Intercellular Transfer of microRNAs in Epithelial Ovarian Cancer

BY

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DISSertation

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ACKNOWLEDGEMENTS

It takes a village to raise a child. I can confidently attest to the fact that a village has been involved in raising this dissertation and me to their current state of completion. It is a great honor to pay tribute to the incredible collective who made this work possible.

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GGM
“Felix qui potest rerum cognoscere causas”

- Virgil, 29 BC
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<td>Description</td>
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<tr>
<td>3’ UTR</td>
<td>Three Prime Untranslated Region</td>
</tr>
<tr>
<td>5’UTR</td>
<td>Five Prime Untranslated Region</td>
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<tr>
<td>AGO</td>
<td>Argonaute</td>
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<tr>
<td>ATR</td>
<td>Ataxia Telangiectasia and Rad3-related protein</td>
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<tr>
<td>BRAF</td>
<td>v-Raf murine sarcoma viral oncogene homolog B</td>
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<tr>
<td>BRCA1</td>
<td>Breast cancer type 1 susceptibility gene</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer type 2 susceptibility gene</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer Antigen 125</td>
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<tr>
<td>CAF</td>
<td>Cancer associated Fibroblast</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CDK12</td>
<td>Cyclin Dependent Kinase 12</td>
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<td>CTNNB1</td>
<td>Catenin Beta 1</td>
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<td>CXCL1</td>
<td>Chemokine (C-X-C Motif) Ligand 1</td>
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<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C Motif) Ligand 12</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
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<tr>
<td>Cy3</td>
<td>Cyanine 3</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine 5</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal transition</td>
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<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
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<tr>
<td>ERM</td>
<td>Ezrin–radixin– moesin</td>
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ESCRT  Endosomal sorting complex required for transport
EV    Extracellular Vesicle
FAK   Focal Adhesion Kinase
FGF1  Fibroblast Growth Factor 1
FGF18 Fibroblast Growth Factor 18
FGF4  Fibroblast Growth Factor 4
GFP   Green Fluorescent Protein
HA    Hyaluronic Acid
HDL   High Density Lipoprotein
HG-SOC High Grade Serous Ovarian Cancer
HMMR  Hyaluronan-mediated motility receptor
HOSE  Human ovarian surface epithelium
HSP   Heat shock protein
ICAM  Intercellular cell adhesion molecule
KRAS  Kirsten rat sarcoma viral oncogene homolog
MAPK  Mitogen-activated protein kinase
Meso-ET Mesothelial to epithelial transition
MFAP5 Microfibrillar Associated Protein 5
miR or miRNA microRNA or micro Ribose nucleic acid
MMP9  Matrix Metallopeptidase 9
MV  Multi vesicular body
NF1   Neurofibromatosis type I
NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells
ORF   Open reading frame
<table>
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<td>p38α</td>
<td>Proline-directed ser/thr MAP kinase</td>
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<tr>
<td>p53</td>
<td>Tumor protein p53</td>
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<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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<tr>
<td>pre – miRNA</td>
<td>precursor- microRNA</td>
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<tr>
<td>pri – miRNA</td>
<td>primary- microRNA</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>RAB</td>
<td>Rab GTPases</td>
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<td>RB1</td>
<td>Retinoblastoma protein</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
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<tr>
<td>RNA BP</td>
<td>Ribose nucleic acid binding protein</td>
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<tr>
<td>RNAase</td>
<td>Ribonuclease</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>SEER</td>
<td>Surveillance, Epidemiology, and End Results Program</td>
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<td>STIC</td>
<td>serous tubal intra-epithelial carcinoma</td>
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<td>TCGA</td>
<td>The cancer genome atlas</td>
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<tr>
<td>TGFBR2</td>
<td>Transforming growth factor beta receptor activity, type II</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TUBB3</td>
<td>Tubulin Beta 3 Class III</td>
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<tr>
<td>VCAN</td>
<td>Versican</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZEB1</td>
<td>Zinc finger E-box-binding homeobox 1</td>
</tr>
<tr>
<td>ZEB2</td>
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SUMMARY

Epithelial ovarian cancer is a leading cause of death from gynecological malignancies. A lot of the roadblocks in effectively treating patients with metastatic disease and preventing chemoresistance can be attributed to the insufficient understanding of the biology and the mechanisms involved in ovarian cancer metastasis.

The tumor microenvironment is a heterogeneous system, characterized by complex interactions between different cell types, which are mediated by several means of cell-to-cell communication. A recently discovered mechanism of intercellular communication is the cell-to-cell transfer of microRNAs. MicroRNAs and their role in cancer have attracted a lot of attention in recent years. They have been identified as key regulators of metastatic progression, tumor response to treatments, and clinical outcomes in ovarian cancer. As the transfer of functional microRNAs between cells could lead to dramatic changes in the fate of the cell-recipient, this dissertation explores their role as mediators of intercellular communication in the ovarian cancer microenvironment.

Using time-lapse confocal microscopy, multi-photon imaging and flow cytometry the process of microRNA transfer between the cells was visualized and its magnitude quantified. Functional intercellular transfer of microRNA-200a was demonstrated across both natural as well as artificial gradients of expression levels. The microRNA-200 family is a versatile player in ovarian
cancer and has been shown to play an important role in epithelial to mesenchymal transition and metastasis. A double negative feedback loop between miR-200 and the ZEB genes taken into context along with the potentially functional transfer of the microRNA between the cells could further the understanding of the reversible EMT process. This project thus aims to characterize intercellular microRNA transfer and its functional significance in epithelial ovarian carcinoma.
Chapter 1: REVIEW OF CURRENT LITERATURE & SCOPE OF THESIS

1.1 Epithelial Ovarian Cancer

1.1.1 Introduction

EOC is a principal cause of death from gynecologic malignancies and one of the deadliest cancers in women [1]. In the United States, an estimated 22,280 new cases of ovarian cancer will be diagnosed in 2016 and an estimated 14,240 will succumb to the disease [2]. Based on data collected from 2006-2012, five year survival of patients diagnosed with ovarian cancer is 46.2% [2]. The relative survival to a great extent is influenced by the stage of the disease at the time of diagnosis and initiation of treatment. If the disease is diagnosed at an early stage and is localized to the primary site the five year survival is can be as high as 92.1% [2]. Whereas, diagnosis at the metastatic stage leads to a five year survival rate of only 28.8% [2]. This drastic fall in survival underlines the connection between late diagnosis and high mortality. Information pertaining to the ovarian cancer statistics is summarized in Figure 1.

One of the early challenges associated with the disease are the nonspecific symptoms. They manifest as fatigue, back pain, abdominal pain, bloating, difficulty eating, and urinary discomfort [3]. The generic nature of the symptoms often causes them to be mistaken for gastro-intestinal diseases, thereby weakening their utility in identifying the disease early [4]. This coupled with the lack of sensitive and accurate screening measures implies that most of the patients are diagnosed at late metastatic stages when there is minimal chance for survival due to the lack of effective anti-metastatic treatments. While current standard of care, a combination of surgery and chemotherapy, is efficient as initial treatment, in most cases EOC
recurs after a few years and becomes resistant to existing treatments [5, 6]. Inability to prolong patient remission is a critical gap in the clinical management of EOC. The underlying cause of this problem stems in part from an insufficient understanding of the biology and mechanisms supporting EOC metastasis.

1.1.2 Clinical Manifestation and Management

Cancer is frequently a disease of age and ovarian cancer is not an exception to that rule [2]. It is most commonly diagnosed in the age range of 55-64 with the median age of diagnosis being 63 [2]. Along with advancing age, a family history of ovarian cancer [7-9] or breast cancer [9, 10] significantly compound the risk of developing the disease. Infertility [11] and nulliparity [12] also contribute to increased risk. Pregnancy [9, 12] and the use of birth control on the other hand exert a protective effect [13].

Effective screening strategies for ovarian cancer are currently unavailable. Individuals who are considered high-risk are screened using a protocol that includes trans-vaginal ultrasounds scanning as a blood test for an antigen called CA125 [14]. The lack of sensitivity and high false positive results from these tests preclude their use in the general population. Confirmatory diagnosis is performed through physical exams, scans and biopsies. The treatment for ovarian cancer is primary surgical debulking of the tumors and drainage of ascitic fluid, followed by aggressive chemotherapy. This is typically IV administered Carboplatin and Paclitaxel in 6 cycles of treatment. Information pertaining to the risk factors and methods used for screening, diagnosis and treatment of EOC summarized in Figure 2.

Carboplatin or Cis diamine (1,1-cyclobutanecarboxylato) platinum (II) belongs to the platinum family of chemotherapeutic drugs, which also include the earlier analogue cisplatin. Carboplatin is favored over cisplatin in the chemotherapeutic regime for ovarian cancer due to comparable efficacy and an improved toxicity profile [15]. The platinum compounds exert
their action by binding to the purines in the DNA bases leading to crosslinking and formation of DNA adducts [16, 17]. This DNA damage subsequently leads to apoptosis with activation of several signaling mediators/pathways including among others, ATR, p53 and MAPK [18].

The taxane family of drugs, which include paclitaxel and docetaxel, are cytotoxic and used to treat several cancers both on and off- label [19]. They cause stabilization of the cellular microtubules and prevent the de-polymerization of the microtubule assembly in cells [19, 20]. This results in the trapping of cells in the metaphase of mitotic cell division, thereby causing cell cycle arrest [21-23]. However, recent studies have shown that the concentrations of paclitaxel required to cause mitotic arrest are higher than the clinically administered levels [24, 25]. In fact, cytotoxic effect of paclitaxel has been found to be associated with formation of multipolar spindles [25-27], as well as unattached kinetochores [19, 28].

Although patients show good response to the platinum-taxane regime initially, the disease recurs and tends to be resistant to platinum therapy [29]. Several targeted therapy options have been identified in the past decade to combat the recurrent disease. VEGF inhibitors and PARP inhibitors have shown a lot of promise in the clinics [30]. The latter especially represents a new era in cancer therapy due to its selective synthetic lethality and the ability to preselect responsive patients [31]. These targeted drugs are the result of our evolving understanding of the pathology. Continued efforts in this direction will be crucial to ensure that the therapeutic interventions in the future will not merely represent a delay of the inevitable for many patients.
Figure 2: Risk Factors and the methods currently used for Screening, Diagnosis and Treatment of Epithelial Ovarian Cancer
1.1.3 Histopathology

Figure 3: The major histologic subtypes of ovarian cancer fall into two sub classifications [32].

Type I tumors are low-grade, slow growing carcinomas that typically arise from well recognized precursors lesions (borderline tumors) that themselves develop from the ovarian surface epithelium, inclusion cysts, or endometriosis. In contrast, Type II tumors are high-grade and rapidly growing carcinomas. Typically, they have spread well beyond the ovary at the time of diagnosis.

(Figure and caption originally published by Jones et al. in Jones PM, Drapkin R. Modeling High-Grade Serous Carcinoma: How Converging Insights into Pathogenesis and Genetics are Driving Better Experimental Platforms. Frontiers in Oncology. 2013;3:217. doi:10.3389/fonc.2013.00217.)
Depending on their growth, progression and genetic stability, ovarian tumors are classified (Figure 3) into:

- **Type I or Low-grade tumors (include Endometrioid, Mucinous, Clear Cell and Serous):** These tumors tend to be slow growing and progress by acquiring a series of gene mutations [32, 33]. The most frequently altered genes are *KRAS, BRAF, PTEN, CTNNB1*, and *TGFBR2* [33-36].

- **Type II or High-grade tumors (include Endometrioid, Clear Cell and Serous):** These are aggressive, cancers which are characterized by TP53 mutations [33, 35]. TCGA data analysis has also shown the association of *NF1, BRCA1, BRCA2, RB1*, and *CDK12* mutations with high-grade tumors [37].

Each of the histotypes has been found to be associated with mutations in specific genes and have different clinical manifestations [32, 38]. The low-grade tumors are invariably easier to treat and present fewer clinical challenges. The high-grade serous ovarian carcinoma (HGSOC) population on the other hand, is characterized by the rapid disease progression, recurrent and chemo-resistant tumors and poor survival prognosis [39]. These are responsible for 60-80% of the deaths from ovarian carcinoma [39].

It is now widely accepted that high grade serous ovarian cancers originate, not as previously thought from the ovarian surface epithelium, but from the fallopian tube fimbriae [33, 40, 41]. *TP53* mutations in these cells are followed by the formation of the serous tubal intra-epithelial carcinoma (STICs), and these lesions metastasize as HGSOC (Figure 4) [42-45].
Figure 4: Early tumor progression within the fallopian tube and the resultant genetic profile of HG-SOC [32].

This illustration depicts the recently identified precursor lesions of HG-SOC that are present in the fallopian tube. Mutations in the TP53 tumor suppressor gene are a very early event in the pathogenesis of HG-SOC, occurring exclusively in benign-appearing secretory cells. These preneoplastic lesions are referred to as ‘p53 signatures’. Acquisition of a neoplastic phenotype and proliferative capacity results in the development of serous tubal intraepithelial carcinoma (STIC). Breaching of the basement membrane and localized dissemination to the ovary and/or peritoneal cavity heralds the development of invasive HG-SOC and the associated clinical scenario. HG-SOCs that involve the ovary or peritoneum are characterized by mutations in TP53 (and BRCA1 in familial cases) and display a complex genomic terrain with widespread copy number alterations throughout the genome.

(Figure and caption originally published by Jones et al. in Jones PM, Drapkin R. Modeling High-Grade Serous Carcinoma: How Converging Insights into Pathogenesis and Genetics are Driving Better Experimental Platforms. Frontiers in Oncology. 2013;3:217. doi:10.3389/fonc.2013.00217.)
1.2 EOC microenvironment

1.2.1 Metastatic dissemination

Ovarian cancer typically follows a route of metastatic dissemination different from the traditionally seen hematogenous route [46]. The human peritoneal cavity represents the space enclosed between the parietal peritoneum present along the abdominal wall and the visceral peritoneum, which forms the lining of the abdominal and pelvic organs [47]. The anatomy of the peritoneal cavity plays a hugely significant role in the preferred mode of metastasis that is observed in ovarian cancer. The cells from the primary tumor are shed into the cavity wherefrom they are disseminated on to the secondary sites in the peritoneal wall, omentum and other adjacent organs (Figure 5) [3, 48]. This dissemination is assisted by the ascitic fluid [3] as well as the negative sub-diaphragmatic pressure generated during respiration [47, 49]. Aside from the single cell suspension of the disseminating cells in the ascitic fluid, the metastasizing cells frequently come together to form clusters that are known as spheroids [50]. The spheroids then implant themselves into the peritoneal wall and the organs within it by using myosin generated force to clear the mesothelial monolayers.

