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Endothelial progenitor cell-breast cancer interaction

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List of Abbreviations

BC	Breast cancer
BM	Basement membrane
BMDCs	Bone marrow-derived cells
CM	Conditioned media
CT	CD8+ cytotoxic T cells
DCIS	Ductal carcinoma in situ
DHFR	Dihydrofolate reductase
ECM	Extracellular matrix
ECs	Endothelial cells
EPCs	Endothelial progenitor cells
ER	Estrogen receptors
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
FGF	Fibroblast growth factor
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factors
HUVECs	Human umbilical vein endothelial cells
ICAM1	Intercellular adhesion molecule 1
IDC	Invasive ductal carcinoma
IFN-γ	Interferon-gamma
IL	Interleukin

ILC	Invasive lobular carcinoma
ILVs	Intraluminal vesicles
LCIS	Lobular carcinoma *in situ*
MCP	Monocyte chemoattractant protein
MCs	Mast cells
MCSF	Macrophage colony stimulating factor
miRNA	MicroRNA
MMPs	Matrix metalloproteinases
MREs	miRNA response elements
mRNA	Messenger RNA
MVB	Multivesicular body
PAF	Platelet-activating factor
PDGF	Placental derived growth factor
PMPs	Platelet-derived microparticles
PR	Progesterone receptor
RT	Room temperature
SCF	Stem cell factor
SDF	Stromal derived factor
SLN	Sentinel lymph node
TABVs	Tumor associated blood vessels
TAMs	Tumor associated macrophages
TASCs	Tumor associated stromal cells
TDLUs	Terminal ductal lobular units

TEM	Transmission electron microscopy
TGF	Transforming growth factor
Th1	CD4+ T helper 1 cells
Th2	CD4+ T helper 2 cells
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer
TNF-α	Tumor necrosis factor-alpha
TNM	Tumor node metastasis
VEGF-A	Vascular endothelial growth factor A
VEGFR-2	VEGF receptor 2

Abstract

Breast cancer remains the second leading cause of cancer death among women in the U.S. Although organ-confined disease is curable, metastatic disease remains incurable and an unmet clinical need. Hence, the development of new therapeutic targets and further understanding of the genesis of malignant progression is needed and to this end analysis of the tumor microenvironment is imperative for novel therapeutic targets in breast cancer. We and others have demonstrated that bone marrow derived endothelial progenitor cells (EPCs) incorporate in the neovasculature around implanted tumors supporting their growth and metastasis. The incorporation of EPCs from the marrow to the tumor is estrogen responsive. We evaluated the effect of estrogen and a naturally occurring anti-estrogen 3, 3'-diindolylmethane (DIM) on precursor progenitor cells of angiogenesis, EPCs, at the cellular and molecular levels using $CD133^{+}/CD34^{+}$ stem cells that were isolated by positive selection from human umbilical cord blood as an *in vitro* model of vasculogenesis. Moreover, we evaluated the secretome of the EPCs and their role in breast cancer progression. Using flow cytometry, we show that the highly proliferative EPCs can differentiate into functional endothelial cells. We concluded that EPCs are estrogen sensitive, and estradiol mediated enhancement of the function of EPCs is abrogated by the anti-estrogen DIM. We further elucidated the significance and function of EPCs in the breast cancer environment using *in vitro* models and attempted to define the interacting determinants. We examined the cell-cell interaction by evaluating the effect of conditioned media from EPCs on breast cancer cell and vice versa. We further isolated secretory exosomes from EPCs and breast cancer and examined their mutual interaction using end point measurements such as cell proliferation, invasion, and migration. Further, we

examined the contents of the exosomes in term of their microRNAs, and the cytokines in the tumor microenvironment. We report that exosomes of EPCs are enriched with oncogenic miRNAs including hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-181a-5p, hsa-miR-21-5p, hsa-miR-142-3p, and their miRNAs are also significant modulators of breast cancer transformation and metastasis. Our studies are consistent with the hypothesis that secretory exosomes and paracrine cytokines are shuttles of cell-cell communication and important targets of novel breast cancer therapy. The exosomes and the microRNAs they carry are the communicating determinants between breast cancer cells and EPCs in the tumor microenvironment and presumably help evolution of breast cancer subsets such as the triple negative breast cancer (TNBC) with gain of metastatic phenotype. Disruption of the cell-cell communication can lead to novel TNBC therapeutic and fulfill a much-needed clinical need.

1 CHAPTER I: INTRODUCTION

1.1 Breast Cancer

Breast cancer is a significant health issue affecting the life of 1 in 8 U.S. women. It is the second most common cause of cancer-related deaths in U.S. women. It is estimated that in 2019, there will be approximately 268,60 new cases of invasive breast cancer and 62,930 new cases of non-in-invasive (in situ) breast cancer. The disease may also affect males; however, it is much less prevalent (1% of total breast cancer cases and deaths) (PDQ Adult Treatment Editorial Board, 2019).

