free survival (months)
P1	62	IIIC	Death	20.53	11.3
P2	56	IIIC	Death	33.25	12.62
P3	42	IV	Asymptomatic	41.89	41.89
P4	65	IIIC	Death	27.5	10.51
P5	59	IIIC	Death	18.1	11.89
P6	52	III/IV	Death	12.12	7.16
P7	46	III/IV	Relapse	34.73	28.48
P8	45	IV	Relapse	34.4	32.99
P9	63	III/IV	Death	29.73	14.13
P10	71	III/IV	Asymptomatic	32.49	32.49
P11	65	IV	Relapse	17.35	17.35
P12	64	IIIC	Relapse	21.13	16.07
P13	64	III/IV	Relapse	18.63	15.9
P14	65	IIIC	Relapse	31.15	26.74
P15	33	III/IV	Relapse	28.71	23.62
P16	61	III/IV	Death	27.27	27.27
P17	53	IIII/IV	Death	7.85	6.74
P18	51	III/IV	Relapse	22.24	15.01
P19	46	III/IV	Asymptomatic	24.48	24.48

performed for 15 primary and 19 metastatic tumor samples. While a membranous cytoplasmic staining pattern was observed for IGF1R, the cases were graded and scored only on the basis of membranous staining as membrane bound IGF1R represents the active form (Figure 2A). Assessment using immunoreactive score or H-score of relative IGF1R expression between the primary and matched metastatic omental tissues revealed no significant differences (Figure 2B-C).

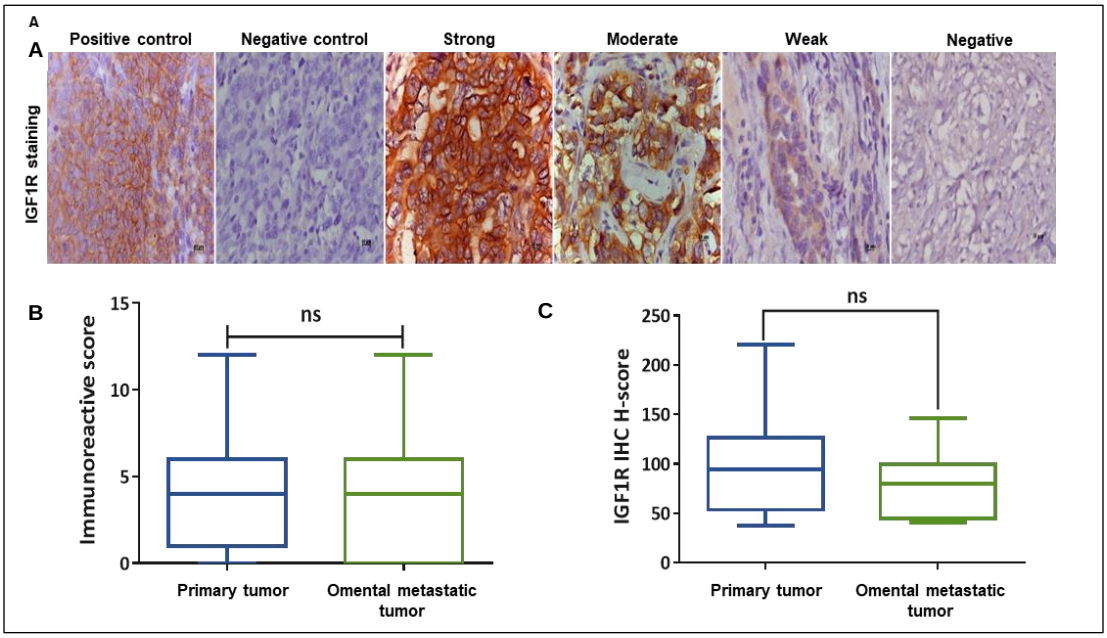

Figure 2. Primary and metastatic tumors exhibit equivalent expression of IGF1R. A) Representative images of IGF1R IHC with differential staining intensities along with the respective positive and negative controls. Average immunoreactive score (B) and average H-score (C) revealed no difference between the membranous expression of IGF1R between the primary and metastatic tumors. ns: non-significant.

Further, to assess the prognostic merit of IGF1R, the cohort was subdivided into IGF1R high (more than median IGF1R expression) and IGF1R low (less than median IGF1R expression) groups, and the OS and DFS were compared between these two groups. The median value of IGF1R expression was used as a cut-off, since the cut-off value for basal levels of IGF1R expression in HGSOC patients is undefined. As determined using the log rank test, patients with an immunoreactive score that was higher than the median for primary tumors showed better OS (OS: 33.2 months) and DFS (15.901 months) than those with an immunoreactive score less than the median [OS: 27.27 months (p = 0.2); DFS: 15.014 months (p = 1.0)]. A similar trend was observed for

metastatic tumors as well {more than median [OS: median not reached; DFS: 26.743 months] vs less than the median [OS: 33.248 months (p = 0.734); DFS: 15.014 months (p = 0.445)], respectively}. However, in each case, the associations were statistically non-significant.

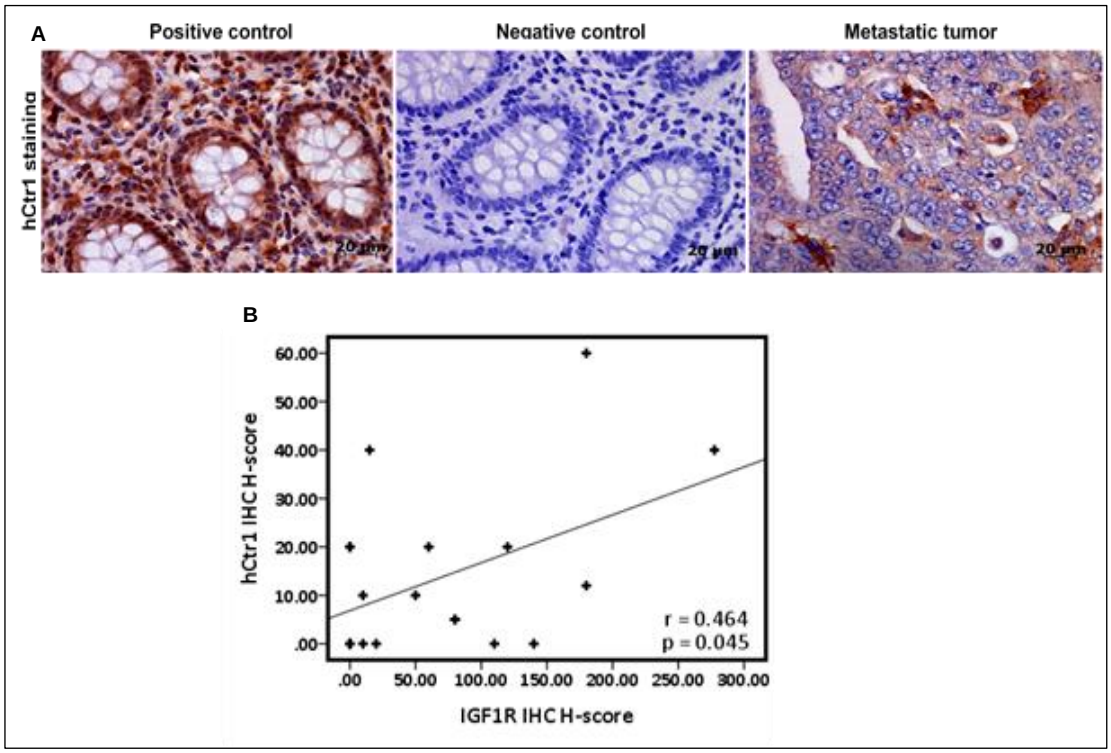

Figure 3.hCtr1 positively correlates with IGF1R. A) Representative images of hCtr1 IHC performed on the metastatic tumor tissue with respective positive and negative controls. B) Bivariate analysis showing significant positive correlation (r = 0.464) between IGF1R and hCtr1 (p = 0.045).