The conjunction of all these events and players contribute to different aspects of the cancer progression, immune cell evasion, drug resistance and survival [3, 51, 52]. The resulting metastatic microenvironment presents breathtaking complexity and relies on the interaction of cells and matrix components from various organ systems with the cancer cells [48, 52]. It is safe to assume that robust mechanisms of intercellular communication are in play to ensure that the tumor can survive and thrive.
At its earliest stage (stage IA/IB), the tumour (shown in the figure as yellow masses) is limited to one or both ovaries, the ovarian capsule is intact, and no tumour is present on the ovarian surface or in ascites or peritoneal washings. Once the capsule is disrupted, the tumour spreads beyond the confines of the ovaries. This can occur by direct extension to, and invasion of, adjacent tissues such as the uterus, fallopian tubes, the mesothelial lining of the pelvic cavity (peritoneum), and the broad ligament (a fold of peritoneum that supports the uterus). Exfoliated tumour cells are transported by peritoneal fluid and implant on the peritoneum and mesothelial linings of pelvic and abdominal organs (serosa). Nests of tumour cells are commonly observed on the omentum (a peritoneal fold connected to the stomach and suspended over the intestines), the mesentery (a peritoneal fold anchoring the intestines to the posterior abdominal wall; not shown) and the diaphragm. Ascites is commonly associated with intraperitoneal dissemination. Tumour cells are also thought to spread through the lymphatics that drain the ovaries to the pelvic and paraaortic lymph nodes. Haematogenous dissemination is a clinically rare phenomenon, and can involve any organ including the brain, although certain organs such as the liver are more frequently involved.

(Figure and caption originally published by Naora et al. in Naora, H. and D.J. Montell, Ovarian Cancer Metastasis: Integrating insights from disparate model organisms. Nat Rev Cancer, 2005. 5(5): p. 355-366.)
**1.2.2 Extracellular matrix environment**

As the name suggests, the extra-cellular matrix (ECM) represent the non-cellular constituents of tissue systems [53]. They form the supporting framework for the cells and nourish them by relaying the chemical and mechanical signals which drive various growth and differentiation processes [53]. The cells in turn are equipped with various receptors and associated signaling pathways that mediate adhesion to the ECM as well as enzymes and other secretions that allow structural remodeling of the ECM [53].

Fundamentally, the ECM is composed of proteoglycans and fibrous proteins (collagen, elastin and fibronectin) [54]. Even as the ECM components remain largely consistent (water, proteins and polysaccharides) [53], there is remarkable variation in the matrix between different tissues [55]. This variation is tailor-made in order to support the needs of different tissues and organ systems, and this becomes very relevant in tumor microenvironments [56].

In ovarian cancer, several signaling mechanisms are involved in the adhesion of the metastatic cells to the mesothelial cell wall covering the peritoneal cavity and its organs. Numerous others are triggered following the adhesion in order to mediate invasion through the matrix and form the secondary metastasis. The ECM constituents play crucial roles in these processes and assist in metastatic cell adhesion, invasion and proliferation [56]. Their roles have been comprehensively reviewed by Cho et al and they summarized their findings in the following table (Table 1) [56].
Table 1: Summary of the ECM proteins and their roles in EOC [56].

<table>
<thead>
<tr>
<th>ECM protein</th>
<th>Role in EOC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrous proteins</td>
<td><strong>Collagen I</strong></td>
<td>Preferential and strong adhesion of primary ovarian cancer cells and spheroids to collagen I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Promotes migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Provides a steering cue for cell migration</td>
</tr>
<tr>
<td></td>
<td><strong>Collagen XI</strong></td>
<td>Expression levels correlate with tumor grade</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated with poor clinical outcome and overall survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Predictor of recurrence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contributes to paclitaxel resistance by upregulating tau</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Expressed in the ECM and ascites</td>
<td>[68, 69]</td>
</tr>
<tr>
<td></td>
<td>Indicator of poor prognosis</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>Mediates migration, invasion, and metastasis</td>
<td>[60, 68, 71, 72]</td>
</tr>
<tr>
<td></td>
<td>Fibronectin fragments enhances adhesion of EOC cells to the peritoneal surface</td>
<td>[73]</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>Promoted increased adhesion and migration</td>
<td>[74]</td>
</tr>
<tr>
<td>Tenascin-X</td>
<td>Levels associated with tumor grade</td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>Strong positive correlation with serum CA-125 levels</td>
<td>[76]</td>
</tr>
<tr>
<td>Laminin</td>
<td>Absent in microinvasive cells and low malignant tumors</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>Significantly higher in EOC ascites than normal peritoneal fluid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No difference in serum levels between</td>
<td></td>
</tr>
<tr>
<td><strong>Proteoglycans</strong></td>
<td><strong>Decorin</strong></td>
<td>Cancer progression associated with reduced or loss of expression in EOC ECM</td>
</tr>
<tr>
<td></td>
<td><strong>Lumican</strong></td>
<td>Downregulation may have role in cancer aggression</td>
</tr>
<tr>
<td></td>
<td><strong>Versican</strong></td>
<td>Elevated levels in EOC ECM correlated with poor disease outcome</td>
</tr>
<tr>
<td></td>
<td><strong>Perlecan</strong></td>
<td>Expression lost in BM which facilitated invasion</td>
</tr>
<tr>
<td></td>
<td><strong>Hyaluronan</strong></td>
<td>Elevated HA levels correlated with tumor grade and metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strong, independent prognostic factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive correlation with invasion and metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Facilitates adhesion of tumor cells to the peritoneum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduces efficacy of chemotherapy and induces chemoresistance in response to chemotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conjugates with chemotherapy increased the efficacy of chemotherapy</td>
</tr>
</tbody>
</table>

1.2.3 Cellular Heterogeneity

The days of visualizing tumors as simple uniform entities are long gone [97]. Cells of different types co-exist within the tumors [51]. Very often their interactions are implicated in supporting the growth and survival (Figure 6) [3]. One can envision a scenario that a deregulated cell further along development of the cancerous phenotype can exert a bystander effect on the surrounding cells. Cellular and molecular cues from the microenvironment can guide tumor progression.

The cancer cells themselves represent a considerably heterogeneous population [98]. There are significant differences the genetic and epigenetic profiles [99, 100]. Their behavior also varies depending on the stages of tumor progression as well as sites of metastasis [99, 100]. In order for them to function in a concerted manner and drive tumor promoting processes, there need to be in place efficient networks of intercellular communication. Several such networks driven by chemokines have been extensively documented in ovarian cancer [51, 101].

Several types of stromal cells, including fibroblasts, adipocytes, endothelial cells, macrophages and other immune cells, following interaction with tumor cells develop tumor supporting phenotypes and express genes that support tumor growth, angiogenesis and metastasis [3, 51, 102-106]. The cross-talk both between the tumor cells themselves and with stromal cells clearly plays a significant role in the progression of the disease. Identifying the
mechanisms that mediate such intercellular cross-talk will be crucial in furthering our understanding of this complex microenvironment.

Figure 6: Cellular cross talk and signaling events in ovarian cancer metastasis [3].

Ovarian cancer progression and metastasis from the primary site to the omental metastatic tumor site are facilitated by the interaction between cancer cells and various stromal components. Left: at the primary tumor site (ovary), ovarian cancer cell motility is enhanced by cancer-associated fibroblast (CAF)-derived secretory proteins. Versican (VCAN) expression is upregulated in CAFs via activation of transforming growth factor (TGF)-β signaling. VCAN then activates NF-κB signaling in ovarian cancer cells and promotes cancer cell motility and invasion potential via upregulation of CD44, hyaluronan-mediated motility receptor (HMMR), and MMP9. At the same time, CAF-derived microfibrillar-associated protein 5 (MFAP5) binds
to the αβ-integrin receptors on the ovarian cancer cell surface, activating the Ca²⁺-dependent FAK/cAMP response element-binding protein/troponin C type 1 signaling pathways. Activation of such signaling stimulates reorganization of the F-actin cytoskeleton and enhances generation of cell traction force, thereby increasing the migration potential of ovarian cancer cells. In addition, chemokine (C-X-C motif) ligand 12 (CXCL12) is a well-reported tumor-promoting factor from CAFs. CXCL12 binds to its receptor, chemokine (C-X-C motif) receptor type 4 (CXCR4), on cancer cells to increase tumor cell proliferation and migration and CXCR4 on endothelial cells to induce tumor angiogenesis. On the other hand, cancer cell-derived fibroblast growth factor 1 (FGF1) and fibroblast growth factor 18 (FGF18), in addition to promoting tumor progression via an autocrine mechanism by binding to its receptor, fibroblast growth factor receptor 4 (FGFR4), on cancer cells, also promote tumor angiogenesis through a paracrine mechanism by binding to FGFR4 on endothelial cells.

Together with cancer cell-derived VEGF, these proangiogenic factors promote the establishment of tumor microvessels, which could subsequently facilitate hematogenous metastasis of ovarian cancer cells. *Right*: in the omentum, a common metastatic site for ovarian cancer, secretory factors from adipocytes and macrophages contribute to a favorable microenvironment for tumor development. Interactions between adipocytes and cancer cells in the omentum promote tumor progression. In addition to providing an energy source for cancer cells, adipocytes also secrete multiple cytokines, including TNF-α, IL-6, and IL-8, which act on ovarian cancer cells and accelerate tumor progression. Macrophages are highly abundant within the omentum, especially at the milky spots. Macrophages actively produce and secrete TNF-α and VEGF into the tumor microenvironment, which promotes tumor progression and tumor angiogenesis, respectively.

*(Figure and caption originally published by Yeung et al. in 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. Am J Physiol Cell Physiol, 2015.)*
1.3 MicroRNAs as mediators of intercellular communication

1.3.1 MicroRNAs

The landmark studies of lin-4 in C. elegans led to the discovery of a new class of molecules called microRNAs (miRNA, miR) [107, 108]. According to the most recent data, 2588 mature human miRNAs have been identified and sequenced [109]. miRNAs are transcribed by RNA polymerases (II and rarely III) to form primary miRNA transcripts (pri-miRNA) [110]. The pri-miRNA is then enzymatically cleaved into pre-miRNA by Drosha and then exported to the cytoplasm. There, it is enzymatically cleaved by Dicer, leading to formation of the mature single-stranded miRNA [110].

miRNAs bind to messenger RNAs as part of the RNA-induced silencing complex (RISC) and serve as post-transcriptional regulators of gene expression [111]. The seed sequences (nucleotides 2–8) of the mature miRNAs bind to the complementary region in the 3′UTR of mRNAs causing their degradation. Alternatively, when perfect complementarity cannot be achieved, or when miRNAs bind to the 5′UTR of the target genes, they inhibit translation [112, 113]. This model of miRNA biogenesis and function is illustrated in Figure 7.

Given the ability of miRNAs to control gene expression [111, 114, 115], they unsurprisingly have become a focal point for their involvement in cancer. In fact, it has been found that miRNAs are frequently dysregulated in cancers [116-119] where they have been shown to contribute to pathogenesis, as well as disease progression and metastasis [120-125]. They may also serve as surrogate markers for clinical response to drug treatments and outcomes
Figure 7: The current model for the biogenesis and post-transcriptional suppression of microRNAs and small interfering RNAs [114].

The nascent pri-microRNA (pri-miRNA) transcripts are first processed into ~70-nucleotide pre-miRNAs by Drosha inside the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into miRNA:miRNA* duplexes by Dicer. Dicer also processes long dsRNA molecules into small interfering RNA (siRNA) duplexes. Only one strand of the miRNA:miRNA* duplex or the siRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target. ORF, open reading frame.

(Figure and caption originally published by He et al. in He, L. and G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet, 2004. 5(7): p. 522-31.)
Furthermore, oncogenic miRNAs that drive tumor progression could potentially be targeted for treatment [128-131]. Consequently, we can glean from these findings that an effective miRNA signature for cancers would be of diagnostic, prognostic, and therapeutic value. Intriguingly, recent studies have presented the evidence that miRNAs can also function as intercellular signaling molecules [132, 133].

(The text in Section 1.3.1 has been previously published by Muralidhar et al. in Muralidhar, G.G. and M.V. Barbolina, The miR-200 Family: Versatile Players in Epithelial Ovarian Cancer. Int J Mol Sci, 2015. 16(8): p. 16833-47.)
1.3.2 MicroRNAs as mobile signaling molecules

The concept of the direct exchange of microRNAs mediating cell-to-cell communication in mammalian cells, emerged following the discovery that microRNAs were present inside nanovesicles [134]. These vesicles carrying microRNA could transfer their cargo out of the cell of origin to recipient cells where they could exert their function of gene silencing [134]. The implication here was that miRNAs represented an entirely new form of mobile signaling distinct from the other receptor-ligand mediated signal transduction. This added a further level of utility to the model of biogenesis and RNA interference (Figure 7) which is not bound by the walls of their originator cell (Figure 8).

The physical properties and functional capabilities of miRNAs could under-line their potential ability to participate in paracrine as well as endocrine mechanisms of cell to cell communication. Buoyed by the probable magnitude of this finding, researchers have vigorously explored the role of microRNAs as mobile signaling molecules [132, 135-138]. Cell to cell transfer of miRNAs has been studied in cancer [139-143], neurodegenerative disease [144, 145], the immune system [146-148], reproduction [149, 150] and cardio-vascular biology [151, 152] to name a few. These studies have shed light on a process that is active in maintaining normal homeostasis and is also involved in various pathologies [132].

In order to live up to the mantle of mobile signaling molecules, miRNAs need to successfully negotiate the following steps [153]:

i. Packaging and release of the miRNAs from the cell of origin
ii. Transferring into the recipient cells without being degraded by RNAase

iii. Retaining their functional capacity in the recipient cells

The cell has developed exquisitely intricate mechanisms with the purpose of accomplishing these steps.

It has been shown that miRNA can be transferred between cells via extracellular vesicles [134, 136, 137, 142, 154-157], HDL particles [158-160], RNA binding proteins [161, 162] and gap junctions [163-165]. Together, these mechanisms constitute the contact dependent and contact independent miRNA transfer. The significance of the mechanism that drives the process is that it dictates the sphere of influence that the miRNAs can exert. The contact dependent mechanisms can be expected to play a major role as paracrine mediators in influencing the neighboring cells. The contact independent processes can be expected to influence sites far away from the miRNA secreting cell. Understanding these different mechanisms not only adds to our knowledge of intercellular communication, but also has great therapeutic and diagnostic potential [132, 139, 166].
In the canonical pathway, primary miRNA transcripts (pri-miRs) are originally transcribed from introns of protein-coding genes. Pri-miRs are subsequently cleaved by the RNase III type endonuclease DROSHA with its partner proteins (Drosha complex), which generates 70-nt stem loop precursor miRNAs (pre-miRNAs). After being exported from the nucleus to the cytoplasm, DICER1, another RNase III type endonuclease, processes pre-miRNAs, generating 22-nt mature form of miRNAs. One of the double-strand mature miRNAs is selectively loaded into the RISC complex, which contains the AGO family protein as a core component. In the cytoplasm, mature miRNAs predominantly bind to the 3′-UTR region of the target mRNA, and repress its expression through mechanisms of both translational repression and mRNA destabilization. A subset of miRNAs have the ability to bind to the ORF and the 5′-UTR of the target mRNA, and activate or repress its translational efficiency. MiRNAs are also secreted to the extracellular space and possibly involved in cell–cell communication. Extracellular miRNAs are stable within vesicles, which include apoptotic bodies and exosomes, or in conjunction with RNA-binding proteins, such as AGO and HDL. In the nucleus, miRNAs bind to gene promoter to regulate gene expression. Alterations of transcriptional activity are associated with histone modification signatures, suggesting that miRNAs are possibly involved in the recruitment of chromatin modifying factors to the promoter region.