Overall mortality rates have declined in both younger and older women since 1989. This fall was primarily due to the availability of better interventions (adjuvant chemotherapy, hormonal, and targeted therapy), early diagnosis by mammography, and a decrease in hormonal replacement therapy prescriptions (PDQ Adult Treatment Editorial Board, 2019). However, invasive metastatic breast cancer, specifically triple-negative breast cancer (TNBC), is still the major challenge.

1.1.1 Anatomy of the breast

The human breast is composed of glandular, adipose, and connective tissues (Figure 1). The glandular tissue consists of a branching structure of epithelial ducts surrounded by adipose tissue (Nickell and Skelton, 2005). The connective tissue connects both the glandular and the adipose tissues together. Cooper's ligaments are multiple bands of connective tissues that connect the breast to the skin dermis (Howard and Gusterson, 2000). The glandular tissue of the breast consists of lactiferous ducts and terminal ductal lobular units (TDLUs). TDLUs are the smallest and the functional units of the breast. TDLUs

encompass a lobule, which is a group of 10-100 pouch-like acini lined with milk-secreting cuboidal cells, and a terminal duct connecting the lobule to the duct system (Maurizio and Fabrizio, 2018).

Two cell layers, luminal epithelial and myoepithelial cells, line the glandular breast tissue. The luminal epithelial layer of the ducts consists of low columnar cells, while the luminal epithelial layer of the ductules consists of cuboidal cells. The myoepithelial cell layer is attached to the basement membrane and is localized between the luminal epithelial cells and the stroma (Sgroi, 2010).

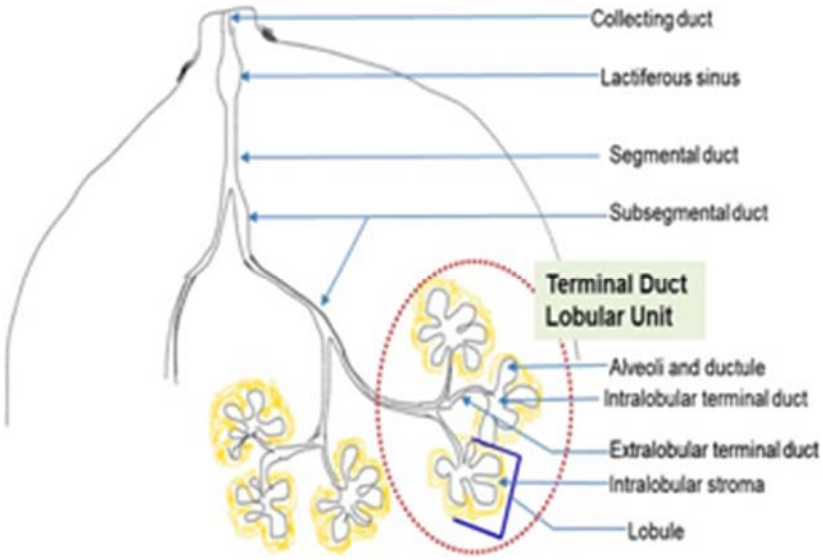


Figure 1. Anatomy of the female breast

(Adapted from (Banik *et al.*, 2017).

1.2 Histological classification of breast cancer

Histologically, breast cancer can be divided into pre-invasive, invasive, and rare breast cancers.

1.2.1 Pre-invasive stage of breast cancer

The pre-invasive lesions of the breast are characterized by neoplastic proliferation of epithelial cells that are contained within the ductal-lobular network of the breast without breaching through the basement membrane into the surrounding breast stromal compartment.

The pre-invasive stages of breast cancer include two histologic subtypes: the lobular and the ductal. Both arise in the same microanatomical site, the TDLU. Differences in cell morphology are the basis for the differentiation between the lobular and the ductal subtype. The lobular subtype typically includes tiny, non-polarized cells that mimic typical breast acini's low-cuboidal luminal cells, while the ductal subtype comprises moderate to large polarized cells identical to typical breast ducts' low-columnar cells (Wellings and Jensen, 1973; Wellings *et al.*, 1975). Atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS) are the two main categories of the pre-invasive lobular breast cancer. The histological differentiation is based on the cell morphology, the extent of proliferation, and the distention of the TDLUs. The lesions of the ductal subtype include flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), and ductal carcinoma in situ (DCIS). According to the American Cancer Society, approximately 20% of breast cancers can be categorized as pre-invasive with DCIS and LCIS, representing 83% and 13% of the total in situ cases diagnosed during 2010-2014, respectively (Breast Cancer Facts & Figures | American Cancer Society. 2017). Pre-invasive breast cancers have the

ability to become invasive as a result of genetic alterations and multistep mutagenic processes.

1.2.2 Invasive breast cancers

Invasive breast cancers have metastatic propensities and histologically distinguished from the pre-invasive lesions by the absence of the myoepithelial layer. The neoplastic cells have the capacity to breach the basement membrane and grow into the surrounding stroma (Pinder and Ellis, 2003). 80% of breast cancers are invasive. These cancers are subdivided into infiltrating or invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas. IDC is the most common histologic type of breast cancer. It accounts for 70-80% of all invasive lesions. According to the mitotic index, nuclear pleomorphism, and glandular tubule formation, IDC is classified into three different differentiation grades (Malhotra *et al.*, 2010). Rare breast cancers include, pure mucinous, tubular, invasive cribriform, neuroendocrine, medullary, adenoid cystic, apocrine, pleomorphic lobular, invasive micropapillary, and metaplastic (Dieci *et al.*, 2014).