5.3.3 Tissue based IGF1R expression positively correlates with hCtr1 levels

The accumulation of chemotherapeutic drugs and therefore the efficacy of adjuvant chemotherapy is often dictated by the expression profiles of drug transporters [462, 463]. hCtr1, which is a human copper transporter protein, is a major transporter responsible for influx of platinum based drugs in tumor cells [464]. Conversely, pre-

clinical and clinical studies of cervical and ovarian cancers demonstrated that the downregulation of hCtr1 leads to resistance against platinum-based drugs and correlates with poor prognosis [456, 465]. In our study, since the upregulation of IGF1R indicated improved survival of HGSOC patients (in terms of OS) with improved therapy response (in terms of DFS), we further assessed its correlation with hCtr1 through IHC. As IGF1R expression was comparable between the primary and metastatic tumor samples, the hCtr1 IHC was performed only on the metastatic tumor samples (Figure 3A) due to larger sample size (n=19). As determined using Pearson's correlation coefficient, the hCtr1 H-score positively correlated with that of IGF1R (r=0.464), and this correlation was found to be significant (p=0.045) (Figure 3B). Further, we also assessed the prognostic role of hCtr1 by comparing OS and DFS between the hCtr1 high (more than median hCtr1 expression) and hCtr1 low (less than median hCtr1 expression) sub-groups of the cohort. As expected, patients, whose IHC scores were greater than the median, exhibited better OS and DFS (OS: median not reached; DFS: 16.066) than patients whose IHC scores were less than the median [OS: 33.248 months (p = 0.724); DFS: 15.901 months (p = 0.365)] However, these differences were statistically insignificant.

5.4 Discussion

Prognostic markers are essential phenotypes that can be used for timely prediction of therapeutic response and survival outcomes, facilitating better management of diseases [466]. Since the discovery of CA125 as a prognostic marker for EOC in 1981, several serum- and tissue-based biomarkers have been explored to predict NACT-related performance status and risk of relapse [450]. However, as standalone markers, none of the currently available biomarkers, including CA125, have been able to provide an unambiguous prospective prediction of disease free interval for HGSOC patients [467, 468]. To address this unmet need, we assessed the prognostic merit of IGF1R in prospectively predicting the overall and disease-free survival outcomes of HGSOC patients who were administered NACT treatment. Our data demonstrated that the primary and omental metastatic tumors harvested from HGSOC patients after NACT exhibit comparable levels of IGF1R expression, and increased IGF1R expression predicts longer OS and DFS, which could likely be due to the positive association between IGF1R and the drug influx protein hCtr1.

Previous studies assessing the prognostic merit of IGF1R have reported contradictory findings. While overexpression of IGF1R correlated with better survival outcomes for patients with soft tissue sarcoma, upregulation of IGF1R in cervical and non-small cell lung carcinoma patients predicted poor prognosis [317, 469, 470]. A similar discrepancy has also been reported regarding the subtypes of breast cancers. These reports indicate that the prognostic nature of IGF1R greatly varies not just within different tumor types but also within the subtypes of the tumors [471, 472]. In the exploratory study that we performed on a homogeneous cohort of patients with HGSOC, we found that high levels of IGF1R expression in the primary or metastatic tumor tissues correlated with improved OS and DFS. However, this association was

statistically insignificant, which was likely a result of the small sample size. Therefore, further investigation with a larger cohort selected with careful consideration of subtype specificity is warranted to accurately establish the prognostic role of IGF1R as a tissue-based biomarker in patients with HGSOC.

To the best of our knowledge, this is the first study to report a direct association between IGF1R and the drug influx protein hCtr1, which may explain the prognostic ability of IGF1R to predict longer survival. In literature, some studies have independently proposed a number of common regulators for hCtr1 and IGF1R genes. For example, the miRNA hsa-mir-98-5p has been shown to negatively regulate both IGF1R and hCtr1 genes [473]. In addition, the transcription factor Sp1, a well-known positive regulator of IGF1R, has been demonstrated to act as a copper sensor by regulating hCtr1 expression through its zinc finger domain [474]. However, the exact molecular mechanism underlying this association between IGF1R and hCtr1 remains to be investigated.

Overall, our study provides a preliminary assessment of the prognostic merit of tissue-based IGF1R for HGSOC patients. In the future, mechanistic studies exploring the positive correlation between IGF1R and hCtr1 may help unravel the protective effects of IGF1R in EOC.

Chapter 6

Summary and Conclusion

Chapter 6. Summary and Conclusion

Emergence of chemotolerant distant metastases is a lingering plight of therapeutic strategies aimed toward management of recurrent cancers, which continues to remain without a defined course of therapeutic intervention [147, 420]. As much as 90% of cancer-related deaths are a result of clinical manifestation of metastases or the repercussions of therapies administered for the treatment of these chemoresistant metastases [5, 365]. Since Stephen Paget's "seed and soil" hypothesis (1889), several attempts have been made to delineate the selective forces that drive the colonization of chemoresistant tumor cells at distant sites, such as liver, bone, lungs, and brain, during relapse [163, 476]. Clinical observations documented over decades and recent pre-clinical experimental findings provide multiple lines of compelling evidence suggesting that systemic chemotherapy accelerates the metastatic dissemination of tumor cells to distant sites [312]. Consequently, the remedial effects of chemotherapy efficaciously rendered at the primary tumor site are counteracted by the pro-metastatic effects of chemotherapy that modulate the intrinsic metastatic properties of the "seed" (tumor cells) and the congenial nature of the "soil" (microenvironment of the distant organs), thus maneuvering the metastatic course of the disseminated tumor cells at these vital organs, leading to dismal clinical outcomes. Understanding the molecular networks underlying this complex phenomenon is imperative in order to impede the double-edged sword effects of chemotherapy and develop targeted intervention strategies for cancers exhibiting overwhelming incidence of metastatic recurrence, such as epithelial ovarian cancer (EOC).

Nearly 70% of patients with EOC show relapse after chemotherapy, owing to the acquirement of chemoresistance, wherein the occurrence of extraperitoneal metastasis is commonly observed in the clinics [163, 476, 477]. Although distant metastasis is a well-observed manifestation of recurrent EOC, the organotropic behavior of EOC cells

and the underlying differential metastatic properties that are perturbed due to acquired chemoresistance remain elusive. Moreover, in the clinical setting, certain limitations hinder the comprehensive investigation into the dissemination patterns of chemoresistant EOC cells. First, patients are immediately treated with second/third-line therapy with non-platinum agents after the confirmation of relapse. Second, biopsy of metastatic tissues located in vital organs, like liver and lung, is extremely difficult. Finally, for patients with multiple recurrences and distant metastases, long-term follow-up is challenging because of extremely poor prognoses. Therefore, the development of animal models in which the unusual progression of chemoresistant EOC cells can be tracked at the cellular and molecular levels is an urgent necessity.