1.3.3 Extracellular vesicle mediated microRNA transfer

The vesicle mediated route is the most frequently studied and reported mechanism of miRNA transfer in different cellular systems. This process is significant for its ability to mediate both paracrine and endocrine signaling. The term ‘extracellular vesicles’ can be used to describe different types of vesicles: exosomes, ectosomes and apoptotic bodies. They can be distinguished on the basis of biosynthesis, size and surface markers (Table 2).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Exosomes</th>
<th>Ectosomes</th>
<th>Apoptotic Vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biogenesis</td>
<td><img src="image" alt="Exosome Biogenesis" /></td>
<td><img src="image" alt="Ectosome Biogenesis" /></td>
<td><img src="image" alt="Apoptotic Vesicle Biogenesis" /></td>
</tr>
<tr>
<td>Diameter</td>
<td>30 - 100 nm</td>
<td>100 nm - 1 μm</td>
<td>1 μm - greater</td>
</tr>
<tr>
<td>Exemplary Surface Markers</td>
<td>RAB proteins (Rab11, Rab27, Rab 35) Tetraspanins (CD63, CD81, CD9)</td>
<td>Phosphatidyl serine</td>
<td>Phosphatidyl serine</td>
</tr>
</tbody>
</table>

Table 2: Different types of Extracellular Vesicles
Figure 9: Long distance transfer of genetic material in extracellular vesicles (EVs) [166]

A. EVs originate through at least three mechanisms. 1) Fusion of multivesicular bodies (MVBs) with the plasma membrane and release of their intraluminal vesicles (ILVs) as exosomes. Neutral sphingomyelinase 2 (nSMase2) is essential for formation of ILVs in the early endosome. Some proteins are channelled by the ESCRT machinery to the MVB route. Rab proteins such as RAB11, RAB27 and RAB35, known to participate in vesicle trafficking between intracellular compartments, have been shown to play a role in exosome secretion. 2) Blebbing of the cellular plasma membrane (ectosomes). 3) Breakdown of dying cells into apoptotic bodies. EVs, which are secreted into the extracellular environment, contain functional mRNA, microRNA and DNA molecules that can be taken up by recipient cells through mechanisms including fusion with the plasma membrane, phagocytosis, or endocytosis. B. All exosomes contain proteins involved in membrane transport and fusion (Rab proteins, Annexins). Cytoskeletal proteins, adhesion molecules and tetraspanins are also abundant. Exosome membranes are enriched in RAFT-lipids (cholesterol, ceramide, sphingolipids). ERM, ezrin–radixin–moesin; HSP, heat shock protein; ICAM, intercellular cell adhesion molecule. Exosomes also contain RNA, mainly microRNA.

Several pathways are responsible for multivesicular body generation, sorting and exosome production. The endosomal sorting complex (ESCRT) machinery is the best characterized among these. It drives the generation of multivesicular bodies in the cell [167], that enclose the vesicles which in turn have been shown to contain miRNAs *(Figure 9)* [166, 168]. Another pathway found to regulate vesicle formation and trafficking is the ceramide dependent pathway [169]. The enzyme neural sphingomyelinase which breaks down sphingomyelin into ceramide appears to be necessary for MVB and exosome production [170].

The endosomes undergo the sorting and maturation process and are directed either towards the formation of lysosomes for degradation or exosomes for release [171]. Different vesicle surface proteins serve as markers for the stages of maturation [172]. The RAB family of proteins is frequently used to identify the stage of progression that an endosomal vesicle is in [171, 173, 174]. Their function is to mediate the traffic of vesicles between different cellular compartments.

Vesicle mediated transfer is an attractive model to study as they represent containers in which miRNAs can be securely packed, without exposure to inactivating enzymes and be transported to other cells where they can exert functions of target gene silencing [153]. It has also been observed that 30% of all extracellular miRNAs are found contained within extracellular vesicles [175]. This robust mechanism has been demonstrated in several models of normal and pathogenic cells [137, 151, 154, 155, 157, 176-179]. In spite of the extensive investigation, the process is not yet entirely understood. A fascinating finding has been that
the contents of the secreted exosomes do not merely reflect the transcriptional profile of the donor cell [166]. The cell thus, specifically synthesizes and packages certain miRNAs for release as opposed to the school of thought that assumed a passive process was in place [180]. Once secreted by donor cells, the vesicles can be taken up by the recipient cells through endocytosis, phagocytosis, micropinocytosis or by direct fusion [181].

Taken together, it is clear that several steps need to be successfully completed in order for vesicular miRNAs to mediate intercellular communication. The desired miRNAs according to the intended message have to be synthesized. Once produced, the miRNAs have to be packaged securely into vesicles and secreted. The recipient cell mechanisms required for vesicle uptake need to fire at the appropriate juncture. And finally, the miRNA has to remain functional and deliver the message to the target in the recipient cell.
1.3.4 Other mechanisms involved in microRNA transfer

Among the other mechanisms that support miRNA transfer are HDLs [158, 159], argonautes and other RNA binding proteins [161]. The theory that one mechanism could serve to compensate for reduced functionality of another is given weight by the finding that when the ceramide synthesis pathway is blocked in cells, miRNAs are packaged into HDLs instead [147, 166]. The vesicular, lipid and protein bound miRNAs are frequently found circulating in the serum and thus represent potential biomarkers for diagnosis and treatment response [127, 161, 182, 183]. Another interesting mechanism is that of miRNAs shuttling through gap junctions. Gap junctions are highly organized structures that form an intercellular bridge when the cell membranes exhibit close contact. They are made up of connexins which can open and close the bridge to allow traffic of small particles, ions and other water soluble cargo [164, 184]. Gap junctions, as they rely heavily on cell-cell contact, would be of great importance to paracrine communication. Several groups have shown that gap junctions and connexin43 in particular, mediate miRNA transfer [163, 165, 185-187].

In addition to the above mentioned routes, several other modes of miRNA transfer have been identified. The exquisitely complex networks that regulate this process suggest that this capacity to communicate via miRNAs was acquired early in the evolutionary process and is likely active across living organisms [146]. Even more interestingly, the sheer number of mechanisms that mediate miRNA transfer implies that several levels of redundancies and safeguards that are active in order to ensure that the necessary messages are delivered at the right time even if one of the systems is malfunctioning.
1.3.5 MicroRNA transfer in EOC

Several studies have focused on different types of vesicles (mainly exosomes) and their payloads in ovarian cancer [188-192]. Given their ability to exert powerful effects on proliferation, migration, invasion and drug resistance, it is understandable that there has been interest in studying the functional role of vesicular miRNA. It may however be crucial to expand one’s attention beyond the functional implications alone. Every novel functional discovery makes it ever more compelling to study the mechanics of the process itself. Remarkably, thorough characterization of miRNA transfer as a process of mediating intercellular communication has not yet been undertaken. This dissertation represents the work done to address that very point.

1.4 MicroRNA-200 Family in EOC [193]

The role of microRNAs (miRNAs or miRs) in the pathology of epithelial ovarian cancer (EOC) has been extensively studied [126, 194-198]. Many miRNAs differentially expressed in EOC as compared to normal controls have been identified, prompting further inquiry into their role in the disease. miRNAs belonging to the miR-200 family have repeatedly surfaced over multiple profiling studies. This section is an attempt to consolidate the data from different studies and highlight mechanisms by which these miRNAs influence progression of metastasis and chemoresistance in EOC.
1.4.1 MicroRNA-200 Family

Owing to their versatile functions, miRNAs can be instrumental in improving our understanding and treatment of EOC [198]. Many miRNAs have been found to be differentially expressed in ovarian carcinomas compared to normal tissues. Due to the high frequency of genomic alterations in miRNA genes in ovarian cancer, a corresponding degree of miRNA dysregulation has also been observed [199, 200]. The dysregulated miRNAs in ovarian cancer, as well as their clinical significance, has been reviewed elsewhere [198]. Recent analysis of the Cancer Genome Atlas (TCGA) data identified a gene network along with the predicted regulatory miRNAs that characterized a pro-malignant mesenchymal phenotype of serous EOC [201]. They showed that 89% of the target genes in the network were regulated by 8 key miRNAs. Two of these key miRNAs, miR-141 and miR-200a, are members of the miR-200 family.

The miRNA-200 family (miR-200 family or miR-200) has repeatedly been implicated for its involvement in EOC as well as other cancers [202-204]. This family consists of miR-200a, 200b, 200c, 141 and 429 (Figure 10). They arise from two different gene clusters: miRs-(200a/200b/429) from chromosome 1 (1p36.33) and miRs-(200c/141) from chromosome 12 (12p13.31) [205]. They share a high degree of sequence homology with a difference of only one nucleotide in their seed sequence (nucleotides 2–8) [205] and regulate expression of many of the same target genes.
miRNA-200 family arises from two gene clusters: miR-200b, miR-200a and miR-429 from chromosome 1 (1p33.36) while miR-200c and miR-141 from chromosome 2 (12p13.31). The highlight indicates that in the seed sequence (nucleotides 2–8) the difference is only in one nucleotide.
1.4.2 Expression Profiles in EOC

One approach to understanding the impact that miRNAs have on cancers is to identify the miRs that are aberrantly expressed in them. With the advent of superior profiling technologies, multiple studies were performed in order to identify the miRNAs that were differentially expressed in EOC and could be linked to pathogenesis and disease progression. Across multiple studies with different detection platforms and over extensive sets of tumor tissues, cell lines, and large sets of normal control samples, differential expression of the members of the miRNA-200 family is a consistent finding. These studies have been summarized in Table 3.

A comparative genomic hybridization study of epithelial cancers, including ovarian cancer, showed frequent alteration in loci containing miRNA genes resulting in aberrant miRNA expression profiles [199]. Specifically, the miRNA-200 family genes showed copy number gains. This indicated an increased expression of miRNA-200 which was also confirmed by another study that compared expression profiles of normal ovarian tissue and ovarian cancer to determine a miRNA signature for ovarian cancer [206]. The major finding was the up-regulation of miR-200a, 141, 200c and 200b. Moreover, miR-200a and miR-200c showed increased expression in serous, endometrioid and clear cell cancer while miR-200b and miR-141 were up-regulated in endometrioid and serous histotypes thereby indicating histotype specificity. Another study with a smaller number of samples also showed that miR-200a expression was increased in ovarian tumor tissues [207]. Their results showed that miR-200a overexpression along with miR-199a* and miR-204 was associated with high grade and late
stage tumors thus suggesting a role in tumor progression. These studies establish a compelling argument for elevated miR-200 family expression as a significant characteristic of ovarian tumors compared to their non-neoplastic counterparts.

The cell of origin in ovarian cancer has been debated [41, 208-210]. This adds to the complexity of interpreting the data from the profiling studies as the results may differ based on what cells are being used as “normal” controls [211, 212]. In the profiling studies undertaken so far, the whole ovary, ovarian surface epithelium (both primary and immortalized), and fallopian tube epithelium have been used as controls. miRNA profiling of serous ovarian cancers compared to fallopian tube epithelial cells showed that miR-200a, 200b, 200c and 141 were up-regulated in low grade serous cancer whereas only miR-200c and 141 were up-regulated in high grade serous tumors [213].

A recent study that utilized parallel pyrosequencing, compared miR expression in normal human ovarian surface epithelium (HOSE) cells and immortalized HOSE cells to that in late stage ovarian tumors [214]. It was found that the miR-200 family expression increased in the HOSE cells following immortalization using E6/E7 viral proteins [214]. This indicated an increase in miR-200 expression in cancer cells while compared to HOSE cells, and this result would have been missed when compared to immortalized HOSE cells [215].

Finally, the data from the cancer genome atlas study showed a down-regulation of miR-200a in the mesenchymal subtype of serous ovarian cancers [201]. These differences in results can
also potentially indicate a mechanistic alteration in expression depending on various factors among which could be the disease stage, the histotype, and whether or not the tumor is metastatic. Information regarding the mechanism of action of miR-200 suggests that the key may lie there.

Following the discovery of the presence of miRNAs in exosomes [134], tumor-derived exosomes became possible surrogate markers for diagnosis, prognosis, and clinical outcomes [216]. Interestingly, exosomes derived from the peripheral circulation of patients with ovarian tumors displayed a similar expression pattern of the miR-200 family as the tumor cells [217]. Elevated levels of miR-200a, 200b and 200c were also observed in the serum of serous ovarian cancer patients [218].
Table 3: miRNA-200 family expression in ovarian cancer profiling studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Samples &amp; Normal Controls</th>
<th>miRNA 200 Family Expression</th>
<th>Conclusions Made by Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iorio et al. [206]; Ohio State Comprehensive Cancer Center microarray, version 2.0 with 460 mature miRNA probes (235 human miRNAs)</td>
<td>Samples: 69 malignant tumor tissues (including serous, endometrioid, clear cell, poorly differentiated and mucinous ovarian carcinoma); Controls: 15 normal ovarian tissue sections</td>
<td>Increased expression of miR-200a, 200b, 200c and 141 in tumor samples vs. normal tissue</td>
<td>MiR-200a, 200b, 200c, and 141 share a common putative target BAP1 (BRCA associated protein 1), a tumor suppressor down-regulated in ovarian cancer</td>
</tr>
<tr>
<td>Yang et al. [207]; Oligonucleotide array, GeneScreen Plus (NEN) membranes printed with 515 human and mouse miRNA probes</td>
<td>Samples: 10 human ovarian epithelial tumors; Controls: Normal ovarian tissue and immortalized human ovarian surface epithelium</td>
<td>43% of primary ovarian carcinomas showed increased miR-200a expression</td>
<td>Increased miR-200a expression was associated with high grade and late stage disease</td>
</tr>
<tr>
<td>Dahiya et al. [215]; miRCURY™ LNA miRNA arrays with 1458 probes for all miRNAs in miRBase Release 8.1 (Exiqon)</td>
<td>Samples: 34 cancer tissues and 10 ovarian cancer cell lines (BG-1, UCI-101, HEY, OVCA420, OVCA432, OVCA433, OVCAR2, OVCAR3, OVCAR5, OV90); Controls: HOSE-B cells (human ovarian surface epithelial cell line immortalized with E6 and E7)</td>
<td>MiR-200a and 141 were found to be down-regulated in the neoplastic samples</td>
<td>Using Target Scan 3.0 miR-200a and 141 were found to share three predicted targets (ZEB2, KLF12 and ZFR)</td>
</tr>
<tr>
<td>Wyman et al. [214]; Parallel pyrosequencing (454 Life Sciences Platform)</td>
<td>Samples: Stage III/IV ovarian tumors including 19 serous, 4 clear cell and 10 endometrioid; Controls: 4 Normal primary human ovarian surface epithelium (HOSE) and E6/E7 immortalized HOSE</td>
<td>MiR-200a, 200b, 200c, 141, and 429 showed increased expression in ovarian tumors and the immortalized HOSE</td>
<td>Normal HOSE expresses low levels of miR-200 family. Immortalization induces their expression</td>
</tr>
<tr>
<td>Study</td>
<td>Samples &amp; Normal Controls</td>
<td>miRNA 200 Family Expression</td>
<td>Conclusions Made by Authors</td>
</tr>
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</tr>
<tr>
<td>Lee et al. [213]; Microarray with 668 Ambion probes (328 known and 154 predicted human miRNA probes)</td>
<td>Samples: 37 serous tumors (including high grade, low grade and borderline serous tumors); Controls: 3 normal fallopian tube epithelium sampled from the fimbriae</td>
<td>In high grade serous tumors miR-200c and 141 were up-regulated; In low grade serous tumors, miR-200a, 200b, 200c, and 141 were up-regulated</td>
<td>MiR-200a, 200b, 200c, and 141 were up-regulated in serous tumors. This was the first study that used fallopian tube epithelium as normal control as opposed to ovarian surface epithelium</td>
</tr>
<tr>
<td>Bendoraite et al. [219]; qRT-PCR using Taqman miRNA assays (Applied Biosciences)</td>
<td>Samples: Stage III/IV malignant ovarian primary tumors from 70 patients (including serous, endometrioid, and clear cell histotypes), 15 ovarian cancer cell lines (A1847, A2780, CaOV3, ES-2, HEY, IGROV1, OVCAR3, OVCAR5, OVCAR10, OV-90, PEO-1, SKOV3, TOV-21G, TOV-112D, 2008); Controls: Non-immortalized early passage primary cell cultures derived from HOSE as normal controls</td>
<td>Expression of all five members of miR-200 family were substantially higher in the primary tumors compared to normal tissues</td>
<td>Low expression of ZEB2 and high expression of miR-200 family in the tumor samples supports mesothelial to epithelial transition model</td>
</tr>
</tbody>
</table>
1.4.3 Metastasis