1.2.3 Paget's disease of the breast

It is a rare histologic type of breast cancer. It accounts for 1%-3% of all breast cancer cases. It is a malignant disease characterized by the presence of an eroding and bleeding ulcer in the nipple associated with an underlying primary invasive or *in situ* carcinoma of the breast. Microscopically, it is characterized by the presence of giant cells called Paget's cells. These cells have an ample amount of cytoplasm and hyperchromatic nuclei with notable nucleoli in the epidermal layer. The disease is

unilateral and starts as an eczematous lesion in the nipple which spreads subsequently to the areola and then the adjacent skin (Dubar *et al.*, 2017).

1.3 Molecular analysis of breast cancer

1.3.1 Genomic and transcriptomic alterations in invasive breast cancer:

Invasive ductal carcinoma (IDC) is distinguished by significant heterogeneity at the histomorphological and clinical levels. Previous IDC's molecular analysis concentrated on the connection of tumor genomic changes and tumor grade. Low-grade IDCs display less chromosomal aberrations as compared with high-grade IDCs (Roylance *et al.*, 1999). Low-grade tumor IDCs exhibit chromosomal loss of 16q (the long arm of chromosome 16) and gain of 1q,16p (the short arm of chromosome 16), and 8q. High-grade tumors display more chromosomal abnormalities including losses of 8p,11q,13q,1p, and 18q, amplifications of 17q12 and 11q13, and gains of 8q,17q,20q, and 16p. Intermediate-grade IDCs exhibit a mixture of low-grade and high-grade genomic abnormalities, which suggests that intermediate-grade IDCs is composed of a combination of 'low-grade-like' and 'high-grade-like' IDCs (Buerger *et al.*, 1999; Roylance *et al.*, 1999).

Transcriptional (gene expression) screening analyses further helped us understand the progression of breast cancer. Gene expression screening conducted by Perou and colleagues (Perou *et al.*, 2000) resulted in the classification of IDCs into four different subtypes. These subtypes consist of two estrogen receptor (ER)-positive and two estrogen receptor (ER)-negative. Estrogen receptor-positive breast cancers include luminal A and luminal B, and estrogen receptor-negative breast cancers include ERBB2 and basal subtypes. The most prevalent subtypes are luminal A and luminal B, which typically represent tumors of low to moderate grades. Luminal A breast cancer has high expression

of genes related to estrogen receptor (ER) and progesterone receptor (PR) and lacks human epidermal growth factor receptor 2 (HER2) expression (Bombonati and Sgroi, 2011; Fragomeni *et al.*, 2018).

On the other hand, luminal B tumors exhibit reduced expression of ER, HER2 overexpression, and increased expression of genes linked to proliferation. The ERBB2 tumor subtype represents high-grade tumors. It is distinguished by HER2 expression and by the absence of ER and PR expression. Lastly, the basal subtype depicts high-grade tumors exhibiting necrosis and striking lymphocytic infiltration. Basal IDCs express cytokeratins, and these tumors are most often distinguished by the absence of ER, PR and HER2 expression (Bombonati and Sgroi, 2011).

Regarding the invasive lobular carcinomas (ILCs), conventional cytogenetic and comparative genomic hybridization analysis have revealed that ILCs are marked by repeated loss of chromosome 16q, the low-grade ductal carcinoma genetic signature. This suggests that ILCs and low-grade IDCs share a similar evolutionary genetic pathway (Simpson *et al.*, 2005). However, ILCs have a different transcription pattern and are characterized by lack of cell-cell cohesion and absence of e-cadherin protein expression (Simpson *et al.*, 2005).

1.3.2 Clinicopathological features of rare breast cancer subtypes

Typically hormone receptor positive

- Pure mucinous carcinoma: It constitutes 1% – 4% of all cancers of the breast and is characterized by excess extracellular and/or intracellular mucin production (over 90% of the tumor mass). The lesion is described as "nests of tumor cells floating in lakes of mucin". Pure mucinous carcinoma is mostly well-differentiated HR+ and HER2− with rare involvement of axillary lymph nodes (Dieci *et al.*, 2014).
- Tubular carcinoma: Among the invasive breast cancers, tubular carcinoma constitutes less than 2% of the total cases and is characterized by tubular architecture in at least 90% of the tumor. Tubular carcinomas are generally HR-positive and HER2-negative with involvement of axillary lymph nodes reported in 4%-17% of the patients (Dieci *et al.*, 2014).
- Invasive cribriform carcinoma: It accounts for 0.1%–0.6% of breast cancers and is identified by invasive components having a cribriform pattern. It is generally HR+, low proliferating, and low grade. Nodal involvement is reported in about 10% of the cases (Dieci *et al.*, 2014).
- Neuroendocrine carcinoma: The presence of morphologic neuroendocrine features characterizes neuroendocrine carcinoma along with the expression of neuroendocrine markers. More than 50% of the cells express synaptophysin or chromogranin. According to the WHO, this rare breast cancer is classified into "three main histologic types: neuroendocrine tumor, well-differentiated neuroendocrine carcinoma, and poorly differentiated/small cell and invasive breast carcinoma with neuroendocrine differentiation". These tumors are low-grade,

estrogen receptor-positive, progesterone receptor-positive, and HER2 negative. Except for the high-grade small-cell variant, neuroendocrine carcinoma has a favorable prognosis (Dieci *et al.*, 2014).