Previously, we developed a cisplatin-paclitaxel dual chemoresistant model of A2780 cell line that mimics the distinct phases of acquired chemoresistance from sensitive (A2780) to onset of resistance (early resistant; A2780-dual^{ER}) to extreme resistance (late resistant; A2780-dual^{LR}) [284]. The striking characteristic of this cellular model is the oscillatory pattern of IGF1R expression, which was observed to escalate in the early stage of resistance with increased MAPK/ERK (A2780-dual^{ER}) signaling and declined in the late stage of resistance (A2780-dual^{LR}) with increased PI3K/AKT signaling [284]. Several researchers, including our group, have demonstrated that the inhibition of IGF1R signaling causes resistance reversal in ovarian cancer [271, 284]. Further, IGF1R signaling has also been implicated in the regulation of organ-selective colonization of several cancers to distant sites *in vivo* [281-283]. Since these studies underline the involvement of IGF1R signaling in the establishment of chemoresistance as well as organ-specific metastasis independently, IGF1R signaling may serve as a common regulatory axis that steers the organ-selective dissemination of EOC cells while governing the chemoresistant phenotype of these cells. Moreover, the role of

IGF1R in the propagation of chemoresistant EOC cells remains to be established. Therefore, in this study, we attempted to decipher the molecular mechanisms driving the evolving landscape of chemoresistant metastases regulated through IGF1R signaling using animal models of sensitive and chemoresistant A2780 EOC cells.

In order to investigate the impact of acquired chemoresistance on the organotropic behavior of EOC cells, we monitored the metastatic dissemination of A2780 EOC cells at various stages of chemoresistance using intra-peritoneal and orthotopic xenograft mouse models through non-invasive optical imaging. This *in vivo* mapping revealed a positive correlation between the chemoresistant stage of A2780 EOC cells and their propensity to metastasize distant sites such as kidneys, spleen, and notably lungs, which is a comparatively rare site of metastasis for EOC cells. A comparative analysis of the secondary sites colonized by wild type A2780 EOC cells and their IGF1R-KD counterparts showed that IGF1R signaling is involved in the colonization process of chemoresistant EOC cells at these sites particularly in the late stage (A2780-dual^{LR}). Further, *in vitro* investigation of the key events of metastasis using primary lung fibroblast cells revealed that the inhibition of IGF1R attenuates the adhesion of chemoresistant cells toward resident lung fibroblast cells also abating their responsiveness to the pro-invasive secretory cues of the lung fibroblast cells. Therefore, IGF1R signaling was found to play a central role in the establishment of initial key events of metastasis of chemoresistant EOC cells, involving the stromal components of the microenvironment at distant sites, like lungs. In addition, to compare between the metastatic properties of acquired and intrinsic resistant EOC cells, we also assessed the metastatic properties, governing the early events of metastasis, of the SKOV3 cell line (inherently resistant to cisplatin) and observed that the metastatic properties of SKOV3 cells resemble those of late-stage chemoresistant EOC cells (A2780-dual^{LR}).

Although the late-stage chemoresistant cells (A2780-dual^{LR}) exhibited relatively lower expression of IGF1R than the early stage chemoresistant EOC cells (A2780-dual^{ER}), IGF1R inhibition more severely affected the organ-selective behavior and the underlying metastatic properties of late-stage chemoresistant cells. Intrigued with this observation, which suggested a possibility of involvement of another signaling pathway, we explored the role of α6β4 integrin, which is known to co-operatively regulate IGF1R signaling by forming a tripartite complex with IGF1R in the presence of IGF1 ligand and activating downstream (MAPK/ERK and PI3K/AKT) pathways [415]. α6 integrin–IGF1R co-localization studies performed as a measure of this tripartite complex formation indicated that the IGF1-induced cooperativity between IGF1R and α6 integrin signaling was highest in the late-stage chemoresistant cells (A2780-dual^{LR}), which may have compensated for the low levels of IGF1R in these cells. Destabilization of this interaction through genetic silencing of either IGF1R or α6 integrin significantly reduced attachment of chemoresistant cells (maximum attenuation in the late stage) to primary lung fibroblast cells and decapacitated their invasiveness under co-culture conditions, indicating that IGF1R–integrin dual signaling is instrumental in establishing the key metastatic events with the resident fibroblast cells. On account of the IGF1R-mediated enhanced responsiveness of chemoresistant EOC cells to the pro-metastatic secretory cues of lung fibroblast cells, a comprehensive analysis of the secretome profile of EOC cell–primary lung fibroblast co-culture was undertaken. These data revealed exclusive secretion of S100A4 from late-stage chemoresistant (A2780-dual^{LR}) and SKOV3 cells, which reciprocally aided in the activation of lung fibroblast cells. Further, genetic and pharmacological inhibition of S100A4 revealed that this reciprocal function of S100A4 was vital for the organotropic

nature of chemoresistant EOC cells, underlining their colonization to distant sites, such as lungs, particularly in the late stage of chemoresistance.

In addition to the role of IGF1R in the regulation of differential metastatic properties of chemoresistant EOC cells, we also measured the IGF1R expression in chemo-treated primary and metastatic tumors harvested from a small cohort of EOC patients with high grade serous subtype, in order to assess the prognostic merit of IGF1R. Although statistically insignificant, high levels of IGF1R expression in the primary or metastatic tumor tissues correlated with improved overall (OS) and disease-free survival (DFS). Interestingly, a positive correlation was observed between the expression of IGF1R and that of hCtr1 (a platinum influx drug transporter) in these tissues, which could rationalize its merit in predicting longer survival in these patients.

In conclusion, this study evinced IGF1R–α6 integrin–S100A4 signaling network as a critical determinant for the organ-specific metastases of platinum-taxol resistant EOC cells. Herein, S100A4 was identified as a key molecule that plays a central role in the colonization of chemoresistant cells to distant sites, such as lungs, which can be therapeutically targeted for improving the clinical outcome of recurrent EOC.

Chapter 6. Summary and Conclusion

SYNOPSIS

Homi Bhabha National Institute

SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Abhilash Nitin Deo

2. **Name of the Constituent Institution:** Tata Memorial Centre – Advanced Centre for Treatment Research and Education in Cancer

3. **Enrolment No. :** LIFE09201504017

4. **Title of the Thesis:** Role of IGF1R in Ovarian Cancer Metastasis

5. **Board of Studies:** Life sciences

Introduction

Ovarian cancer (OC) is the third leading cause of cancer related deaths in India and seventh worldwide among women, with the epithelial subtype being predominant in more than 90% of cases [1, 2]. The peculiar feature of epithelial ovarian cancer (EOC) that makes it stand out among other epithelial cancers is the nature of its metastasis. Owing to the absence of physical boundaries, EOC primarily disseminates in the peritoneal cavity, as the tumor cells are shed into the surrounding peritoneal fluid [3]. The circulatory movement of peritoneal fluid mediates the dispersal of tumor cells across the abdomen, making it highly metastatic in nature. Moreover, in majority of the patients, the disease is diagnosed at an advanced stage (III/IV) due to diffuse symptoms and a lack of early detection markers [4]. As a result, nearly 70% of patients with advanced stage EOC exhibit vast spread of tumor cells with colonization in the omentum, colon, and pelvic organs at the time of diagnosis [5]. Due to this expansive spread and immense tumor burden, these patients are treated with neoadjuvant chemotherapy and radical surgery in succession to achieve optimal tumor debulking [6].