There is a very high degree of sequence homology between the members of the miR-200 family [220] (see Figure 1). Due to the homology in their seed sequences, they share several targets. Two established targets of miR-200 are the zinc finger transcriptional repressors: ZEB1 (TCF8/ZFHX1A/δEF1) and ZEB2 (SIP1/ZFHX1B/SMAD1P1) [201, 205, 221-224]. The ZEB transcription factors bind to the E-boxes in promoter regions of E-cadherin and cause transcriptional repression of E-cadherin expression [225-228]. E-cadherin is a critical protein for maintenance of the epithelial phenotype. ZEB-mediated loss of E-cadherin causes cells to develop spindle-shaped morphology and express greater migratory and invasive potential [229].

The ability to manipulate ZEB expression makes miR-200 ideally positioned to influence the process of epithelial to mesenchymal transition (EMT) [203, 230, 231]. Further, there exists a double negative feedback loop between miR-200 and the ZEB genes [232, 233]. ZEB binds to E-boxes in the miR-200 promoter and thereby suppress their expression. While miR-200 causes post-transcriptional repression of ZEB, the latter regulates transcriptional repression of the miRNAs [234]. This double negative feedback loop allows greater flexibility over cell fate, but complicates attempts to understand the reversible EMT process, especially in terms of isolating the initiating events. Also, some degree of variation in the effect on EMT has been reported between the different members of the miR-200 family [235]. There has been evidence supporting a mesothelial to epithelial transition (MET) in normal cells during ovarian tumorigenesis that involves increased miR-200 expression [219]. The ovarian cancer cells
could later undergo the traditional EMT during metastatic dissemination. This raises the possibility of a dual expression profile of miR-200 during tumor progression [219].

It has been shown that TGF-β mediated down-regulation of miR-200 in mesothelial cells promotes cancer cell attachment and proliferation [236]. Additionally, reduced miR-200 expression causes increased activity of its targets, Interleukin-8 and chemokine ligand CXCL1, secreted by both endothelial as well as tumor cells [237] resulting in increased angiogenesis and metastasis. Both studies demonstrated that delivery of miR-200 in mouse models as therapy caused suppression of metastatic dissemination.

Even as more information regarding the potential of miRNA-based therapeutics is gathered [238], development of successful miRNA delivery systems remains a challenge [239, 240]. Yet, in light of their involvement in ovarian carcinoma metastasis, a miR-200-based therapeutic strategy [241] could prove to be promising.
1.4.4 Effect on Chemotherapeutic Response and Clinical Outcomes

Current treatment options for ovarian cancer include surgical resection followed by chemotherapy. The drugs used for first line therapy include a combination of carboplatin (a platinum-containing alkylating agent) and paclitaxel (a microtubule-targeting agent). Since most cases are diagnosed at a late stage, the high rate of response of stage I patients to therapy is overshadowed by the relapse and mortality of patients diagnosed late. The relapse of the cancer is mediated by loss of sensitivity to the chemotherapeutic agents. Unsurprisingly, miRNAs seem to be significant players in therapy resistance [195].

Alteration in the expression of class III β-Tubulin (TUBB3) is one of the mechanisms by which ovarian cancer cells gain resistance to microtubule-targeting agents [242-244]. It has been shown that miR-200c binds to the 3′UTR of TUBB3 and down-regulates its expression thereby robustly sensitizing the cells to paclitaxel as well as other microtubule targeting agents, such as vincristine and epothilone [245, 246]. In a follow-up study, all the other members of the miR-200 family were also shown to be regulating TUBB3 levels [247]. In addition, low expression of miR-200 was shown to be a marker for poor survival and resistance to paclitaxel in ovarian cancer patients [247, 248].

However, further investigation of the interaction of miR-200c and TUBB3 along with the involvement of an RNA Binding Protein-HuR exposed the complexity of the underlying
mechanism. It has been shown that cytoplasmic HuR causes stabilization and increases the levels of TUBB3 [249] in conjunction with miR-200c [250] leading to poor survival. This is in direct contrast to the previous findings and prompted the researchers to propose a model that describes the two different mechanisms by which miR-200c regulates TUBB3 mRNA in ovarian cancer. According to this model, when HuR is located in the nucleus, high levels of miR-200c are favorable and cause down-regulation of TUBB3. On the other hand, cytoplasmic HuR causes a miR-200c-mediated increase in TUBB3 leading to paclitaxel resistance and poor outcomes [250]. This could also potentially explain the recent findings that showed high miR-200a, 200b and 200c expression correlated with poor overall survival [251].

Studies performed in mouse models showed that increased expression of miR-141 and miR-200a increased tumor growth [252]. However, the miRNAs were also responsible for repressing p38α that produces oxidative stress response, which was shown to improve clinical outcomes [252]. Reactive oxygen species (ROS) have been shown to play a crucial role in sensitizing cells to paclitaxel treatment, and the cells producing the oxidative stress response showed better response to paclitaxel [253, 254]. This paradox led the investigators to propose a model that explains the cross-talk between miR-200, p38α, and ROS. In normal cells, there is a balance between these players [252]. In a neoplastic cell that is still in the early stages of transformation, there is an increased concentration of ROS prompting up-regulation of the miRNAs, which in turn represses p38α. These conditions produce a state of oxidative stress,
which improves sensitivity to paclitaxel. As the tumor progresses, down-regulation of the miRNAs restores p38α expression thereby causing cells to become resistant to paclitaxel.

The miR-200 family has also been identified in ovarian cancer survival and clinical outcomes studies [247, 248, 255-257]. It was shown that higher expression of miR-200a, 200b, 200c and 141 was part of a miRNA signature that significantly correlated with decreased progression-free survival and overall survival in ovarian cancer [251, 255]. Conversely, results from other studies showed that higher expression of miR-200a was predictive of better outcomes and survival in ovarian cancer [237, 256, 257] and that the expression decreased with stage [257]. Similar results with the associations between miR-200c with overall survival and progression-free survival have also been shown [248, 258]. These discrepancies in the data from previous findings were suggested to be related to differences in profiling platforms [256] and insufficient staging information at the time of diagnosis [257]. In a large study, serum from 74 ovarian cancer patients, 19 borderline patients and 50 healthy controls were extracted and the levels of miR-141 and 200c were measured. While their elevated expression could be used to distinguish patient from healthy controls, higher expression also correlated with increased survival [259]. Some of the studies that investigated the relationship between miR-200 and clinical outcomes have been summarized in Table 4.
### Table 4: Predictive value of miRNA-200 family expression for disease outcomes.

<table>
<thead>
<tr>
<th>Study</th>
<th>Samples</th>
<th>miRNA 200 Family Expression</th>
<th>Conclusions Made by Authors</th>
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</thead>
<tbody>
<tr>
<td>Nam <em>et al.</em> [255]; Microarray with 377 (314 human) mirVana miRNA probes (Ambion)</td>
<td>Samples: 20 serous ovarian cancer tissues: 9 chemo-resistant, 11 chemo-sensitive tumors; Controls: 8 normal ovarian tissues</td>
<td>Increased expression of miR-200a, 200b, 200c and 141 in tumor samples vs. normal tissue</td>
<td>High expression of miR-200a, 200b, 200c and 141 were significantly correlated with decreased progression-free survival as well as overall survival</td>
</tr>
<tr>
<td>Hu <em>et al.</em> [256]; qRT-PCR miRNA assays (Applied Biosystems)</td>
<td>55 patients: 48 epithelial ovarian carcinomas and 7 primary peritoneal carcinomas</td>
<td>Disease recurrence and poor overall survival were associated with low miR-200a, 200b and 429 expression</td>
<td>miR-200b-429 cluster expression has prognostic value in EOC</td>
</tr>
<tr>
<td>Eitan <em>et al.</em> [257]; Custom microarray slide (Nexetrion®) with 900 miRNA probes</td>
<td>57 patients who had undergone surgery for tumor resection: 19 Stage I patients, 38 Stage III patients; All received platinum based chemotherapy</td>
<td>miR-200a expression was higher in Stage I ovarian cancer compared to Stage II</td>
<td>The data set shows significantly higher expression of miR-200a in early stage disease correlating with improved survival</td>
</tr>
<tr>
<td>Marchini <em>et al.</em> [248]; G4470B human miRNA microarray (Agilent Technologies) with probes for 723 human miRNAs</td>
<td>144 patients with Stage I EOC out of which 29 patients relapsed</td>
<td>Tumors with lower miR-200c levels seen in patients who relapsed</td>
<td>miR-200 expression could be used as an indication of relapse in Stage I tumors</td>
</tr>
<tr>
<td>Leskela <em>et al.</em> [247]; qRT-PCR using the miRCURY™ LNA miRNA assay kits (Exiqon)</td>
<td>72 patients were studied for overall survival analysis; A subgroup of 57 patients with both advanced tumor stage and serous carcinoma histotype were studied for treatment response</td>
<td>miR-200 expression correlated with β-Tubulin III levels</td>
<td>Low miR-200 expression was seen in patients without complete response to paclitaxel when compared to patients with complete response; Low miR-200 expression had a trend towards poor survival</td>
</tr>
</tbody>
</table>
In spite of such extensive investigation, the expression and role of miR-200 in EOC remains a point of contention. Available data indicate that these miRNAs are subject to dynamic changes depending on the stage of tumor progression, EMT, nuclear or cytoplasmic localization of interacting proteins and the cellular ROS content; it will, to an extent, explain the discordant data in the profiling studies. Additionally the double negative feedback loop between miR-200 and the ZEB genes taken into context along with the possibility of functional transfer of the microRNA between the cells could help us further our understanding of the variability in the data. A compilation of some of the published findings pertaining to miR-200 [219, 250, 252] lends itself to a possible model of tumor progression in ovarian cancer as shown in Figure 11. Depending on where and when the tumors are sampled from, they may exhibit very contradictory expression patterns. It might be beneficial to perform preliminary studies in animal models in order to standardize the normal cell controls, tumor stage, site and histotype among other variables. Effectively establishing the role of miR-200 in EMT and chemo-resistance will hopefully open new avenues for therapeutic intervention. Irrespective of the exact associations, it is quite clear that these miRNAs are indeed versatile players in the EOC microenvironment.
Figure 11: Model for the expression and mechanisms of action of miR-200.
This figure has been adapted from Bendoraite et al. [219], Mateescu et al. [252] and Prislei et al. [250]. miR-200 could regulate tumorigenic and metastatic transformation by Mesothelial to Epithelial Transition (MET) and Epithelial to Mesenchymal Transition (EMT) respectively. miR-200 expression aided by ROS represses p38α and increases sensitivity to paclitaxel. In cancer cells with low miR-200 expression, this process is not active leading to paclitaxel resistance. Another mechanism involved is the miR-200 mediated down-regulation of TUBB3 in cells with nuclear HuR leading to better clinical response and treatment outcomes.
1.5 Motivation

Epithelial ovarian carcinoma (EOC) is a leading cause of death from gynecological malignancies [260]. A lot of the roadblocks in effectively treating patients with metastatic disease and preventing chemo-resistance can be attributed to the insufficient understanding of the biology and the mechanisms involved in EOC metastasis. The EOC microenvironment is characterized by complex interactions between the different cell types, which are mediated by several means of cell-to-cell communication. A recently discovered mechanism of intercellular communication is the cell-to-cell transfer of microRNAs (miRNAs or miRs) [134, 165, 186, 261, 262]. microRNAs and their role in cancer have attracted a lot of attention in recent years [117, 118, 120, 121, 263]. They have been identified as key regulators of metastatic progression, tumor response to treatments and clinical outcomes in EOC [194, 196, 201, 206, 264, 265].

When a cell or subsets of cells gain differential expression of miRNAs, it would result in tumor heterogeneity. If miRNA could be transferred between neighboring cells, the effect of deregulation of miRNA expression in one cell could be significantly amplified to the scale of an entire tissue. As the transfer of functional microRNAs between cells could lead to dramatic changes in the fate of the cell-recipient, it is worth exploring their role as mediators of intercellular communication in the EOC microenvironment. It is possible that miRNA transfer could play a key role in regulation of the function of the bystander cells through changes in their gene expression, and possibly leading to development of more malignant phenotype in EOC, as well as conversion of the stromal cells toward tumor-supporting phenotype. This could
lead to reprogramming of the cell fates of both tumor and stromal cells, resulting in induction of resistance to therapies and formation of cancer stem cells among other potential outcomes. Before that can be established, it will be vital to characterize the phenomenon of cell-to-cell microRNA transfer, which this thesis aims to accomplish.
1.6 Scope and Organization of Thesis

The objective of this work was to characterize the process of cell-to-cell transfer of miRNAs as an apparatus of intercellular communication in the context of the epithelial ovarian cancer microenvironment. To that end, several experiments were performed which have been organized in this thesis as follows:

- **Chapter 2** presents the microscopic as well as flow-cytometric evidence that allow the visualization and quantification of miRNA transfer in EOC cell lines.

- **Chapter 3** examines the functional significance of miRNA transfer by measuring the levels of transferred miRNA as well as the effect of the transferred miRNA on its putative targets.