Typically hormone receptor negative

- Medullary carcinoma: It affects younger women and most often found in women with BRCA1 mutations. It constitutes less than 2% of breast carcinomas. The tumor cells are highly proliferative and poorly differentiated. The presence of prominent vesicular nuclei and visible nucleoli, arranged in syncytial format with circumscribed margins are the main features of the medullary carcinoma cells (Dieci *et al.*, 2014).
- Adenoid cystic carcinoma of the breast: It accounts for less than 1% of breast cancers and is distinguished by the existence of epithelial cells and myoepithelial cells organized in tubular and cribriform structures. The estrogen receptor and HER2 are absent in adenoid cystic carcinoma. The axillary lymph node metastasis is reported in 0%-8% of the cases (Dieci *et al.*, 2014).

The use of the molecular classification for the design of individualized treatments led to extreme changes in disease-free survival. The majority of luminal tumors respond well to hormonal interventions. Anti-HER2 therapies effectively control HER2+ tumors. However, basal-like tumors, lacking hormone receptors and HER2 and referred to as triple-negative breast cancer (TNBC), are still the primary concern. There is currently no specific molecular-targeted therapy for TNBC. Conventional chemotherapy is currently used for the treatment of those patients; however, only 20 percent of the patients respond adequately. The development of new therapies for TNBC is one of the top targets in the

ongoing work in breast cancer (Breast Cancer Facts & Figures | American Cancer Society. 2017).

If a patient presents with features of malignant tumors, including a mass, architectural distortion, asymmetry, and microcalcifications, then, a complete diagnostic examination of the patient needs to be done. The evaluation consists of clinical examination, breast imaging (diagnostic mammogram and ultrasound), core needle biopsy, and sometimes breast magnetic resonance imaging (O'Sullivan, C. C. *et al.*, 2018).

There are a number of options for treating breast cancer patients. Therapeutic decisions are taken on the basis of disease stage and clinical factors, such as levels of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2/neu) in the tumor tissue, menopausal condition, tumor grade, and overall health status of the individual (PDQ Adult Treatment Editorial Board, 2019). To define breast cancer, the American Joint Committee on Cancer utilizes the tumor, node, metastasis (TNM) classification which stages the disease based on the primary tumor characteristics, presence or absence of lymph node involvement, presence or absence of metastases outside primary tumor area as indicated in Table 1.

Table 1 TNM Classification

Stage	TNM	Features
0	Tis, N0, M0	- Paget disease of the nipple NOT associated with invasive carcinoma and/or DCIS in the underlying breast parenchyma - No regional lymph node metastases - No clinical or radiographic evidence of distant metastases
IA	T1, N0, M0	-Tumor ≤20 mm in greatest dimension - No regional lymph node metastases - No clinical or radiographic evidence of distant metastases
IB	T0, N1mi, M0	- No evidence of primary tumor - Micrometastases (approximately 200 cells, >0.2 mm, but ≤2.0 mm - No clinical or radiographic evidence of distant metastases
	T1, N1mi, M0	- Tumor ≤20 mm in greatest dimension - Micrometastases (approximately 200 cells, >0.2 mm, but ≤2.0 mm - No clinical or radiographic evidence of distant metastases
IIA	T0, N1, M0	- No evidence of primary tumor - Metastases to movable ipsilateral Level I, II axillary lymph nodes - No clinical or radiographic evidence of distant metastases
	T1, N1, M0	- Tumor ≤20 mm in greatest dimension - Metastases to movable ipsilateral Level I, II axillary lymph nodes - No clinical or radiographic evidence of distant metastases

Stage	TNM	Features
	T2, N0, M0	- Tumor >20 mm but ≤50 mm in greatest dimension - No regional lymph node metastases - No clinical or radiographic evidence of distant metastases
IIB	T2, N1, M0	- Tumor >20 mm but ≤50 mm in greatest dimension - Metastases to movable ipsilateral Level I, II axillary lymph nodes - No clinical or radiographic evidence of distant metastases
	T3, N0, M0	- Tumor >50 mm in greatest dimension - No regional lymph node metastases - No clinical or radiographic evidence of distant metastases
IIIA	T0, N2, M0	- No evidence of primary tumor - Metastases in ipsilateral Level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases - No clinical or radiographic evidence of distant metastases
	T1, N2, M0	- Tumor ≤20 mm in greatest dimension - Metastases in ipsilateral Level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases - No clinical or radiographic evidence of distant metastases