Although most EOC patients initially respond to the cornerstone therapeutic regimen of cisplatin and paclitaxel in neoadjuvant settings, nearly 70% of them develop resistance to platinum after administration of adjuvant therapy [7]. Consequently, these patients relapse with metastatic disease with a five-year survival rate of less than 30%. At relapse, in nearly 85% of the patients, the metastatic spread is confined to the peritoneal cavity encompassing the peritoneum, omentum, and pelvic organs [8]. However, in some cases, the tumor cells seed in liver parenchyma, spleen, lungs, breast, and even brain, developing into distant metastases that show worst prognosis [8-20]. **Despite the development of targeted therapies and multidisciplinary**

approaches over the last decade, neither a significant improvement in the rate of relapse nor a cure for the recurrent disease have emerged. The cellular processes and underlying mechanisms responsible for distant or organ specific metastasis of chemoresistant ovarian cancer cells remain inadequately understood. In clinical settings, once relapse is confirmed, the patients are immediately treated with second/third line therapy. Biopsy of the metastatic tissues located in organs like liver, lung, and brain is extremely difficult. Moreover, for patients with multiple recurrences and/ distant metastases, long-term follow up is challenging due to extremely poor prognosis. Therefore, there is a dire need to develop animal models in which the unusual progression of chemoresistant OC cells can be studied at the cellular and molecular levels in order to discern the underlying mechanisms and facilitate the development of therapeutic intervention strategies for metastatic recurrent ovarian cancer.

We previously established a clinically relevant cisplatin–paclitaxel dual chemoresistant model in the A2780 OC cell line that mimics the dynamic stages of acquired chemoresistance. As reported earlier, this model shows an oscillatory expression of insulin like growth factor 1 receptor (IGF1R), wherein the basal expression of IGF1R in sensitive cells escalates in the early stage and declines in the late stage of resistance [21]. IGF1R, a well-known tyrosine kinase receptor, regulates various normal developmental pathways and malignant properties, such as proliferation, migration, invasion, and chemoresistance across several types of cancer, including ovarian cancer [22, 23]. Our previous findings have demonstrated that the inhibition of IGF1R signaling causes sensitization of resistant tumor cells in ovarian cancer [21]. IGF1R signaling has also been implicated in the colonization of colon

cancer cells to liver, osteosarcoma cells to lung or liver, and breast cancer cells to lungs *in vivo* [24-26].

Rationale

In order to target the metastatic tumor population that is indolent to the chemotherapeutic treatment, it is important to decipher the signaling axis that imparts differential metastatic properties to chemoresistant OC cells. Since IGF1R signaling is known to regulate both these phenotypes, it may serve as a regulator for differential metastatic properties of chemoresistant OC cells. Moreover, in depth understanding of how IGF1R controls organ specific colonization chemoresistant OC cells might reveal therapeutically relevant molecular targets.

Hypothesis

We hypothesized that IGF1R signalling, perturbed owing to chemoresistance, imparts differential metastatic properties to chemoresistant ovarian cancer cells, leading to metastatic organotropism.

The study has been designed with the following key questions:

Key questions

1. Does acquirement of chemoresistance influence the metastatic properties of ovarian cancer cells? If so, which steps of peritoneal metastasis (adhesion, invasion, and organ homing potential) are perturbed due to resistance?

2. Does IGF-1R regulate metastatic properties of chemoresistant ovarian cancer cells?

3. What is the status of IGF-1R expression in primary and metastatic tumor specimens from advanced stage ovarian carcinoma patients?

Based on these key questions, the following specific objectives were designed:

Objective 1: Monitor the metastatic potential of sensitive vs. early and late resistant cells *in vitro* and *in vivo* using optical imaging

To understand the cellular and molecular processes underlying the course of acquired chemoresistance, a cisplatin–paclitaxel dual chemoresistant model in the A2780 OC cell line has been previously established in our lab. The model was developed by treating drug sensitive A2780 cells stably expressing tdTomato-firefly luciferase bi-fusion reporter with incremental doses of cisplatin–paclitaxel combination treatment. The resistant sublines thus obtained were characterized as early (A2780-dual^{ER}; onset of resistance) and late resistant (A2780-dual^{LR}; extreme resistance) cells, according to their viability upon treatment with the IC50 of the combination and resistance index. To investigate the influence of chemoresistance on organ specific colonization of ovarian cancer cells, we studied the pattern of metastatic spread of sensitive vs. chemoresistant OC cells using an IP xenograft mouse model. The findings of this experiment revealed that A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells colonize colon, kidney, and liver with similar frequency [A2780 group: 40% (n=15); A2780-dual^{ER} group 47% (n=15); and A2780-dual^{LR} group: 40% (n=15)]. In addition, while spleen metastasis was observed in the mice injected with A2780-dual^{ER} and A2780-dual^{LR} cells, lung metastasis was seen exclusively in the mice injected with A2780-dual^{LR} cells.

The IP xenograft model clearly demonstrated the tendency of chemoresistant cells to colonize distant organs. To further delineate the intriguing pattern of organ selective metastasis, the initial key events of the metastatic cascade such as adhesion and invasion of sensitive vs. resistant cells were compared *in vitro*. In these experiments, the SKOV3 cell line, which is inherently resistant to cisplatin, was also used to compare acquired and intrinsic resistance. The adhesion potential of sensitive vs.

chemoresistant cells was measured on three matrices—Matrigel, NIH3T3 fibroblast cells, and immortalized surface epithelial (IOSE) cells—using an indigenously developed adhesion assay. While both the sensitive and resistant cells exhibited similar attachment kinetics on acellular Matrigel matrix, resistant cells displayed significantly increased attachment kinetics on the NIH3T3 fibroblast cell matrix compared to sensitive cells. At 2 hours, SKOV3, A2780-dual^{LR}, and A2780-dual^{ER} cells showed 27%, 23.6%, and 14.4% higher attachment, respectively, than A2780 cells. Intriguingly, the temporal kinetics for maximal attachment varied with degree of resistance. While 90% of SKOV3 and A2780-dual^{LR} cells showed attachment on NIH3T3 cells within 2 h, attachment of A2780-dual^{ER} cells took 4 h, whereas A2780 cells showed relatively slower kinetics with maximum attachment (around 80%) at 8 h. Notably, chemoresistant cells exhibited adhesion kinetics similar to those of sensitive cells on IOSE cells, suggesting that increased adhesion properties are specific toward fibroblast cells.