- **Chapter 4** probes the mechanistic details of the intercellular miRNA transfer through live-cell imaging data as well as immunofluorescence imaging methods.

- **Chapter 5** summarizes the key findings of this thesis and outlines future directions of research that could merit investigation on the strength of the data compiled here.
Chapter 2: VISUALIZATION & QUANTIFICATION OF MICRORNA TRANSFER

2.1 Introduction

At the outset, in order to determine whether intercellular shuttling of miRNAs occurs in the EOC cell model, visual evidence was sought. Effective capture of the transfer of miRNAs from one cell to another required the use of a dual fluorophore model. This was set up using different tags to distinguish between donor and recipient cell population.

To this end, donor cells were transfected with cy3/cy5 labeled miRNAs and recipient cells were tagged to express GFP. This differential labelling was particularly crucial for the microscopic experiments (performed both in-vitro and in-vivo) where it achieved the goals of:

- Adequately distinguishing between the donor and the recipient cells and
- Tracking the movement of the miRNAs.

After miRNA transfer was successfully observed using microscopy, this was followed up by attempts to quantify the frequency of the transfer process. The dual fluorophore model was readily adapted to flow cytometric measurements and the frequency was quantified as a function of the recipient cells positive for the miRNA signal.
Taken together the data in this chapter demonstrates proof of the occurrence of the transfer process as well as provides an estimate of its ability to occur at measurable frequencies under varying conditions.
2.2 Materials & Methods

2.2.1 Cell Lines: Human ovarian carcinoma cell lines SKOV3, ES-2 and OVCAR4 with serous histotype that originated from malignant cells in the ascites fluid were purchased from the National Cancer Institute (NCI) Tumor Cell Repository (Detrick, MD). Another human ovarian carcinoma cell line, CAOV3, that originated from malignant cells in the ascites of HGSC patients was obtained from Dr. M. S. Stack (University of Notre Dame, IN). SKOV3, CAOV3 and ES-2 were cultured according to manufacturer’s instructions using minimal essential media (MEM) (Corning) supplemented with 10% fetal bovine serum (FBS) (SIGMA-ALDRICH), 0.5% penicillin/streptomycin (Corning), 0.4% amphotericin B (Corning) and 0.22% g/ml sodium bicarbonate (Santa Cruz Biotechnology) for less than 20 consecutive passages. OVCAR4 was grown in RPMI (Corning) containing 10% FBS, 0.5% penicillin/streptomycin, 0.4% amphotericin B and 0.2% g/ml sodium bicarbonate. All cells were kept at 37°C and 5% CO₂ in a humidified incubator, and were routinely tested for Mycoplasma. Cell line authentication was performed for all the cell lines using STR analysis.

2.2.2 Transfection of GFP plasmid with EndoFectin™ Max: The cell lines were transfected with GFP plasmid using the EndoFectin™ Max Transfection Reagent to generate the recipient GFP positive cell populations for the dual fluorophore model. The manufacturer’s recommended transfection protocol

(http://www.genecopoeia.com/wp-
2.2.3 **miRNA mimics**: mirVana™ miRNA Mimic, Negative Control #1 from Thermo Fischer (Cat No 4464058) was used. This mimic is a random sequence miRNA mimic molecule which as such does not effect any observable changes in cell behavior or morphology.

2.2.4 **Fluorescent (Cy3/Cy5) labeling of miRNA mimics:**

Mirus Label IT® miRNA Labeling Kits were used in order to covalently label the miRNA mimics with fluorescent tags (either cy3 for the imaging experiments or cy5 for the flow
cytometric measurements) using the manufacturer’s recommended protocol (https://www.mirusbio.com/assets/protocols/ml053_label_it_mirna_labeling_kit.pdf).

2.2.5 Transfection of labeled miRNAs using DharmaFECT: The transfection reagent DharmaFECT was used to transiently transfect the labeled miRNAs into the donor cell populations using the manufacturer’s recommended protocol (http://dharmacon.gelifesciences.com/uploadedfiles/resources/basic-dharmafect-protocol.pdf).

2.2.6 In-vitro model to study miRNA transfer using fluorescence microscopy: Donor cells transfected with fluorescently (cy3) labeled miRNA mimics were co-cultured with recipient cells tagged with GFP on glass coverslips at 50% confluency. Following 24 hours the cells were fixed with 4% Paraformaldehyde solution and incubated with 4’,6-Diamidino-2-phenylindole (DAPI) at a concentration of 10 μg/ml for 15 minutes. Cells were washed, air dried, and mounted on glass slides using ProlongGold (Invitrogen, Carlsbad, CA). Fluorescent imaging was performed using a Zeiss AxioObserverD.1 fluorescent microscope.

2.2.7 In-vivo model to study miRNA transfer using multiphoton microscopy: 1.5 million donor cells transfected with fluorescently (cy3) labeled miRNA mimics were mixed with 1.5 million recipient cells tagged with GFP and injected intra-peritoneally into nude mice. After 48 hours the mice were sacrificed using CO₂ and
cervical dislocation. The peritoneal wall was excised and multiphoton imaging was performed using the Ultima In-Vivo Multiphoton Laser Scanning Microscopy System. The 3D and orthogonal reconstruction were performed using the Imaris viewer.

2.2.8 Coating of cell culture plates with different modified supports: Tissue culture-treated plates were pre-coated with 10 μg/ml human collagen type I (Corning, Ref354236), Matrigel (diluted 1:100), 0.5mg/ml Poly-D-Lysine (MP Biomedicals, 102694 for 1 h at 37 °C. The plates were subsequently rinsed with PBS and air dried before use.

2.2.9 Ovarian cancer spheroid models: The ovarian cancer spheroids were generated by culturing the cells on plates with agarose overlay. The overlay was prepared by pouring 0.5% agarose on the culture plates and then allowing them to solidify and cool at room temperature for at least 30 min.

2.2.10 Flow cytometric quantification of miRNA transfer frequency: Donor cells transfected with fluorescently (cy5) labeled miRNA mimics were co-cultured with recipient cells tagged with GFP. After 254 hours the cells were trypsinized, washed and re-suspended in PBS and were then analyzed by using the BD acuri C6 flow cytometer. The number of GFP (+) recipient cells that were positive for the cy3 (+) miRNA were measured as a percentage of the total number of GFP (+) recipient cells in order to determine the frequency of the miRNA transfer process.
2.3. Visualizing miRNA transfer

2.3.1. In-vitro (Homotypic and Heterotypic)

The transfer of miRNAs was tested both between donor and recipient populations of the same parental cell line (homotypic transfer) as well as between the donor and recipient populations belonging to different cell lines (heterotypic transfer). A dual fluorophore model was utilized. Cy3 labeled miRNAs were transfected into donor cells and co-cultured with the GFP-tagged recipient cells. Following 24 hours of co-culture, the cells were fixed and stained with DAPI and imaged by fluorescence microscopy. Homotypic transfer of the cy3-miRNA to the GFP tagged recipient cells was observed in SKOV3 cells (Figure 13). 14-30% of the GFP tagged cells were seen to be recipients of the cy3-miRNA in the images taken. The process of homotypic miRNA transfer was also observed with the OVCAR4 cell line (Figure 14). Using the same model and method, heterotypic miRNA transfer of miRNA from CAOV-3 donor cells to ES2 recipient cells (Figure 15) as well as from OVCAR4 donor cells to ES-2 recipient cells (Figure 16) was observed.

Figure 12: Dual-fluorophore model setup to visualize miRNA transfer
Figure 13: Transfer of miRNAs between SKOV3 cells.
SKOV3 cell line was used to create both the donor and recipient cell populations. The donor cells were transfected with Cy3 (red) labelled miRNA mimics and recipient cells were tagged with GFP (green). Following co-culture for 24 hours, the green recipient cells show evidence of the homotypic transfer of red labelled miRNAs.
OVCAR4 cell line was used to create donor and recipient cell populations. The donor cells were transfected with Cy3 (red) labelled miRNA mimics and recipient cells were tagged with GFP (green). Following co-culture for 24 hours, the green recipient cells show evidence of the homotypic transfer of red labelled miRNAs.
Figure 15: Transfer of miRNAs from CAOV-3 cells to ES-2 cells.

CAOV-3 and ES-2 cell lines were used to create the donor and recipient cell populations respective. The donor CAOV-3 cells were transfected with Cy3 (red) labelled miRNA mimics and recipient ES-2 cells were tagged with GFP (green). Following co-culture for 24 hours, the green ES-2 recipient cells show evidence of the heterotypic transfer of red labelled miRNAs.
Figure 16: Transfer of miRNAs from OVCAR4 cells to ES-2 cells.

OVCAR4 and ES-2 cell lines were used to create the donor and recipient cell populations respective. The donor OVCAR4 cells were transfected with Cy3 (red) labelled miRNA mimics and recipient ES-2 cells were tagged with GFP (green). Following co-culture for 24 hours, the green ES-2 recipient cells show evidence of the heterotypic transfer of red labelled miRNAs.
2.3.2. **In-vivo (Homotypic transfer)**

While the in-vitro data demonstrates transfer of the labeled miRNA to the recipient cells it does not distinguish between miRNAs internalized into the recipient cells and miRNAs simply adhering to the cell surface. In order to address that shortcoming as well as to test the process in-vivo, the donor and recipient cell mixture was injected into athymic nude mice (Figure 17). Following 48 hours, the peripheral peritoneum was excised out and imaged using multi-photon microscopy to generate a 3-dimensional reconstruction of the tissue (Figure 18). The reconstruction also demonstrates several instances of miRNA transfer as seen in-vitro. The orthogonal view of the imaged section shows the cy3-miRNA internalized within the GFP tagged donor cells in the x, y and z axis (Figure 19). These results demonstrate the intercellular transfer of miRNAs in-vitro as well as in-vivo in ovarian cancer cell lines.

![Figure 17: Setup to test miRNA transfer, in-vivo](image)
Figure 18: Three-dimensional reconstruction of the cells adhered to the peritoneal wall. GFP tagged SKOV-3 cells (1.5 million cells) mixed with SKOV-3 cells transfected with cy3-miRNA (1.5 million cells) were injected intra-peritoneally into athymic nude mice. After 48 hours the mice were sacrificed using CO$_2$ and cervical dislocation. The peritoneal wall was excised and analyzed using two-photon microscopy and the 3D images were reconstructed using the Imaris viewer. The 3-dimensional reconstruction of the images revealed GFP tagged cells showing the transferred cy3-miRNA.
Figure 19: Orthogonal view of the three-dimensional reconstruction of the cells adhered to the peritoneal wall, confirming internalization of transferred miRNAs. The 3D reconstruction from Figure 4 can be analyzed by using these instances of the orthogonal view in order to confirm internalization of the transferred miRNAs in the GFP tagged recipient cells. The white intersecting axis lines traced over the x, y and z-axes indicates complete internalization of the red-labelled miRNA inside the green recipient cell.
2.4. Quantifying the Frequency of miRNA transfer

In order to quantify the frequency, fluorescent activated cell sorting (FACS) was used in conjunction with the dual-fluorophore model as previously described (Section 3.1). For flow cytometry the miRNAs were labelled with cy5. Following 24 hour co-culture the donor and recipient SKOV-3 cell population was sorted out into four sub-populations with distinct fluorescent markers (Figure 20). This allowed comparison of the frequency of miRNA transfer on modified supports coated with different proteins of the extracellular matrix specific for the epithelial ovarian cancer microenvironment. Different populations of fluorescent labelled and un-labeled cells were used as FACS controls to set the fluorescent thresholds (Figure 21). Frequency of the miRNA transfer events was determined in cells cultured on tissue culture treated plates, poly-d-lysine, collagen and matrigel coated plates (Figure 22(A)). The ovarian cancer cells are known to form multicellular clusters or spheroids. So the ovarian cancer spheroid model was tested as well (Figure 22(A)). Overall, the data indicated that miRNA transfer events occur consistently over several different culture conditions, both adherent and non-adherent (Figure 22 (B)). Another in-vitro experimental parameter to be queried was seeding cell density, and whether it affected the frequency of the process. To that end the donor and recipient cells were co-cultured at densities of 25, 50 and 75% on tissue culture treated plates. The miRNA transfer at 50% cell density (which was used for all the previous imaging as well as FACS experiments) was significantly higher than at 25 and 75% (Figure 23).
Figure 20: Experimental design to measure the frequency of miRNA transfer

The donor and recipient cell population with their respective fluorescent tags were co-cultured for 24 hours. Using FACS analysis, the number of transfer positive recipient cells could then be measured as a fraction of the total recipient cell population and serve as a quantitative measure of miRNA transfer.

Figure 21: FACS Controls to set the fluorescent thresholds for measurement and analysis

Populations of fluorescent labelled and un-labeled cells were used as FACS controls to set the fluorescent thresholds.
Figure 22: Effect of different culture conditions on the frequency of miRNA transfer

(A) Raw flow cytometry data. The thresholds for analysis were set using the controls shown in Figure 21.
(B) % miRNA transfer in different in-vitro culture conditions.
Figure 23: Effect of different cell seeding densities on the frequency of miRNA transfer

(A) FACS Controls to set fluorescent thresholds
(B) Raw flow cytometry data.
(C) % miRNA transfer in different seeding densities.
Chapter 3: FUNCTIONAL SIGNIFICANCE OF MICRORNA TRANSFER

3.1. Introduction

For the previous experiments we utilized a dual fluorescent label approach with confocal microscopy and flow cytometry (in-vitro) and two-photon microscopy (in-vivo) to study the miRNA transfer process. While the results did serve to clarify our hypothesis, they are not necessarily the primary readout of transfer. It is possible that the transfer we measured was that of free dye (cy3/cy5) used to label the miRNA being shuttled between cells. More importantly they do not provide information regarding the functional nature of the transferred miRNA. Hence the experiments in this chapter were designed to improve the model system and obtain confirmation that miRNA is indeed being transferred between the cancer cells and remains functional in the recipient cell.

As described in chapter 1, section 1.4, the miR-200 family members are versatile and important players in EOC. Analysis of The Cancer Genome Atlas revealed that miR-141 and miR-200a, both members of the miR-200 family are among 8 miRNAs that were predicted to be master regulators of at least 89% targets in a miRNA regulatory network characteristic of the pro-malignant phenotype of serous EOC [201]. Given its relevance in the EOC pathology, miR-200a was chosen as the candidate miRNA to study the functional significance of miRNA transfer in EOC.

In order to study the transfer of miR-200a a gradient in the expression of miR-200a between the donor and the recipient cell populations is required. To this end, either an artificial
gradient was created by over-expressing miR-200a in EOC cells or a cell line with little or no basal expression of miR-200a was co-cultured with a cell line having high basal expression of miR-200a (Figure 24). Following co-culture the recipient cells were collected and analyzed to test evidence of functional miRNA transfer.