Stage	TNM	Features
	T2, N2, M0	- Tumor >20 mm but ≤50 mm in greatest dimension - Metastases in ipsilateral Level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases - No clinical or radiographic evidence of distant metastases
	T3, N1, M0	- Tumor >50 mm in greatest dimension - Metastases to movable ipsilateral Level I, II axillary lymph nodes - No clinical or radiographic evidence of distant metastases
	T3, N2, M0	- Tumor >50 mm in greatest dimension - Metastases in ipsilateral Level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases - No clinical or radiographic evidence of distant metastases
IIIB	T4, N0, M0	- Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules); invasion of the dermis alone does not qualify as T4 - No regional lymph node metastases - No clinical or radiographic evidence of distant metastases

Stage	TNM	Features
	T4, N1, M0	- Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules); invasion of the dermis alone does not qualify as T4 - Metastases to movable ipsilateral Level I, II axillary lymph nodes - No clinical or radiographic evidence of distant metastases.
	T4, N2, M0	-Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules); invasion of the dermis alone does not qualify as T4 - Metastases in ipsilateral Level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases - No clinical or radiographic evidence of distant metastases
IIIC	Any T (Tis, T1, T0, T2, T3, T4; N3, M0)	- Any T - Metastases in ipsilateral infraclavicular (Level Ill axillary) lymph node(s) with or without Level l, II axillary lymph node involvement; or in ipsilateral internal mammary lymph node(s) with Level l, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement - No clinical or radiographic evidence of distant metastases
IV	Any T (Tis, T1, T0, T2, T3, T4; Any N = N0, N1mi, N1, N2, N3, M1)	- Any T - Any N - Distant metastases detected by clinical and radiographic means

Information in the Table.1 adapted from: Breast Cancer Treatment (Adult) (PDQ®) on Sept 1, 2019; (PDQ Adult Treatment Editorial Board, 2019).

1.4 Treatment modalities in breast cancer

The principal objectives of the treatment in non-metastatic breast cancers are to eliminate the disease from the breast and the lymph nodes and to prevent the metastatic recurrence. Local and systemic therapies are available for non-metastatic breast cancer. Local therapies include surgical resection and sampling or excision of axillary lymph nodes. Postoperative radiation is considered for some patients. Systemic treatment could be either preoperative (neoadjuvant), postoperative (adjuvant), or both. The subtype of breast cancer defines the option of prescribed systematic therapy. It consists of endocrine therapy for all hormone receptor (HR)-positive tumors (some patients may need chemotherapy), trastuzumab-based antibody treatment with chemotherapy for all ERBB2+ breast cancers (with additional endocrine therapy, if the tumor is HR-positive), and the only available option for treating triple-negative breast cancer patients is chemotherapy due to the lack of hormone receptors expression and absence of molecular targets (Waks and Winer, 2019).

Contrary to the goals of treatments in non-metastatic breast cancers, the treatments in metastatic breast cancers aim at prolonging survival and relieving the complication. Currently, there is no effective treatment for metastasizing breast cancers, and the disease remains incurable. Once metastasis happens, the patients will receive the same categories of systemic and local therapies. However, the treatments given aim to help patients live longer and to relieve their pain (Waks and Winer, 2019).

1.4.1 Surgical treatment of breast cancer

In the past decades, breast cancer surgical treatment has advanced dramatically. Such improvements sought to reduce the long-term cosmetic and functional side effects of

local therapy. After a biopsy indicates the existence of a malignancy, the primary tumor surgical management includes the following:

- Breast-conserving surgery plus radiation therapy: This approach can be used to treat all histological forms of invasive breast cancer (PDQ Adult Treatment Editorial Board, 2019), in which the breast segment containing the malignant tumor is excised together with some healthy tissues and adjacent lymph nodes (Akram *et al.*, 2017). This procedure is usually used for the initial phase of cancer. However, other types of treatments, such as radiation therapy, chemotherapy, or hormone replacement therapy, are also required (Akram *et al.*, 2017).
- Radical mastectomy: is a procedure in which most or all of the lymph nodes under the arm, the lining over the chest muscles, and part of the chest wall muscles are excised (PDQ Adult Treatment Editorial Board, 2019). This is considered the most successful approach to dealing with an already diffused case of breast cancer, for which a lumpectomy was not sufficient.

The main goal of this procedure is to decrease the risk of the development of breast cancer (Voogd *et al.*, 2001); however, even bilateral prophylactic mastectomy may not eliminate the risk of developing primary or recurrent cancers entirely (Akram *et al.*, 2017). Tamoxifen dramatically decreases the risk of contralateral breast cancer in ER-positive breast cancer. Inhibitors of aromatase enzyme can reduce the rates of contralateral disease to the same level or even better than tamoxifen (Tuttle *et al*., 2007). Clinical trials have shown that adjoining systemic therapies (adjuvant systemic chemotherapy, endocrine

therapy, and anti-HER2 directed therapy) with local interventions decreases the risk of recurrence dramatically and increases overall survival (Anampa *et al.*, 2015).