The intrinsic invasive properties of sensitive vs. resistant cells were compared by measuring their ability to invade matrigel (Boyden chamber assay) and secrete MMPs (gelatin zymography). As revealed by the Boyden chamber assay, acquired chemoresistance was found to have no effect on the intrinsic invasiveness of the A2780 sensitive and chemoresistant cells. However, inherently resistant SKOV3 cells exhibited approximately 2.3-times higher invasion than did the A2780 cells. Since resistant cells exhibited higher propensity of adherence to fibroblast cells than A2780 cells, we further tested the influence of fibroblast cells in modulating the invasive properties of sensitive vs. chemoresistant EOC cells under co-culture conditions. For this purpose, sensitive and chemoresistant OC cells suspended in fibroblast conditioned medium (FCM) were subjected to Boyden chamber assay, and the

invaded fraction was compared with that of the control (suspended in incomplete medium). Incubation with FCM significantly increased the invasion potential of A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells by 1.8-, 3.2-, and 2.5-fold, respectively. Although A2780 cells showed about 1.4-fold increase in their invasiveness after incubation with FCM, this increase was statistically insignificant. To understand whether this increase in invasiveness was caused by the increased activity of secretory MMPs, the co-culture supernatants were subjected to gelatin zymography, and the relative activities of these supernatants were compared to that of the FCM. Under co-culture conditions, the relative MMP9 and MMP2 proteolytic activities in the A2780-dual^{LR} cells showed an overwhelming increase of 105.4% and 289.9%, respectively, while those in A2780-dual^{ER} cells showed an increase of 66.4% and 20.5%, respectively. In contrast, A2780 cells showed only modest increases of 24.8% and 15.1% in the MMP2 and MMP9 activities, respectively. Overall, these data suggest that chemoresistant OC cells exhibit a stronger metastatic phenotype that may promote the early events of metastasis. Remarkably, the *in vitro* metastatic properties of intrinsically resistant SKOV3 cells were found to closely resemble those of acquired chemoresistant cells in the late stage than early stage.

Although EOC is thought to arise from tubal epithelial cells, it primarily develops in the ovary from where it further metastasizes through peritoneal fluid, lymphatics, and the blood stream [27]. Therefore, to study the role of IGF1R in organ specific colonization and metastatic dissemination of EOC, an orthotopic tumor xenograft mouse model was established. This model revealed a significant advancing metastatic trend of OC cells with the progression of chemoresistance (p_{trend}=0.013). The frequency of metastasis was observed as follows: A2780-dual^{LR} (100%)> A2780-dual^{ER} (90%) > A2780 (50%). In this orthotopic tumor model, all three cell types

(A2780, A2780-dual^{ER}, and A2780-dual^{LR}) were found to colonize in intestines, kidneys, liver, spleen, and lungs but at different frequencies. In comparison with that of A2780 cells, the frequency of colonization of A2780-dual^{LR} cells was significantly higher in all organs studied, while for A2780-dual^{ER} cells it was significantly higher only in the intestines. Interestingly, the incidence of lung metastasis was found to increase with increasing degree of chemoresistance: A2780 (10%), A2780-dual^{ER} (40%), and A2780-dual^{LR} (80%) cells (p_{trend}=0.0039). Taken together, results obtained from IP and orthotopic xenograft mouse models explicitly highlight the tendency of late stage resistant cells to colonize in the lungs suggesting metastatic organotropism.

Objective 2: To monitor the effect of IGF-1R knock-down on metastatic potential of sensitive v/s early and late resistant cells

As previously reported, the chemoresistant A2780 model exhibits a pulsatile pattern of IGF1R expression [21]. The basal IGF1R expression and membrane localization in A2780 sensitive cells escalates in the early stage of resistance (A2780-dual^{ER}) and declines as the cells become highly resistant (A2780-dual^{LR} cells) [21]. The intrinsically resistant SKOV3 cells also exhibit higher IGF1R expression than do the A2780 cells [21]. To understand whether IGF1R regulates differential metastatic properties of chemoresistant A2780 and SKOV3 cells, the gene was silenced using a lentivirus-mediated approach in both sensitive and resistant cells, and the metastatic properties were assessed. IGF1R KD resulted in a modest (~20%–10%) decrease in the initial (2 h) and maximal period (12 h) of adherence for A2780-dual^{LR} and SKOV3 (~16%) cells on fibroblast cells. This decrease in adherence of A2780-dual^{ER} cells was statistically insignificant, and no change was observed for A2780 cells. Similarly, IGF1R KD did not affect the intrinsic invasiveness of sensitive or resistant cells. However, IGF1R KD caused a significant reduction in the invasiveness of

resistant cells under co-culture conditions. After IGF1R KD, the invaded fractions of A2780-dual^{LR}, SKOV3, and A2780-dual^{ER} cells decreased by 2-, 2.3-, and 1.7-fold, respectively, compared to the respective wild type controls under co-culture conditions. Although IGF1R silencing resulted in a 1.5-fold decrease in the invaded fraction of A2780 cells compared to the wild type, this decrease was statistically insignificant. To test whether this decreased invasiveness was a result of the altered secretory MMP activity, the relative MMP9 and MMP2 activities of WT and IGF1R KD cells under co-culture conditions were compared. After IGF1R silencing, MMP9 activities of A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells decreased by 11.3%, 56.2%, and 66.8%, respectively, while the MMP2 activities decreased by 16.9%, 33.3%, and 54.6%, respectively. These data indicate that IGF1R silencing likely impairs the interaction between chemoresistant tumor cells and fibroblast cells and affects their adhesion and invasion properties. After IGF1R KD, the colonization of A2780-dual^{LR} cells in the kidneys, spleen, and lungs was significantly reduced as revealed by the orthotopic xenograft mouse model. While a similar trend was observed for A2780 and A2780-dual^{ER} cells, the decrease was statistically insignificant.

Since the enhanced incidence of lung metastasis was found to be associated with the degree of resistance and IGF1R signaling, we further aimed to investigate the role of primary mouse lung fibroblasts in the key events of lung colonization. To address this, the adhesion and invasion properties of sensitive vs. chemoresistant OC cells were tested against primary mouse fibroblast cells. A2780-dual^{LR} cells showed about 26% and 11% higher adhesion on primary lung fibroblast cells than did the A2780 and A2780-dual^{ER} cells, respectively. Similarly, SKOV3 cells showed approximately 30% higher attachment than A2780 cells. Additionally, A2780-dual^{LR} cells exhibited 1.8-

and 1.6-times higher invasion than did the A2780 and A2780-dual^{ER} cells, respectively, under co-culture conditions with primary lung fibroblast cells, and SKOV3 cells showed 3.2-times higher invasion than A2780 cells. IGF1R silencing also had a substantial impact on the adhesion and invasion properties of A2780-dual^{LR} cells. While IGF1R KD resulted in a >50% and 33% decrease in the adhesion of A2780-dual^{LR} and A2780-dual^{ER} cells, respectively, its effect on the adhesion potential of A2780 cells was negligible. Similarly, SKOV3 cells exhibited about 45% decrease in adhesion and an almost two-fold decrease in invasion after IGF1R silencing. These data indicate that lung fibroblast cells play a central role in effectuating the pre-metastatic events along with chemoresistant cells in mouse lungs.