Figure 24: Experimental design to study transfer of miR-200a
3.2. Materials & Methods

3.2.1. Cell lines: Human ovarian carcinoma cell lines SKOV3, ES-2 and OVCAR4 with serous histotype that originated from malignant cells in the ascites fluid were purchased from the National Cancer Institute (NCI) Tumor Cell Repository (Detrick, MD). Another human ovarian carcinoma cell line, CAOV3, that originated from malignant cells in the ascites of HGSC patients was obtained from Dr. M. S. Stack (University of Notre Dame, IN). OVSAHO and Kuramochi were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). SKOV3, ES-2 and CAOV3 were cultured according to manufacturer’s instructions using minimal essential media (MEM) (Corning) supplemented with 10% fetal bovine serum (FBS) (SIGMA-ALDRICH), 0.5% penicillin/streptomycin (Corning), 0.4% amphotericin B (Corning) and 0.22% g/ml sodium bicarbonate (Santa Cruz Biotechnology) for less than 20 consecutive passages. Kuramochi, OVCAR4 and OVSAHO were grown in RPMI (Corning) containing 10% FBS, 0.5% penicillin/streptomycin, 0.4% amphotericin B and 0.2% g/ml sodium bicarbonate. All cells were kept at 37°C and 5% CO2 in a humidified incubator, and were routinely tested for Mycoplasma. All cell lines were authenticated using STR analysis.

3.2.2. Measuring expression levels of miR-200a: miRNA expression levels for miRNA-200a were be determined by qRT-PCR. The miRNA isolation was performed by lysing 1 x 10^6 cells in 1ml of trizol (Life technologies, California) and precipitating with isopropanol and chloroform. Reverse transcription was performed using Universal cDNA Synthesis kits (Exiqon) according to the recommended protocol.
ExiLENT SYBR® Green master mix was used to perform qPCR for miRNA 200a (Exiqon LNA primer 204539) normalized to controls. UniSp6 and miR-103 were used as normalization controls. Data was analyzed using the 2-ΔΔct method and student’s t-test.

3.2.3. Transfection of miRNA Expression plasmids: The ES-2 cell line was transfected with hsa-mir-200a miExpress™ Precursor miRNA Expression Clone, (Genecopoeia Product ID: HmiR0002, precursor sequence:

ccgggccccugugagcaucuuaccggacagugcuggauuucccagcuugacucuaacugucugguaacgauguu caaaggugacccgc) using the EndoFectin™ Max Transfection Reagent to generate the GFP positive miRNA-200a over-expressing cells, ES2-miR200OE. The manufacturer’s recommended transfection protocol (http://www.genecopoeia.com/wp-content/uploads/oldpdfs/product/reagent/pdf/GeneCopoeia-EndoFectin_Max_Datasheet.pdf) was utilized.

3.2.4. miRNA-200a transfer experimental setup: The donor cells with high miR-200a expression were co-cultured with recipient cells that expressed little or no miR-200a for 48 hours. The donor and recipient cell populations were distinguished by tagging one of the populations with GFP. Using the GFP label, the cells were sorted out using the Moflo cell sorter and the recipient cells were collected for further analysis by qRT-PCR.
3.2.5. Measuring expression levels of miR-200a target genes: Total RNA isolation was performed with the SV Total RNA isolation system (Promega, Wisconsin). Reverse transcription was be performed using High capacity reverse transcription kit (ABI) according to the recommended protocol. Q-PCR was performed using SYBR green (ABI) for ZEB1, ZEB2, CTNNB1 and TGFβ2 (all predicted gene targets of miR 200a). The primer sequences are as follows.

**ZEB1**
Forward Primer: TTACACCTTTGCATACAGAACCC
Reverse Primer: TTTACGATTACACCCAGACTGC

**ZEB2**
Forward Primer: AATGCACAGAGTGTGGCAAGGC
Reverse Primer: CTGCTGATGTGCGAACTGTAGG

**CTNNB1**
Forward Primer: CATCTACACAGTTTGATGCTGCT
Reverse Primer: GCAGTTTTGTCAGTTCAGGGA

**TGFβ2**
Forward Primer CCATCCCGCCCACTTTCTAC
Reverse Primer AGCTCAATCCGTTGTCAGG
*RPL19* and *EEF1A1* were used as housekeeping gene controls. Data was analyzed using the -2ΔΔct method and student’s t-test.
3.3. Transfer across an artificial gradient

Using a miR-200 expression plasmid we generated ES-2 cells that over express miR-200a. The elevated basal expression of miR-200a in the ES2-miR200OE cells was confirmed using q-RT-PCR, with the parental ES-2 cells as controls (Figure 25(A)). The targets of miR-200a were significantly downregulated in the ES2-miR200OE cells compared to the parental ES-2 cells (Figure 25(B)). The ES2-miR200OE cells were then co-cultured with the parental ES-2 cells for 48 hours. The ES2-miR200OE cells were tagged with GFP in order to facilitate sorting of the cells using the Moflo cell sorter.

Following the co-culture, cells were sorted (Figure 26 (C)) and the parental ES-2 cells were collected and analyzed for miR-200a levels. We observed that following co-culture, miR-200a levels in the naïve parental cells increased significantly (Figure 26(A)). We also tested for the mRNA expression some of the miR-200a target genes (ZEB1, ZEB2 and CTNNB1) and found them to be significantly downregulated thereby indicating that the transferred miRNA remains functional (Figure 26(B)).
In order to create an artificial gradient in the miR-200a expression, ES-2 cells were transfected with miR-200 expression plasmid. 4 days following transfection the expression levels of miR-200a (A) and its targets (B) were measured in the ES2-miR200OE cells and compared to the naïve parental ES-2 cells using qRT-PCR. The miR-200a expression in the over-expresser cell line was found to be significantly higher than the naïve, non-transfected cells. The targets of miR-200a were found to be significantly downregulated compared to the naïve, non-transfected cells. Analysis by t-test showed significant difference where p value is <0.0001.
Naïve ES2 cells were co-cultured with the GFP (+) ES2-miR200OE cells (that over-express miR200a, see Figure 1). After 48 hours, the GFP (-) ES2 cells were sorted out of the population and analyzed.

(A) qRT-PCR measurements showed following co-culture, there was significant increase in the miR-200a expression in the ES2 cells co-cultured with ES2-miR200OE cells, compared to the naïve parental ES-2 cells. Analysis by t-test showed significant difference where p-value is <0.0001

(B) Furthermore the targets of miR-200a in the recipient cells were significantly downregulated, thereby indicating that the transferred miR-200a remains functional. Analysis by t-test showed significant difference where p-value is <0.0001
Naïve ES2 cells were co-cultured with the GFP (+) ES2-miR200OE cells (that over-express miR200a, see Figure 1). After 48 hours, the GFP (-) ES2 cells were sorted out of the population using a Moflo Cell sorter.

(C) Representative data obtained from Moflo cell sorter that indicates how the cell populations were separated on the basis of GFP expression and collected for analysis.
3.4. Transfer across a natural gradient

Different EOC cell lines were profiled for the expression of miR-200a (Figure 27). Based on the profiling data, SKOV-3 cells expressed significant levels of miR-200a, whereas ES-2 and Kuramochi cells had little or no basal expression. SKOV-3 cells (stably expressing GFP) were then co-cultured with Kuramochi and ES-2 cells for 48 hours. Following the co-culture, cells were sorted based on the GFP expression and the GFP (-) ES-2 and Kuramochi cells were collected and analyzed for miR-200a levels. Naïve ES-2 and Kuramochi cells were used as controls for basal miR-200a expression.

We observed that following co-culture miR-200a levels in the recipient cells increases significantly (Figure 28(A) and Figure 29(A)). We also tested for the mRNA expression some of the miR-200a target genes (ZEB1, ZEB2, TGFβ2 and CTNNB1) and found them to be significantly downregulated thereby indicating that the transferred miRNA remains functional (Figure 28(B) and Figure 29(B)).
Several EOC cell lines were profiled, using qRT-PCR, in order to determine their basal miR-200a expression levels. Based on this data, the candidate cell lines to be used to create a natural gradient were selected. SKOV-3 was chosen to be the donor cell line due to its high basal expression of miR-200a. ES-2 and Kuramochi were selected to be the recipient cell lines due to their little or no basal expression of miR-200a.
ES-2 cells, with very low basal expression of miR-200a, were co-cultured with the GFP (+) SKOV-3 cells that express high levels of miR200a (see Figure 3). After 48 hours, the GFP (-) ES-2 cells were sorted out of the population and analyzed.

(A) qRT-PCR measurements showed following co-culture, there was significant increase in the miR-200a expression in the ES-2 cells co-cultured with SKOV-3 cells, compared to the naïve parental ES-2 cells. Analysis by t-test showed significant difference where p-value is <0.0001.

(B) Furthermore the targets of miR-200a in the recipient ES-2 cells were significantly downregulated, thereby indicating that the miR-200a remains functional in the recipient cells, following transfer. Analysis by t-test showed significant difference where p-value is <0.005.
Kuramochi cells, with very low basal expression of miR-200a, were co-cultured with the GFP (+) SKOV-3 cells that express high levels of miR200a (see Figure 3). After 48 hours, the GFP (-) Kuramochi cells were sorted out of the population and analyzed.

(A) qRT-PCR measurements showed following co-culture, there was significant increase in the miR-200a expression in the Kuramochi cells co-cultured with SKOV-3 cells, compared to the naïve parental Kuramochi cells. Analysis by t-test showed significant difference where p-value is <0.0001.

(B) Furthermore the targets of miR-200a in the recipient Kuramochi cells were significantly downregulated, thereby indicating that the miR-200a remains functional in the recipient cells, following transfer. Analysis by t-test showed significant difference where p-value is <0.05.
Chapter 4: MECHANISMS OF MICRORNA TRANSFER

4.1. Introduction

The experimental results from Chapter 2 and 3 represent observations made at a terminal time point. While they demonstrate the occurrence of the transfer process itself, they do not provide insights as to how the process actually occurs. As presented in the Chapter 1, several processes have been implicated for their role in intercellular transfer of miRNAs in different cell models. These include but are not limited to, gap junctions (Figure 30), apoptotic bodies, HDLs and intercellular transfer of vesicles (Figure 31).

![Figure 30: Gap Junction Mediated miRNA transfer](image)
In order to determine which process was largely contributing to the mobile miRNAs in the EOC cell lines, live-cell imaging was utilized with the dual fluorophore model. A time-lapse live-cell microscopy approach allowed for constant monitoring of the cells in their native environment that would elicit normal physiology and interactions. These experiments shed light on the mechanistic intricacies of miRNA transfer. Based on the live-cell imaging data, the prime suspects mediating the miRNA transfer were gap junctional activity and vesicular mechanisms. This chapter presents the data obtained on the role of these mechanisms.
4.2. Materials and Methods

4.2.1. Cell Lines: Human ovarian carcinoma cell lines SKOV3 with serous histotype that originated from malignant cells in the ascites fluid were purchased from the National Cancer Institute (NCI) Tumor Cell Repository (Detrick, MD). SKOV3, was cultured according to manufacturer’s instructions using minimal essential media (MEM) (Corning) supplemented with 10% fetal bovine serum (FBS) (SIGMA-ALDRICH), 0.5% penicillin/streptomycin (Corning), 0.4% amphotericin B (Corning) and 0.22% g/ml sodium bicarbonate (Santa Cruz Biotechnology) for less than 20 consecutive passages. All cells were kept at 37°C and 5% CO₂ in a humidified incubator, and were routinely tested for Mycoplasma. Cell line authentication was performed using STR analysis.

4.2.2. Transfection of GFP plasmid with EndoFectin™ Max: The cell lines were transfected with GFP plasmid using the EndoFectin™ Max Transfection Reagent to generate the recipient GFP positive cell populations for the dual fluorophore model. The manufacturer’s recommended transfection protocol (http://www.genecopoeia.com/wp-content/uploads/oldpdfs/product/reagent/pdf/GeneCopoeia-EndoFectin_Max_Datasheet.pdf) was utilized. mirRNA mimics: mirVana™ miRNA Mimic, Negative Control #1 from Thermo Fischer (Cat No 4464058) was used. This mimic is a random sequence miRNA mimic molecule which as such does not effect any observable changes in cell behavior or morphology.
4.2.3. **Fluorescent (Cy3/Cy5) labeling of miRNA mimics:** Mirus Label IT® miRNA Labeling Kits were used in order to covalently label the miRNA mimics with fluorescent tags (either cy3 for the imaging experiments or cy5 for the flow cytometric measurements) using the manufacturer’s recommended protocol.


4.2.4. **Transfection of labeled miRNAs using DharmaFECT:** The transfection reagent DharmaFECT was used to transiently transfect the labeled miRNAs into the donor cell populations using the manufacturer’s recommended protocol.

4.2.5. **Live-cell Imaging microscopy:** Donor cells transfected with fluorescently (cy3) labeled miRNA mimics were co-cultured with recipient cells tagged with GFP on Mattek™ glass 35mm plates at 50% confluence. The cells were then imaged using the Olympus Viva View FL Incubator Microscope. Images were acquired in the green, red and DIC channels every 10 minutes for 24 hours over multiple z-stacks. The acquired images were collapsed and processed using the Metamorph image analysis software.

4.2.6. **Flow cytometric quantification of miRNA transfer frequency:** Donor cells transfected with fluorescently (cy5) labeled miRNA mimics were co-cultured with recipient cells tagged with GFP. After 254 hours the cells were trypsinized, washed and re-suspended in PBS and were then analyzed by using the BD acuri C6 flow cytometer. The number of GFP (+) recipient cells that were positive for the cy3 (+) miRNA were measured as a percentage of the total number of GFP (+) recipient cells in order to determine the frequency of the miRNA transfer process.

4.2.7. **Gap junction inhibition and Scrape loading dye transfer assay:** 18 Beta Glycyrrhetinic acid (18BGA), Carbeneoxonolone (CBX) and 1-Octanol were used as biochemical inhibitors of gap junction activity. The cells were treated with the inhibitors (at 50μM concentrations) in serum free conditions. Inhibition of gap junction activity was tested using the scrape loading dye transfer assay. For this assay, the cells were grown in complete monolayers. Following overnight treatment with the inhibitors, the
cells were rinsed 3X with PBS and a 0.5 mg/ml solution of Lucifer Yellow was added to the cells. Scrapes were made in the monolayer using a scalpel blade and the plates are left undisturbed, in the dark for 3 minutes. Cells are then washed with PBS and fixed with 4% paraformaldehyde. The extent of the permeation of the Lucifer yellow dye from the scrape line into the adjacent cell monolayer was observed by using the Zeiss AxioObserverD.1 fluorescent microscope. The mean fluorescence intensity was quantified using ImageJ.