1.4.2 Axillary lymph node management

The status of the axillary lymph nodes continues to be the most important predictor of outcome in breast cancer. Staging of axillary lymph nodes helps in determining not only prognosis but also treatment plan (PDQ Adult Treatment Editorial Board, 2019).

In women with invasive breast cancer, sentinel lymph node (SLN) biopsy is the primary procedure for axillary staging. Any node receiving direct drainage from the primary tumor is known as SLN. Radioactive substances are used in the identification of the SLN. Such compounds are drained to the axilla after their injection around the tumor or in the subareolar region, which results in the SLN identification. Using this procedure, SLN is detected (Figure 2) in 92 to 98% of the patients (Kern, 1999; PDQ Adult Treatment Editorial Board, 2019; Rubio *et al.*, 1998).

Axillary lymph node dissection (ALND) was the standard approach to axilla before clinical trials found no substantial difference in local recurrence and overall survival between women who had complete ALND and women who were subjected to sentinel lymph node biopsy (SLN), in those with clinical node-negative (cN0) breast cancer. ALND was recommended only if the SLN was positive; however, recent studies indicated that ALND is not necessary in patients with limited SLN-positive breast cancer if the patient is treated adequately with systemic and local therapies (PDQ Adult Treatment Editorial Board, 2019).

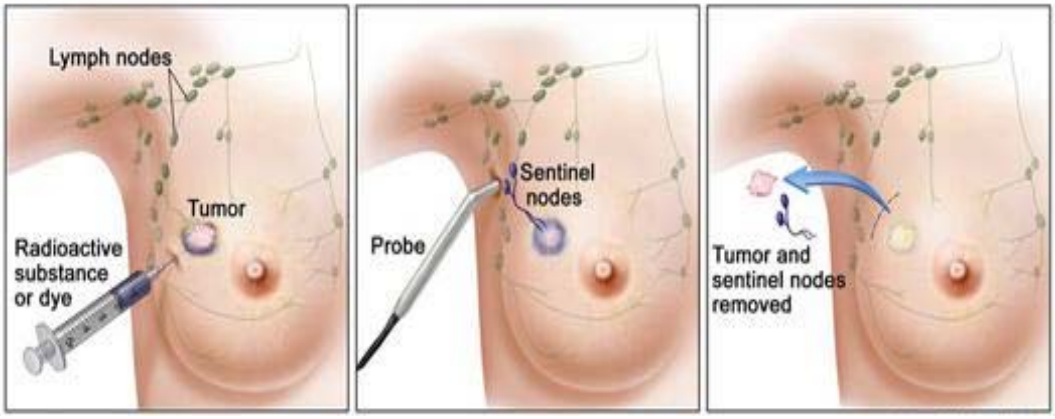


Figure 2. Sentinel lymph node biopsy of the breast

Adapted from (PDQ Adult Treatment Editorial Board, 2019).

1.4.3 Radiation therapy

The main objective of adjuvant radiation therapy is to eliminate the remaining disease, thus decreasing local recurrence. It is regularly employed after breast-conserving surgery. A meta-analysis of 10,801 patients showed that the administration of whole-breast radiation therapy following lumpectomy was associated with a major decline in the 10-year risk of recurrence and the 15-year risk of breast cancer death compared with breast-conserving surgery alone (Waks and Winer, 2019).

1.4.3.1 Dosing and schedule

Whole-breast radiation dosage: traditional whole-breast radiation is administered to the whole breast (with or without regional lymph nodes) after lumpectomy in 1.8 Gy to 2 Gy daily amounts with a cumulative dosage of 45 Gy to 50 Gy over 5 to 6 weeks (PDQ Adult Treatment Editorial Board, 2019). Recent studies indicate that a shorter period of 42.5 Gy over 3 to 4 weeks is as efficient for the risk reduction of local recurrence and equally if not more efficacious for cosmesis (Whelan *et al.*, 2010). Therefore, a shorter fractionation schedule is now "preferred" per current guidelines (Slamon *et al.*, 2011).

1.4.3.2 Radiation boost

The local recurrence of tumors tends to occur mostly at the site of the primary tumor probably due to the presence of residual microscopic tumor cells. Therefore, an extra dose (boost) of radiation is commonly delivered to the initial tumor site since radiation can eliminate the microscopic tumor cells (Kindts *et al.*, 2017). Two randomized trials have shown that using boosts of 10 Gy to 16 Gy was associated with local recurrence risk reduction from 4.6% to 3.6% at three years and from 7.3% to 4.3% at five years. Results

were close after a median follow-up of 17.2 years (PDQ Adult Treatment Editorial Board, 2019). The radiation boost is used for women at high risk of local recurrence. However, it is not recommended to be used for women at low risk of local recurrence (Kindts *et al.*, 2017). Physicians use four major features to identify high-risk patients. If the patient has one of these features, she will be in the category of a high risk of local recurrence. These features are: four or more positive axillary nodes, clearly visible extracapsular nodal expansion, massive primary tumors, or quite close or positive borders of primary tumor resection (Fowble *et al.*, 1988; Overgaard *et al.*, 1997; Ragaz *et al.*, 1997). In this high-risk group, radiation therapy can minimize locoregional recurrence, even between patients who receive adjuvant chemotherapy (Early Breast Cancer Trialists' Collaborative Group, 2000).