Objective 3: Investigate the role of differentially expressed adhesion molecules in driving the events of metastasis

Even though A2780-dual^{ER} cells express higher levels of IGF1R than do A2780-dual^{LR} cells, the metastatic properties of A2780-dual^{LR} cells were clearly superior to those of A2780-dual^{ER} cells. Therefore, it is likely that another signaling pathway that is highly active in A2780-dual^{LR} cells, besides the IGF1R signaling pathway, may contribute to the regulation of their metastatic properties. Since A2780-dual^{LR} cells exhibited high adhesion potential, the expression of integrins in A2780-dual^{LR} cells was profiled, as the integrin and IGF1R signaling pathways are redundant in nature. Of all the integrins whose expression was profiled in this model (α1, α2, α5, α6, β1, β4, and β5), the expressions of α2, α6, and β4 integrins were highly upregulated in A2780-dual^{LR} cells in comparison with those in A2780 and A2780-dual^{ER} cells. These integrins were also found to be downregulated across the model after IGF1R silencing, with maximum reduction observed in A2780-dual^{LR} cells. The α6 and β4 integrins together constitute a functional heterodimer and are known to form a

tripartite complex with IGF1R after stimulation with the IGF1 ligand [28]. Therefore, the co-localization of α6 integrin and IGF1R was evaluated in the model in the presence and absence of the IGF1 ligand as a measure of this complex formation using overlap coefficients. A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells were found to have an overlap coefficient of 0.45, 0.56, and 0.57, respectively, at the basal level (serum starved conditions). After IGF1 treatment, the overlap coefficient for A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells increased by 1.2-, 1.3-, and 1.5-fold, respectively. After IGF1R silencing, the overlap coefficient of A2780, A2780-dual^{ER}, and A2780-dual^{LR} cells decreased by 1.4-, 1.4-, and 1.7-fold, respectively, in the presence of the IGF1 ligand. This experiment revealed that although, IGF1R levels are lower in A2780-dual^{LR} cells than those in A2780-dual^{ER} cells, A2780-dual^{LR} cells exhibit higher signaling relayed through the IGF1R–α6 integrin complex. To further investigate the involvement of α6 integrin in the regulation of adhesion properties of sensitive vs chemoresistant cells, the *ITGA6* gene was silenced across the A2780 model as well as in SKOV3 cells. *ITGA6* silencing resulted in a decrease in the adhesion of both sensitive and resistant cells to lung fibroblast cells. While SKOV3 and A2780-dual^{LR} cells exhibited 50% and 59% reduction in adhesion, respectively, A2780-dual^{ER} and A2780 cells showed a respective reduction of 16% and 12% in adhesion after α6 integrin silencing, implying the important role of α6 integrin in the adhesion of SKOV3 and A2780-dual^{LR} cells to lung fibroblast cells.

Since both sensitive and resistant cells showed lung colonization, it was clear that both cells can infiltrate lung tissues. However, the A2780-dual^{LR} cells showed a much higher incidence of lung colonization than did the A2780 and A2780-dual^{ER} cells. The higher rate of successful colonization by A2780-dual^{LR} cells could be because of specific factors secreted by these cells upon their interaction with lung fibroblast cells,

thus leading to the formation of a favorable pre-metastatic niche. Therefore, in order to identify the major factors influencing increased lung colonization by chemoresistant cells, an extensive and systematic secretome analysis of tumor cell-primary lung fibroblast co-culture was conducted. The secretome analysis was performed in 5 sets as follows: Set A, lung fibroblast cell supernatant (fibroblast cell control); Set 2, A2780/A2780-dual^{ER}/A2780-dual^{LR} cell supernatant (cancer cell control); Set 3, A2780/A2780-dual^{ER}/A2780-dual^{LR} IGF1R KD cell supernatant (KD control); Set 4, A2780/A2780-dual^{ER}/A2780-dual^{LR} cell-lung fibroblast co-culture supernatant; and Set 5, A2780/A2780-dual^{ER}/A2780-dual^{LR} IGF1R KD cell-lung fibroblast co-culture supernatant. To identify the proteins secreted under co-culture conditions (upon induction with lung FCM), whose secretion was abrogated after IGF1R KD, the co-culture secretome profiles (sets 4 and 5) were compared with the control profiles (sets 1, 2, and 3) and the overlapping candidates were excluded. In this analysis, a total of 8 candidates were found to be exclusively secreted by A2780-dual^{LR} cells under co-culture conditions, of which S100A4 protein, known to be involved in the metastatic processes such as invasion through MMP secretion, ECM remodeling, and angiogenesis, was chosen for further studies [29].

To further elucidate the role of secretory S100A4 in pre-metastatic niche formation, primary lung fibroblast cells were incubated with the co-culture supernatants of wild type and S100A4-KD A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, and the activation of fibroblasts was measured. α-SMA and vimentin were used as activation markers for lung fibroblasts cells. After incubation of fibroblast cells with co-culture supernatants of A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, α-SMA transcript levels were observed to be 1.6-, 2-, 2.6-, and 3.2-fold higher, respectively, than the basal levels. Notably, when the fibroblast cells were incubated

with supernatants of S100A4-KD A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, a decrease to the order of 1.3-, 1.5-, 2-, and 1.7-fold, respectively, was observed in the α-SMA transcript levels. Vimentin expression also demonstrated a similar trend. After incubation with the co-culture supernatants of wildtype A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, the vimentin expression in lung fibroblast cells was, respectively, 1.5-, 2.1-, 2.4-, and 2.8-fold higher than the basal levels, whereas upon incubation with the co-culture supernatants of S100A4-silenced A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells, the vimentin expression in lung fibroblast cells showed a decrease of 1.2-, 1.8-, 1.8-, and 2.1-fold, respectively. It is well known that the activation of fibroblasts in turn leads to an increase in their invasiveness. Therefore, primary lung fibroblast cells were incubated with the co-culture supernatants of WT and S100A4-KD A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells and then subjected to Matrigel invasion, while fibroblast cells incubated in incomplete DMEM were used as control. A 2-fold higher invasion was shown by lung fibroblast cells incubated with co-culture supernatants of A2780-dual^{LR} and SKOV3 cells than the control, while ~1.5- and 1.3-fold higher invasion rates were observed when the same fibroblast cells were incubated with the co-culture supernatant of A2780-dual^{ER} and A2780 cells, respectively. Intriguingly, silencing of S100A4 in A2780, A2780-dual^{ER}, A2780-dual^{LR}, and SKOV3 cells led to 1.5-, 1.8-, 1.8-, and 1.7-fold lower invasion by fibroblast cells, respectively, in the above-mentioned setting. As S100A4 turned out to be major regulator of pre-metastatic niche formation for chemoresistant cells *in vitro*, S100A4-KD A2780-dual^{LR} cells were injected orthotopically in mice, and the incidence of lung metastasis was assessed. Upon S100A4 KD, the overall metastatic frequency reduced significantly to 50% (p=0.0325), with no incidence of lung metastasis (p=0.0007). Interestingly,

S100A4 KD also caused a 70% reduction in spleen and liver metastasis (p=0.0031) and a 60% reduction in kidney (p=0.0108) and intestinal metastasis (p=0.0198). These data imply that S100A4 is a crucial player that dictates distant colonization of chemoresistant OC cells, and its intracellular and extracellular functions are indispensable for lung homing.

Niclosamide, a common anti-helminthic drug, is also a potent transcriptional inhibitor of S100A4 [30]. Since our findings highlighted the implications of S100A4 in distant metastasis of chemoresistant OC cells, we further evaluated the therapeutic effects of niclosamide on the metastatic spread of A2780-dual^{LR} cells using the orthotopic xenograft mouse model. As compared to the untreated control group, the overall metastatic frequency was significantly reduced by 57% in the niclosamide treatment group (p=0.0147). Moreover, while niclosamide treatment resulted in 57% reduction in intestinal colonization (p=0.0147), it caused a 29% reduction in colonization to kidney (p=0.0034) and liver (p=0.0034). Remarkably, the niclosamide treatment group did not show any incidence of spleen (p=0.0001) or lung metastasis (p=0.0023). These results underline the efficacy of niclosamide as a therapeutic drug in attenuating distant metastasis of chemoresistant OC cells.