4.2.8. Immunofluorescence microscopy: Donor cells transfected with fluorescently (cy3) labeled miRNA mimics were co-cultured with recipient cells tagged with GFP on glass coverslips at 50% confluency. Following 24 hours the cells were fixed with 4% Paraformaldehyde solution and permeabilized with 0.1% Triton-X-100. Primary antibodies for EEA1 (Life Technologies), RAB4, RAB5, RAB7, RAB9 and RAB11 (Cell Signaling) were used to stain for the early, sorting and late endosomes at a dilution of 1:500 and incubated overnight at 4 degrees. Secondary antibodies FITC-anti-mouse (for EEA1) and Alexa488-anti-rabbit (for the RAB family) were incubated with the cells at a dilution of 1:1000 for 1 hour at RT. Following which, they were incubated with 4’,6-Diamidino-2-phenylindole (DAPI) at a concentration of 10 μg/ml for 15 minutes. Cells were washed, air dried, and mounted on glass slides using Prolong Gold (Invitrogen, Carlsbad, CA). Fluorescent imaging was performed using a Zeiss AxioObserverD.1 fluorescent microscope.
4.3. Live-cell imaging

In order to visualize the entire process of miRNA transfer, the donor and recipient SKOV-3 cell populations were co-cultured and live-cell imaging was performed. The cells were co-cultured in glass bottom plates, coated with either poly-d-lysine or collagen and imaged for 24 hours. Distinct mechanisms of miRNA transfer were observed.

Intercellular transfer of cy3-miRNAs was seen when the cell membranes made close contact with one another (Fig. 32 (A) and 32 (B)). MiRNA transfer was also mediated by vesicular structures (Fig. 33). The time points chosen here are representative of the transfer process. These have been generated from time lapse videos corresponding to the three miRNA transfer events.

The frequency of the transfer events in the time-lapse movies recorded were calculated using the number of the GFP tagged cells that were recipients of the cy3-miRNA, as a percentage of the total number of GFP tagged cells in view. A higher frequency of transfer events i.e. a higher % miRNA transfer with matrigel and collagen than the poly-d-lysine coated plates was observed (Fig. 34).
Figure 32 (A): Time-lapse imaging demonstrating intercellular transfer of miRNAs through extensive cell contact

SKOV3 donor cells were transfected with Cy3 (red) labelled miRNA mimics and recipient cells were tagged with GFP (green). Live cell imaging was utilized to visualize evidence of the intercellular transfer of miRNAs.
Figure 32 (B): Time-lapse Imaging demonstrating intercellular transfer of miRNAs through extensive cell contact

SKOV3 donor cells were transfected with Cy3 (red) labelled miRNA mimics and recipient cells were tagged with GFP (green). Live cell imaging was utilized to visualize evidence of the intercellular transfer of miRNAs.
Figure 33: Time-lapse imaging demonstrating vesicle-mediated intercellular transfer of miRNAs.

SKOV3 donor cells were transfected with Cy3 (red)-labelled miRNA mimics, and recipient cells were tagged with GFP (green). Live cell imaging was utilized to visualize evidence of the intercellular transfer of miRNAs.
The frequency of the transfer events during the live-cell imaging experiments on plates coated with different modified supports was determined by quantifying the number of the GFP tagged cells that were recipients of the cy3-miRNA, as a percentage of the total number of GFP tagged cells within view.
4.4. Gap junction mediated transfer

In order to test their contribution to the miRNA transfer process, SKOV-3 donor cells transfected with cy3-miRNA were co-cultured with GFP tagged SKOV-3 recipient cells in presence of the gap junction inhibitors. 18-beta-glycyrrhetinic acid (18-βGA), 1-octanol and carbenoxolone (CBX) are commonly biochemical gap junction inhibitors [266-269]. To verify the inhibition of gap junction activity following treatment with 18BGA, CBX and Octanol, the scrape-loading dye transfer assay was performed on treated cells and untreated controls. The assay is performed by measuring the penetration of a fluorescent dye Lucifer Yellow, which migrates through the gap junction channels, into scrapes made in the cell monolayers (Figure 35). The assay confirmed that there was a significant reduction (40-50%) in the gap junction activity in the treated cells vs the controls as evidenced by penetration of the Lucifer yellow dye into scratches made in the cell monolayers which was quantified using the mean fluorescence intensity (Figure 36).

Having established the activity of the inhibitors, the miRNA transfer was tested in the presence of gap junction inhibition, using the flow cytometry experimental setup described in Chapter 2. The % miRNA transfer results, indicated that gap junction inhibition does not impair the miRNA transfer activity (Figure 37). This evidence suggested that:

- Either, gap junctions are not the principal mediators of the miRNA transfer in EOC cell lines
- Or, other mechanisms get activated and compensate for the lack of gap junction activity
This assay measures the effect of treatment with three biochemical inhibitors, CBX, 18BGA and 1-Octanol on gap junction activity. The ability of a low molecular weight fluorescent dye, Lucifer yellow, to permeate through scrapes made on cell monolayers is used as a surrogate for the gap junction activity.
Figure 36: Activity reduction following treatment with gap junction inhibitors

The results from the scrape loading dye transfer assay were quantified by measuring the mean fluorescence intensity from the fluorescence images (Figure 35). There is a significant reduction (40-50%) in the gap junction activity as evidenced by the decrease in the mean fluorescence intensity.
Figure 37: % miRNA Transfer following treatment with gap junction inhibitors

FACS analysis in conjunction with the dual fluorophore model (Previously described in Chapter 2) was used to measure effect of gap junction inhibition on the % miRNA transfer. There was no observable change in the transfer efficiency in spite of the significant reduction in the gap junction activity. This implies a limited role played by the gap junction channel or the presence of compensatory mechanisms.
4.5. Vesicle mediated transfer

As the biochemical inhibition of gap junctional activity did not affect the intercellular transfer of miRNAs, the focus shifted to vesicle mediated transfer mechanisms. Firstly, the intent was to determine the contribution of vesicle mediated, contact independent mechanisms. The natural gradient setup described in Chapter 3 was modified to introduce a physical barrier between the donor and recipient cell population that could selectively allow passage of vesicles of size <0.4 μm (Figure 38). The donor cells with high miR-200a basal expression (SKOV-3) were cultured on trans-well porous membranes (0.4μm pore size) and the recipient cells with no basal expression of miR-200a were cultured in the lower chamber.

![Figure 38: Setup to study vesicular miRNA transfer](image)

Using a porous membrane barrier, miRNA transfer through indirect contact can be tested. This setup would block the transfer processes mediated by cell membrane contact, microvesicles, apoptotic bodies and any other carriers larger than the 0.4 μm diameter.

Following 48 hours of culture, the cells in the lower chamber were collected and analyzed for their miR-200a expression levels. It was observed that allowing traffic of exosomes (30nm-100nm), while inhibiting passage of micro-vesicles and apoptotic bodies (>1 μm) significantly blocked the transfer of miR-200a as compared to the transfer observed through
direct contact (Figure 39). However the process was not completely abrogated and there was about a 10 fold increase in miR-200a in the recipient ES-2 cells in spite of the indirect contact through the porous barrier (Figure 39).

![Figure 39: Comparison of miRNA-200a transfer with and without direct cell-cell contact](image)

As previously shown in Chapter 3, when ES2 and SKOV-3 were co-cultured, in direct contact with each other, on an average, a nearly 40 fold increase in miR-200a is observed in the recipient ES-2 cells. However when the same cells were indirectly cultured together, separated by a porous membrane (0.4 μm pore size), the transfer was inhibited but not entirely blocked. Vesicles smaller than 0.4 μm (i.e. exosomes) seem to contribute to an average 10 fold increase in miR-200a in recipient cells.
Having observed a significant contribution of exosomes to miRNA transfer, the next step was to probe the nuances of the endocytic recycling, packaging and release pathways. In order to ascertain whether the miRNAs are packaged and released via endocytic pathways the following experimental setup with the SKOV-3 cell line was utilized (Figure 40 (A)). The donor cells were transfected with Cy3 (red) labelled negative control miRNA mimic and recipient cells were tagged with GFP (green). Following co-culture for 24 hours, different markers of early, sorting and late endosomes (Figure 40 (B) [171, 173, 270-274]) were probed using immunofluorescence. The endosome markers were stained using a FITC (green) labelled secondary antibody.

Figure 40 (A): Setup to test the role of endocytic packaging and recycling pathways in miRNA transfer
Confocal microscopy revealed co-localization between fluorescently labeled miRNAs and markers of:

- Early Endosomes: EEA1 and Rab4
- Sorting Endosomes: Rab5
- Late Endosomes: Rab7, Rab9 and
- Recycling Endosomes: Rab11

Figure 40 (B): Markers of Sorting, Early, Late and Recycling Endosomes [171, 173, 270-274]
This co-localization presents evidence that the miRNAs are shuttled by endocytic trafficking. In the donor cells, they are packaged into the Rab4 positive early endosomes (Figure 41) which then progress into the Rab5 positive sorting endosome (Figure 42), then maturing into the Rab7/9 positive later endosomes (Figure 43 and 44). A fraction of the miRNA population is finally guided into the Rab11 positive vesicles (Figure 45) which can then be released by the cell through exocytosis. These exocytosed vesicles are then internalized by the recipient cell and re-enter the endocytic pathway as evidenced by the co-localization of the miRNA with the early endosome marker EEA1 and Rab4 in the recipient cells (Figure 46 and Figure 41 respectively).
Rab5 is associated often with the sorting endosomal compartment. Rab5 vesicles mediate recruitment of Rab7 to the early endosomes as well as maturation to late endosomes. Rab5-miRNAs in both the donor and recipient cells represents the miRNA containing vesicles entering the sorting endosomes.

Figure 41: Co-localization of cy3-miRNA with Rab5 positive sorting endosomes
Figure 42: Co-localization of cy3-miRNA with Rab4 positive early endocytic vesicles

Rab4 is associated with the early endosomal compartment. Rab4 vesicles could enter the traffic into either recycling or degradative pathways. Rab4-miRNAs in both the donor and recipient cells represents the miRNA containing vesicles entering the endocytic pathway.
Figure 43: Co-localization of cy3-miRNA with EEA1 positive early endosomes

EEA1 is an early endosome marker. Here we see that immediately following transfer of miRNA into the recipient cell, it is enclosed within the EEA1 positive early endosome.
Figure 4: Co-localization of cy3-miRNA with Rab7 positive late endosomes

Rab7 is believed to control the late endocytic stages as well as the degradative pathways. Aside from mediating hydrolytic degradation of its vesicular contents, it has also been shown to fuse with the plasma membrane and release exosomes. Rab7-miRNAs in the donor cells could potentially represent the vesicles that are released in order to mediate miRNA transfer.
Figure 45: Co-localization of cy3-miRNA with Rab9 positive late endosomes

Rab9 is often associated with the late endocytic stages and the vesicles leading into the trans-golgi network. Here the donor cells show cy3-miRNA enclosed in Rab9 positive late endosomes.
Rab11 has been shown to control exocytosis at the cell membrane. It regulates the release of the recycling endosomes. Here the Rab11 positive vesicles co-localized with the cy3-miRNA could represent the vesicles being prepared for exocytosis.
Figures 41-46 (where a non-functional negative control miRNA was used), displayed a pattern wherein a larger fraction appeared to be shuttling into the Rab7 positive endosomes which represent the endocytic pathway stage prior to degradation. This presented a hypothesis that the distribution pattern through the different endocytic pathways could change depending on whether the miRNA is functional or not. In order to test that, donor cells were transfected with Cy3 (red) labelled functional miRNA-200a mimic and recipient cells were tagged with GFP (green). Following co-culture for 24 hours, different markers of early, sorting and late endosomes were probed using immunofluorescence as described previously (Figure 40 (B)). The immunofluorescence data indicated that a much larger fraction of the miRNA-200a mimic was packaged into the Rab11 positive recycling endosomes as compared to its non-functional control counterpart (Figure 47). This could suggest that the cell possesses the ability to distinguish between functional and non-functional sequences and responds to their presence differently using the endocytic pathways.
Figure 47: Co-localization of cy3-miRNA-200a with Rab11 positive late endosomes

In comparison to the co-localization observed with the non-functional/negative control miRNA mimic a much larger degree of co-localization is observed between functional miRNA-200a mimic and Rab11 positive recycling endosomes. This indicates the ability of the endocytic pathways to distinguish between functional and non-functional miRNAs.
5.1. Summary of results

The concept of miRNAs acting as mobile signaling molecules is very appealing simply due to their versatility and ability to exert powerful control over gene regulation. The experimental design to test this idea was driven largely to create solutions for the thorny problem of studying transfer. In order to trace the movement of an entity from one location to another, the entity needs to be labeled in a manner that distinguishes it from the surroundings. Also necessary is the creation of a gradient in basal expression in order to study the transfer. That was the thought process behind utilizing the cells exogenously transfected with fluorescently tagged miRNA. The gradient enabled the determination of the transfer and the fluorescence was handy to visualize it.

The data presented in chapter 2 demonstrated the occurrence of intercellular transfer of miRNAs in several EOC cell lines. Using a dual fluorophore model and microscopic techniques, we compiled visual evidence of miRNA transfer both in-vitro and in-vivo. The FACS analysis allowed the quantification of the process and implied that its efficiency can be affected by parameters like culture conditions, extracellular matrix environment and cell density. In these experiments, labeled miRNAs were exogenously transfected into cells using a transfection reagent. One concern that arises is that remnants of the transfection reagent could be aiding and abetting the miRNA transfer process. To reduce the artifact generated by such an event there was a time lag built into the experimental protocol.
between transfection and co-culture. Additionally the donor cells were washed to remove any miRNAs enclosed in transfection reagent that may be adherent to the cell surfaces having escaped transfection. In spite of such precautionary measures, these experiments however, do not necessarily reflect on the transfer of endogenous miRNAs. They merely suggest that the model with artificial introduction of visible miRNAs provides a read out that may very well be applicable to endogenous miRNAs.

Visual evidence, while rather compelling, is only a part of the larger answer that was sought. Measuring the transfer of a candidate miRNA and analyzing the functional capabilities of the transferred miRNA was necessary to understand the biological significance of this exquisite mechanism of cell signaling. The experimental setup in chapter 3 was aimed at studying the absolute miRNA levels transferred as opposed to the fluorescence emitted by the dye labeled surrogate as the measure of transfer and thus address some of the shortcomings of the models used in the earlier chapter.

MiR-200a represented a very attractive candidate for study, not merely because of its consistent involvement in ovarian cancer, but also due to its role in the EMT process. Extensive attempts to profile miR-200 family expression in EOC (Chapter 1.4.2) produced discordant results. These to an extent can be explained by susceptibility to dynamic changes depending on the stage of tumor progression, EMT, localization of interacting proteins to the nucleus or cytoplasm and cellular ROS content. Functional transfer of miR-200a would add an interesting level of complexity to the mechanisms controlling the fluctuations of its
expression and help in further understanding the variability observed in the profiling studies. The increased levels of miR-200a as well as the decrease in the target mRNA levels provided a phenotypic read-out that coincided with the increased miRNA expression in recipient cells, characterized by little or no endogenous expression of miR-200a. The suggestion that this increase is due to miRNA transfer is bolstered by the fact that it was observed consistently over both a natural as well as an artificial gradient in expression.