1.4.4 Chemotherapy

Many randomized trials evaluated the role of adjuvant chemotherapy in breast cancer. The first was in 1958, which reported in 1968 that giving thiotepa (an alkylating agent) reduced the recurrence rate significantly in premenopausal women with four or more positive axillary lymph nodes (Anampa *et al.*, 2015). Many randomized trials followed, and in 2001, the National Institute of Health consensus panel in the US concluded: “Because adjuvant polychemotherapy improves survival, it should be recommended to the majority of women with localized breast cancer regardless of lymph node, menopausal, or hormone receptor status” (Abrams, 2001; Anampa *et al.*, 2015).

The widespread adoption of the polychemotherapy regimen contributed to the declining of the breast cancer mortality rate; however, it also resulted in the unintentional overtreatment of many patients with chemotherapy (Anampa et al., 2015). Prognosis and selection of therapy may be influenced by the clinicopathological features such as anatomic

stage, tumor grade, and histologic type (PDQ Adult Treatment Editorial Board, 2019). However, they are not perfect in the assessment of risk and chemosensitivity (Gluz *et al.*, 2016; Pan *et al.*, 2017; Sparano *et al.*, 2015; Waks and Winer, 2019).

Several gene profile tests have been developed to estimate prognosis and predict chemotherapy benefit (Anampa *et al.*, 2015). In order to estimate the prognosis and predict the efficacy of chemotherapy, various gene profile tests were developed (Anampa *et al*., 2015). The American Society of Clinical Oncology suggests the use of the 21-gene recurrence score and 70-gene assay to direct decisions on the administration of adjuvant chemotherapy for HR-positive, ERBB2-negative, node-negative breast cancer patients to assess which of these patients will benefit most from adjuvant chemotherapy (Harris *et al.*, 2016; Krop *et al.*, 2017). Gene profile tests include the following:

- **MammaPrint**: MammaPrint is the first gene profile test to have the U.S. Food and Drug Administration approval. It depends on the expression level of 70 genes to generate scores that classify the patients into high-risk and low-risk groups (PDQ Adult Treatment Editorial Board, 2019).
- **Oncotype DX (21-gene assay)**: It is used to determine prognosis and chemotherapy effectiveness in patients with hormone receptor-positive breast cancer. It depends on the expression level of 21 genes, which classifies the patients into high-risk (the Recurrence score ≥31), intermediate-risk (the Recurrence score ≥18 and <31), and low-risk (the Recurrence score <18) (PDQ Adult Treatment Editorial Board, 2019).

According to the NSABP B-20 trial, which assessed the use of Oncotype Dx to predict benefits from chemotherapy in patients with hormone receptor-positive, node-negative breast cancer, the high-risk group gained benefits from the addition of

chemotherapy to tamoxifen. The 10-year distant disease-free survival (DFS) improved from 60% to 88%. Nevertheless, the low recurrence score group did not gain any benefits (PDQ Adult Treatment Editorial Board, 2019). More limited evidence indicates that the gene profile assays in HR-positive, ERBB2– negative, node-positive breast cancer can evaluate the chemotherapy gain and prognosis (Waks and Winer, 2019).

Adjuvant! is widely used in clinical practice as a Web-based decision aid. This allows physicians and patients to better consider the possible benefits of adjuvant treatment, especially chemotherapy. Adjuvant chemotherapy regimens are categorized by Adjuvant! into a first, second, and third generation. Typically, such regimens containes anthracyclines (doxorubicin, epirubicin) and/or taxanes (paclitaxel, docetaxel), two most powerful groups of cytotoxic substances for breast cancer at the early and the advanced stages (Anampa *et al.*, 2015).

Anthracyclines interfere with redox cycling, which results in DNA damage due to reactive oxygen species (ROS) production. A number of cellular oxidoreductases reduce the quinone portion of anthracyclines to a semiquinone radical. The semiquinone auto-oxidizes in the presence of molecular oxygen to form the original anthracycline and a superoxide anion. The pathway is non-enzymatic and permits a self-perpetuating redox cycle to be initiated, which results in the buildup of superoxide anions. Free cellular iron and ferrous-ferric cycling of molecular iron can also elevate the ROS levels. The complexes of doxorubicin-iron produce free radicals and reactive nitrogen species, leading to greater nitrosative stress and dysfunction of the mitochondria.