Objective 4: To analyse the IGF-1R expression in matched paired primary and omental metastatic tumor specimens obtained from patients with advanced stage ovarian carcinoma

HGSOC is the most common subtype of EOC that is often detected at an advanced stage (III/IV), in which the patients exhibit vast metastatic dissemination. In this objective, we conducted a comparative analysis of IGF1R expression in primary and metastatic omental tumor samples obtained from high grade serous ovarian cancer (HGSOC) patients. A total of 19 high grade serous ovarian carcinoma (HGSOC)

patients scheduled for neo-adjuvant chemotherapy (NACT) were included in the study with informed consent. Post NACT, paired primary tumor and metastatic omental tumor samples were collected during radical debulking surgery. Of the 19 paired samples that were collected, 4 primary tumor samples had no viable tumors. Therefore, IGF1R IHC was performed on 15 primary and 19 omental metastatic tumor samples. IGF1R expression was compared using immunoreactive score given by an experienced pathologist and H-score calculated using IHC profiler. The results of this analysis indicated that there was no significant difference between IGF1R expression of primary and metastatic tumors.

To further investigate the prognostic merit of IGF1R for HGSOC, the tissue base IGF1R expression was correlated with the overall (OS) and disease-free survival (DFS) of the cohort. For this purpose, based on the median immunoreactive score, the cohort was subdivided into two groups (less than median and more than median), and a log-rank test was performed to evaluate the correlation between active IGF1R staining and survival. Patients with immunoreactive scores more than the median in primary tumors had better OS (OS: 33.2 months) and DFS (15.901 months) than those with scores less than the median [OS: 27.27 months (p = 0.2); DFS: 15.014 (p = 1.0)]. A similar trend was observed when the metastatic tumor immunoreactive score was considered [more than median (OS: median not reached; DFS: 26.743) vs less than the median (OS: 33.248; DFS: 15.014; p = 0.734 and p = 0.445, respectively). These data indicated that the chemo-treated primary and metastatic tumor tissues from HGSOC patients have equivalent IGF1R expression. Although statistically insignificant, increased IGF1R expression in the primary and metastatic tissue samples was found to indicate improved OS and DFS in these patients. This statistical insignificance could be a result of the small cohort size.

Conclusion

Herein, using indigenously developed cellular and animal models, we demonstrated that extreme chemoresistance augments tumor intrinsic metastatic properties of OC cells through the regulation of IGF1R signaling, which results in an increased propensity toward colonization in the lungs. Our findings also evince the IGF1R-α6 integrin-S100A4 signaling network as a critical determinant for organ specific metastases of platinum-taxol resistant EOC cells, thus highlighting the translational prospects of S100A4 inhibition as a therapeutic intervention for the treatment of recurrent metastatic EOC.

References

1. Zhang, X.-Y. and P.-Y. Zhang, *Recent perspectives of epithelial ovarian carcinoma.* Oncology letters, 2016. **12**(5): p. 3055-3058.

2. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA: a cancer journal for clinicians, 2018. **68**(6): p. 394-424.

3. Yeung, T.-L., et al., *Cellular and molecular processes in ovarian cancer metastasis. A review in the theme: cell and molecular processes in cancer metastasis.* American Journal of Physiology-Cell Physiology, 2015. **309**(7): p. C444-C456.

4. Smolle, E., V. Taucher, and J. Haybaeck, *Malignant ascites in ovarian cancer and the role of targeted therapeutics.* Anticancer research, 2014. **34**(4): p. 1553-1561.

5. Thomakos, N., et al., *Rare Distant Metastatic Disease of Ovarian and Peritoneal Carcinomatosis: A Review of the Literature.* Cancers, 2019. **11**(8): p. 1044.

6. Sato, S. and H. Itamochi, *Neoadjuvant chemotherapy in advanced ovarian cancer: latest results and place in therapy.* Therapeutic advances in medical oncology, 2014. **6**(6): p. 293-304.

7. Ushijima, K., *Treatment for recurrent ovarian cancer—at first relapse.* Journal of oncology, 2010. **2010**.

8. Cormio, G., et al., *Distant metastases in ovarian carcinoma.* International Journal of Gynecologic Cancer, 2003. **13**(2): p. 125-129.

9. Longo, R., et al., *A late, solitary brain metastasis of epithelial ovarian carcinoma.* BMC cancer, 2014. **14**(1): p. 543.

10. Achariyapota, V., T. Chuangsuwanich, and M. Benjapibal, *Inflammatory breast cancer from metastatic ovarian cancer.* Case reports in obstetrics and gynecology, 2016. **2016**.

11. Zhang, M. and J. Sun, *Bone metastasis from ovarian cancer.* Saudi Med J, 2013. **34**(12): p. 1270-1273.

12. Kumar, H., et al., *Multiple metastases from ovarian cancer: case report.* South African Journal of Obstetrics and Gynaecology, 2014. **20**(3): p. 94-95.

13. Amzerin, M., et al., *Case report: Mammary and rectal metastases from an ovarian cancer: Report of two cases and review of literature.* F1000Research, 2014. **3**.

14. Klein, R.L., et al., *Ovarian cancer metastatic to the breast presenting as inflammatory breast cancer: a case report and literature review.* Journal of Cancer, 2010. **1**: p. 27.

15. Robinson, W.R., et al., *Extraperitoneal metastases from recurrent ovarian cancer.* International Journal of Gynecologic Cancer, 2012. **22**(1): p. 43-46.

16. Bacalbasa, N., et al., *Liver resection for ovarian cancer liver metastases as part of cytoreductive surgery is safe and may bring survival benefit.* World journal of surgical oncology, 2015. **13**(1): p. 235.

17. SAVESCU, A., I. BALESCU, and N. BACALBASA, *The surgical treatment of thoracic metastases in ovarian cancer.* Romanian Journal of Medical Practice, 2015. **10**(2).

18. Sood, A.K., et al., *Distant metastases in ovarian cancer: association with p53 mutations.* Clinical Cancer Research, 1999. **5**(9): p. 2485-2490.

19. Sahdev, A., et al., *Computed tomography features of recurrent ovarian carcinoma according to time to relapse.* Acta Radiologica, 2007. **48**(9): p. 1038-1044.

20. Hirose, S., et al., *Retrospective analysis of sites of recurrence in stage I epithelial ovarian cancer.* Journal of gynecologic oncology, 2018. **29**(3).

21. Singh, R.K., et al., *IGF-1R inhibition potentiates cytotoxic effects of chemotherapeutic agents in early stages of chemoresistant ovarian cancer cells.* Cancer letters, 2014. **354**(2): p. 254-262.

22. Eckstein, N., et al., *Hyperactivation of the insulin-like growth factor receptor I signaling pathway is an essential event for cisplatin resistance of ovarian cancer cells.* Cancer research, 2009. **69**(7): p. 2996-3003.

23. Denduluri, S.K., et al., *Insulin-like growth factor (IGF) signaling in tumorigenesis and the development of cancer drug resistance.* Genes & diseases, 2015. **2**(1): p. 13-25.