The heterotopic transfer of miR-200a from SKOV-3 cells to both ES2 and Kuramochi cells addressed to a great extent any misgivings one may have about artificially over-expressing miRNAs to study transfer. Furthermore, due to the involvement of the miR-200 family in the mesothelial-to-epithelial transition (Meso-ET), the functional transfer of miR-200a could be involved in the transformation of the cells in the secondary tumor sites following metastasis. A double negative feedback loop between miR-200 and the ZEB genes taken into context along with the potentially functional transfer of the microRNA between the cells could help us further understand the reversible EMT process.

This information however, complicates the use of miRNAs as diagnostic/prognostic markers. There has been considerable interest in using miRNA profiles as clinical biomarkers of various pathologies [123, 127, 183, 256, 259, 275]. This can be attributed to a building consensus on the use of miRNA expression within extracellular vesicles as proxies or cellular business cards. The data in chapter 3 suggests that the picture put forth by profiling experiments could represent a state or expression pattern that is ephemeral and depending
on when the picture is taken, wildly different conclusions can be reached. Therefore, functional transfer of miRNAs could potentially undermine the argument for using them as clinical biomarkers.

Having accumulated proof of occurrence of miRNA transfer, the next step was to probe the mechanistic underpinnings. In order to accomplish that, in chapter 4, the experimental models validated in chapters 2 and 3 were adapted and used. Live cell imaging provided the view previously reserved for the proverbial fly on the wall of the cell culture incubator. The cellular dynamics necessary for miRNA transfer were visualized. Based on the imaging data, transfer favored extensive cell-cell membrane contact or was assisted by the packaging of miRNAs into vesicular structures. Using biochemical inhibitors, it was observed that the contribution of the gap junction channels was either insubstantial or easily substituted. Blocking vesicular transport on the other hand was a different matter. Selectively allowing passage of vesicles under 0.4 μm (which largely constitute exosomes) resulted in a significant transfer of miRNAs. It however, represented only a fraction of the transfer that was observed with direct unhindered contact. Further, the immunofluorescence staining experiments suggested that the miRNAs enter the pathways of endocytic packaging and recycling. This evidence, taken collectively, shed light on the cellular mechanics involved in miRNA transfer.

Endocytosis is a mechanism by which cells internalize matter [172]. This matter is then sorted through the endosomal pathways which involve formation of internal vesicles that
pass the parcel in a manner of speaking to different destinations. The sorting (RAB5 [276]) and the early endosomes (RAB4 [272] and EEA1 [273]) characterized by the surface presentation of proteins shuttle their contents towards one of three possible fates:

- Degradation in lysosomes through RAB7 late endosomes [270]
- To the golgi apparatus through RAB9 late endosomes [271]
- For extracellular release through RAB11 recycling endosomes [274]

When the co-localization of miRNAs with the different endosomal compartments was tested in chapter 4, the data pointed at differences between how a non-functional control miRNA is shuttled in comparison to the functional miR-200a mimic. While a significant portion of the negative control seems to be directed towards degradation through the RAB7 positive late endosomes, the functional miR-200a mimic was queued up for extracellular release in the RAB11 positive recycling endosomes. This could be considered as a nod to mechanisms and the supporting apparatus that allow a cell to read and identify a miRNA sequence.

Based on this information one could hypothesize that the cell receives a message, interprets it, and makes a determination on whether it wishes to act on it, ignore it and/or pass it along to another cell. If miRNAs are indeed involved in such a sophisticated system of communication, then the implication would be that the miRNA transfer process is not merely a passive event where the cell discards excess miRNAs into its surroundings forcing the neighboring cells to accept and act on the nucleotide sequence based instructions. It
could very well be an active process where a cell is intentionally releasing certain miRNA mediated cues which the recipient cells process and make a determination on their response to those cues. That is not to say that passive transfer does not occur. The suggestion quite simply is that the active processes could be a nod toward sophisticated machinery that allows precise communication between a cell and its microenvironment.

In conclusion, this dissertation is an account of the visualization and characterization of a novel mechanism of intercellular communication mediated by miRNAs in the context of epithelial ovarian cancer.
5.2. Significance of the findings

The EOC microenvironment represents a formidable, heterogeneous and ever-evolving adversary. John Donne reflected in his 1621 tome (*Devotions upon Emergent Occasions*), that “no man is an island” [277]. He may not have had the ovarian cancer cell in mind but given the influence that the different players within the microenvironment have on the ability of the cancer cell to thrive in an unfavorable environment, the musing is definitely relevant here. For the sake of its own survival, the cancer cell cannot forever remain a lone crusader and has to coax other partners to work with it in concert and hijack processes active in normal cells. Several mechanisms of intercellular communication are involved in the interactions between cancer cells [278]. The data compiled here suggests that mobile miRNAs belong in those ranks.

The acquired data shows that the process of miRNA transfer can occur between EOC cells. The occurrence of the functional transfer of miR-200a and its ability to affect targets in the recipient cells is another interesting facet of the findings here. This raises further questions regarding the underlying mechanisms and the functional relevance of this process in EOC, as well as the likelihood of controlling clinically relevant phenomena of metastasis and resistance to treatment. Better understanding of the basic biological phenomena of miRNA transfer and their mechanisms in EOC may lead to development of more rational treatment approaches, uncover fundamental basis of poor clinical outcomes, development of resistance, and may overall influence future approaches to the treatment of metastatic...
disease. The significance of the implications (Figure 47) associated with miRNAs in EOC warrants extensive studies of the miRNA-related processes.

Figure 48: Significance of intercellular transfer of miRNAs
Intercellular miRNA transfer means that miRNAs possess the power to modulate gene expression in target cells and act like mobile silencing signals. This mechanism is thus particularly relevant in cancer as it allows a dysregulated cell to affect the genotype and phenotype of the surrounding cells. As a result, impaired functioning in a single cell is transmitted to several others, thereby amplifying the effect. Heterotypic transfer of miRNAs would allow the process to regulate tumor-stromal interactions [133].

In addition to their ability to influence the cells in the vicinity, the functional transfer of miRNAs in many ways, bestows on them the capabilities of hormones [132]. They are secreted entities which can effect significant changes in sites distant from the cell of origin. Understanding the networks that mediate miRNA trafficking could go a long way to improving the penetration of nucleic acid therapeutics into tumor tissues. These are only a few significant reasons to pursue this line of research.

The following excerpts from the work of other groups, taken together, provide a nuanced summary of the importance of this line of research and the caveats associated with it:

“RNAi has been shown to be conserved in almost all species and to possess a common, fundamental mechanism of regulating gene expression.” [175]

“The issue of miRNA movement remains a contentious one. A number of studies have addressed the ability of miRNAs to move from cell to cell, revealing a seemingly cell-autonomous activity. Experiments using sensors of miRNA activity or expressing artificial miRNAs that provide a phenotypic read-out document an apparent overlap of miRNA-
mediated gene silencing and activity of the promoters driving precursor expression. Such reporter assays assume that small RNA accumulation resulting from mobility is accurately reported; an inference that, considering the dose-dependent activity of miRNAs, may not be correct. The efficacy of the miRNA, its abundance, and the expression level of the target are all factors that might prevent detection of non-cell-autonomous activity. Also, the possibility that miRNAs move over small cellular distances or in a context-dependent manner cannot be ruled out based on these studies.” [279]

“The robustness and exquisite control of biological processes that leverage small RNA mobility is undoubtedly achieved through mechanisms that have yet to be discovered. Now that the movement of small RNAs has been established, we can focus on their biology. It is perhaps not so surprising that plants have, in so many instances, mobilized silencing signals, as they provide a flexible platform to transmit highly specific information; the current task at hand is to be aware not only of non-cell-autonomous small RNA activity, but to characterize it thoroughly to the point that we know precisely what moves, how and why.” [279]

Studies that uncover the inner workings of basic cellular biology in normal and pathological conditions enable the existence of translational science. The author hopes that this work is a small addition to the larger collective enterprise of basic science.
5.3. Recommendations for future work

Having characterized the transfer process and having developed validated experimental models for its study, this work could serve as a primer for several follow-up lines of study.

- **Tumor- Stromal Interactions:** It is well known that stromal cells such as fibroblasts, adipocytes and macrophages play important roles in EOC tumor progression. Whether the cellular cues from tumor cells that trigger their cancer promoting behavior are mobile miRNAs would be worth exploring.

- **Other secreted miRNAs:** It is quite likely that not all miRNAs are produced and packaged for secretion. A broad profiling analysis following co-culture of different cells, could determine which miRNAs are functionally transferred. This could go a long way into understanding the type of messages the donor cells seek to deliver by means of miRNAs.

- **Effect of micro-environmental cues on miRNA transfer:** The flow cytometry quantification of miRNA transfer on different culture conditions suggested that certain modified supports favor transfer over others. This could greatly increase the importance of micro-environmental cues and extra-cellular matrix conditions on transfer efficiency. The implication would then be that certain organs within the peritoneal cavity may allow this mode of intercellular communication to a greater extent than others.
Chapter 6: CITED LITERATURE


Jabbari, N., A.N. Reavis, and J.F. McDonald, Sequence variation among members of the miR-200 microRNA family is correlated with variation in the ability to induce hallmarks of mesenchymal-epithelial transition in ovarian cancer cells. J Ovarian Res, 2014. 7: p. 12.


277. Donne, J. and W. Marshall, *Devotions upon emergent occasions, and severall steps in my sicknesse*
digested into 1. Meditations, upon our humane condition, 2. Expostulations, and debatements with God,
3. Prayers, upon the severall occasions, to him. 1634, Printed by A.M. and are to be sold by
Charles Greene.; S.l. p. 1 online resource.

278. Kandouz, M. and SpringerLink (Online service), *Intercellular Communication in Cancer*. 2015,
Springer Netherlands.; S.l. p. 1 online resource.

415-9.
Chapter 7: APPENDIX I

7.1. Cell line authentication

**Cell Line Authentication (STR)**

Name: Hilal Gurler  
Request number: 13439  
Date initiated: 06/08/2016  
Date finalized: 06/08/2016

**Quality Controls**

**QC Qubit**

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Page 2 of 2
7.2. Approved animal use protocol

12/18/2014

Maria Barbolina
Biopharmaceutical Sciences
M/C 865

Dear Dr. Barbolina:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and renewed on 12/18/2014.

Title of Application: Chemokine-Dependent Control of Survival in Ovarian Carcinoma
ACC NO: 12-222
Original Protocol Approval: 1/2/2013 (3 year approval with annual continuation required).
Current Approval Period: 12/18/2014 to 12/18/2015

Funding: Portions of this protocol are supported by the funding sources indicated in the table below.

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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/Ag
cc: BRL, ACC File, Randi Zilmer
Chapter 8: APPENDIX II

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SEER Cancer Statistics Review (CSR) 1975–2013

Updated September 12, 2016 (Revision History)

The SEER Cancer Statistics Review (CSR), a report of the most recent cancer incidence, mortality, survival, prevalence, and lifetime risk statistics, is published annually by the Surveillance Research Program of the NCI. The scope and purpose of this work are consistent with a report to the Senate Appropriations Committee (Breslow, 1988) which recommended that a broad profile of cancer be presented to the American public on a routine basis. This edition includes statistics from 1975 through 2013, the most recent year for which data are available.

There are three ways to access the statistics in the CSR:

1. **Browse the Tables and Figures** – statistics are presented in HTML format based on user selections.
2. **Access Contents in PDF** – statistics are provided in sections by cancer site and topical groupings.
3. **Generate Custom Reports** – individual pages can be extracted and grouped into custom-made PDFs.

**Suggested Citation**


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Keywords: extracellular matrix, ovarian cancer, proteins, collagen, proteoglycan


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Edited by:
Ben Davidson, Oslo University Hospital, Norway

Reviewed by:
Noam Rechat, Hebrew University of Jerusalem, Israel
Bjørn Åsa Helberg, Oslo University Hospital, Norway

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*Correspondence: Vive M. Howell, vwhowell@sydney.edu.au

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Title: Cellular and molecular processes in ovarian cancer metastasis. A Review in the Theme: Cell and Molecular Processes in Cancer Metastasis
Author: Tsz-Lun Yeung, Cecilia S. Leung, Kay-Pong Yip, Chi Lam Au Young, Stephen T. C. Wong, Samuel C. Mok
Publication: Am J Physiol-Cell Physiology
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The miR-200 Family: Versatile Players in Epithelial Ovarian Cancer

Goda G. Muralidhar and Maria V. Barbolina

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(This article belongs to the Special Issue RNA Interference)

Abstract

The role of microRNAs (miRNAs or miRs) in the pathology of epithelial ovarian cancer (EOC) has been extensively studied. Many miRNAs differentially expressed in EOC as compared to normal controls have been identified, prompting further inquiry into their role in the disease. miRNAs belonging to the miR-200 family have repeatedly surfaced over multiple profiling studies. In this review, we attempt to consolidate the data from different studies and highlight mechanisms by which these miRNAs influence progression of metastasis and chemoresistance in EOC.

Keywords: ovarian carcinoma; miR-200 family; metastasis; chemoresistance; expression

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Chapter 9: APPENDIX III

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EDUCATION:

2011- Ph.D., Biopharmaceutical Sciences (Advisor: Professor Maria V. Barbolina)
University of Illinois at Chicago, College of Pharmacy, Chicago, IL

2007-2011 B. Pharmacy
University of Mumbai, BV’s College of Pharmacy, Navi Mumbai India

FELLOWSHIPS & HONORS

2015-2016 Chancellor’s Graduate Research Fellowship
University of Illinois at Chicago

2014 Pre-doctoral Education for Clinical and Translational Scientists Fellowship
University of Illinois at Chicago - Center for Clinical and Translational Science

2011 Best Student Award
Indian Pharmaceutical Association (IPA-MSB)

2010 Rajiv Gandhi Fellowship
Jawaharlal Nehru Centre for Advanced Scientific Research

2010 Summer Research Fellowship Award
Jawaharlal Nehru Centre for Advanced Scientific Research

2005-2010 NPCIL Scholarship
Nuclear Power Corporation India Limited

2007 Venkata Memorial Award
Atomic Energy Junior College

2004 Green Olympiad Merit Award
Ministry of Environment and Forests, Government of India
EXTRA-CURRICULAR ACTIVITIES

2016    American Association for Advancement of Science (AAAS)
         Member & National Meeting Student Aide

2015-2016 American Association of Pharmaceutical Scientists (AAPS)
         Member & Membership Strategic Oversight Committee Student Representative

2015-2016 Expanding Your Horizons (EYH)
         Organizing Committee Member

2015-2016 National Ovarian Cancer Coalition- Illinois Chapter
         Volunteer

2015-2016 Student Science Policy and Outreach Group
         Founding Member

2013-2015 Graduate Student Committee, College of Pharmacy at UIC
         Member

2013-2015 UIC Graduate Student Council
         Biopharmaceutical Sciences Dept. Representative

2014    Pharmaceuticals Graduate Student Research Meeting
         Organizing Committee Co-Chair

PUBLICATIONS


SELECTED CONFERENCES AND TECHNICAL SYMPOSIA

• Goda G. Muralidhar & Maria V. Barbolina, “MicroTalks: Intercellular transfer of microRNAs in Epithelial Ovarian Cancer” UIC Cancer Center Research Forum (2016)