24. Reinmuth, N., et al., *Impact of insulin-like growth factor receptor-I function on angiogenesis, growth, and metastasis of colon cancer.* Laboratory investigation, 2002. **82**(10): p. 1377-1389.

25. Wang, Y.H., et al., *Increased expression of insulin-like growth factor-1 receptor is correlated with tumor metastasis and prognosis in patients with osteosarcoma.* Journal of surgical oncology, 2012. **105**(3): p. 235-243.

26. Sachdev, D., et al., *The type I insulin-like growth factor receptor regulates cancer metastasis independently of primary tumor growth by promoting invasion and survival.* Oncogene, 2010. **29**(2): p. 251.

27. Shih, I.-M., Y. Wang, and T.-L. Wang, *The origin of ovarian cancer species and precancerous landscape.* The American Journal of Pathology, 2020.

28. Fujita, M., et al., *Cross-talk between integrin α6β4 and insulin-like growth factor-1 receptor (IGF1R) through direct α6β4 binding to IGF1 and subsequent α6β4-IGF1-IGF1R ternary complex formation in anchorage-independent conditions.* Journal of Biological Chemistry, 2012. **287**(15): p. 12491-12500.

29. Garrett, S.C., et al., *S100A4, a mediator of metastasis.* Journal of Biological Chemistry, 2006. **281**(2): p. 677-680.

30. Sack, U., et al., *Novel effect of antihelminthic Niclosamide on S100A4-mediated metastatic progression in colon cancer.* Journal of the National Cancer Institute, 2011. **103**(13): p. 1018-1036.

Publications in Refereed Journals:

a) Published:

1. Deo, Abhilash, et al. "IGF1R predicts better survival in high-grade serous epithelial ovarian cancer patients and correlates with hCtr1 levels." Biomarkers in medicine 13.07 (2019): 511-521 (From thesis).

b) Accepted: Not applicable

c) Communicated: IGF1R-α6 integrin-S100A4 network governs the metastatic organotropism of chemoresistant epithelial ovarian cancer cells.

Other Publications:

a) Review articles:

1. Dhadve, A., **Deo, A**., Bishnu, A., Mukherjee, S. and Ray, P., 2017. MOLECULAR IMAGING IN CANCER: HOW THE HALLMARKS AID IN HUNTING. *International Journal of Drug Research and Technology*, *7*(1), p.1.

2. **Deo, Abhilash**, et al. "Subtype specific biomarkers associated with chemoresistance in epithelial ovarian cancer." Indian Journal of Pathology and Microbiology 63.5 (2020): 64.

b) Book Chapter

1. Aniketh Bishnu, **Abhilash Deo**, Ajit Dhadve, Bhushan Thakur, Souvik Mukherjee, Pritha Ray. Non Invasive Imaging in Clinical Oncology: A Testimony of Current Modalities and a Glimpse into the-

Future." *Molecular Medicines for Cancer: Concepts and Applications of Nanotechnology*, CRC Press, Taylor & Francis Group, 2018, pp. 217–262.

c) Conference/Symposium

1. "Upregulation of IGF-1R-PIK3CA/AKT signalling potentiates adhesion potential of chemoresistant ovarian cancer cells" at All India Cell Biology conference and International Symposium on Functional Genomics and Epigenomics, Jiwaji University, Gwalior, 2016.

2. "Understanding the influence of acquired chemoresistance on metastatic properties of ovarian carcinoma cells" at International Congress of Cell Biology, Hyderabad, 2018.

3. "Acquirement of chemoresistance augments metastatic properties in ovarian cancer cells steered by IGF1 receptor" at 38th annual convention of Indian Association for Cancer Research, Chandigarh, 2019.

4. "Chemoresistance progressively enhances the metastatic phenotype of ovarian cancer (OC) cells imparted through IGF1R signalling" at 2nd joint EACR-MRS Conference on Seed and Soil: Mechanisms of Metastasis, Berlin-Germany, 2019.

Signature of Student: dhaleo

Date: 29/12/2020

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1.	Dr. Neelam Shirsat	Chairperson		29/12/2020
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Thesis Highlight

Name of the Student: Abhilash Nitin Deo

Name of the CI/OCC: TMC – ACTREC **Enrolment No.:** LIFE09201504017

Thesis Title: Role of IGF1R in Ovarian Cancer Metastasis

Discipline: Life Sciences **Sub-Area of Discipline:** Cancer biology (Therapy resistance)

Date of viva voce: 25th May 2021

Treatment of recurrent metastatic epithelial ovarian cancer (EOC) is challenging and associated with limitations, as the mechanisms governing the metastatic behavior of chemoresistant EOC cells remain elusive. Herein, we studied the mechanistic role of insulin like growth factor 1 receptor (IGF1R) signaling in organ-specific metastasis of EOC cells at different stages of chemoresistance to elucidate potential target/s for treatment of recurrent metastatic EOC.

Herein, we show that aquired chemoresistance augments organotropic metastasis of EOC cells via hematogenous route. This augmented metastatic potential is partly regulated through the IGF1R-α6 integrin network. Further, through extensive profiling and subtractive analysis of EOC cells – lung fibroblast co-culture secretome, we reveal that that interaction with lung fibroblast cells induces the secretion of S100A4 from highly resistant EOC cells, which reciprocally activates lung fibroblasts. S100A4 inhibition completely abrogates lung metastasis of chemoresistant EOC cells. In summary, this study evinces that the IGF1R–α6 integrin–S100A4 signaling network is a critical determinant for the organ-specific metastases of platinum-taxol resistant EOC cells. Herein, S100A4 was identified as a key molecule that plays a central role in the colonization of chemoresistant cells to distant sites, such as lungs, which can be therapeutically targeted for improving the clinical outcome of recurrent EOC.

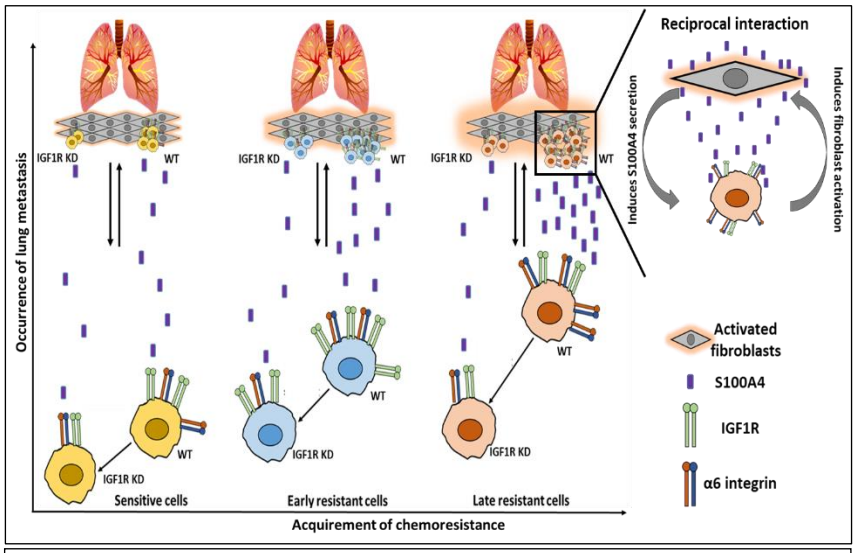

Figure 1. IGF1R-α6 integrin-S100A4 network governs the metastatic organotropism of chemoresistant epithelial ovarian cancer cells.