Anthracyclines have different mechanisms of actions, and more recently, topoisomerase II poisoning by anthracyclines has been suggested to be one of the mediators

of cardiotoxicity and cancer cell killing (Yang, F. *et al.*, 2014). Topoisomerases are highly conserved enzymes that are present in all life forms. Topoisomerases cause transient single or double-stranded breaks to control topological changes during DNA replication, transcription, recombination, and remodeling of chromatin (Yang *et al.*, 2014). In humans, topoisomerase II exists in two isoforms, topoisomerase IIα and topoisomerase IIβ. Topoisomerase IIα is the most abundant and is extensively expressed in dividing cells. On the other hand, top IIβ is more prevalent in quiescent cells, such as cardiomyocytes (Yang *et al.*, 2014). For chromosomal segregation, topoisomerase IIα is critical, and its expression differs over the cell cycle, reaching the highest during G2/M phases. Contrarily, topoisomerase IIβ expression remains constant during the cell cycle (McGowan *et al.*, 2017).

Attachment of the topoisomerase enzyme to DNA supercoils and entangled DNA breaks both DNA strands resulting in the passage of the other duplex through the resulting gap. Next, the gap is resealed. The resulting effect is the release of torsional stresses created during replication and transcription of DNA. Doxorubicin holds topoisomerase II at the break site. This effect results in the stabilization of the cleavage complex and prevention of DNA resealing (McGowan *et al.*, 2017; Yang *et al.*, 2014).

When doxorubicin binds topoisomerase IIα, the complex inhibits DNA replication resulting in cell cycle arrest at G1/G2 and apoptosis (Figure 3) induction as proposed in proliferating malignant cells. On the other hand, when doxorubicin binds topoisomerase IIβ, mitochondrial dysfunction is initiated by the inhibition of peroxisome proliferator-activated receptor (PPAR). PPAR controls oxidative metabolism; thus, the binding of doxorubicin to topoisomerase IIβ in cardiomyocytes results in the activation of the altered

P53 tumor suppressor pathway, β-adrenergic signaling, impaired handling of calcium, mitochondrial dysfunction and increased apoptosis (McGowan *et al.*, 2017) (Figure 3).

Furthermore, doxorubicin exerts its cytotoxic effect by intercalating DNA (Figure 4). It binds DNA adjacent to GC base pairs and forms hydrogen-bond with guanine. Doxorubicin-DNA adducts activate DNA damage and induce cell death independent of topoisomerase II (Yang *et al.*, 2014). Epirubicin and doxorubicin are the most commonly used medicines in breast cancer (Akram *et al.*, 2017). Doxorubicin is one of the most highly used cytotoxic agents in the management of metastatic breast cancer. However, administering the drug to the patients resulted in congestive cardiomyopathy which necessitated limiting the cumulative lifetime dose to lessen the toxicity effect. Epirubicin is an epimer of doxorubicin. The difference in the orientation of the C4 hydroxyl group on the sugar differentiates the two anthracyclines (Figure 5). Epirubicin is a less cardiotoxic treatment than doxorubicin (Anampa *et al.*, 2015).

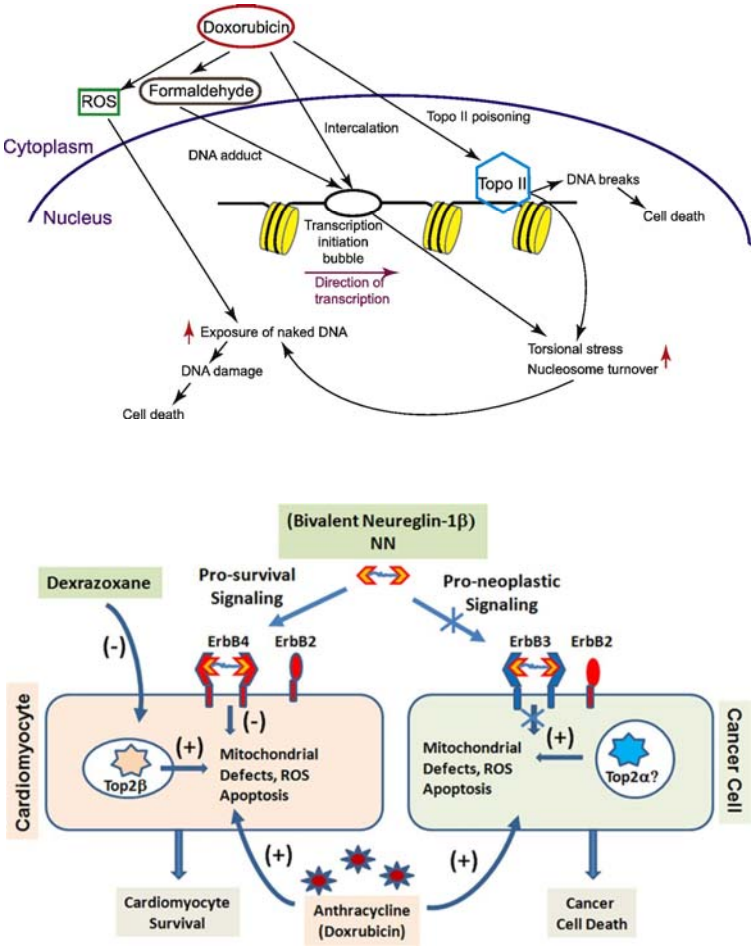


Figure 3. Anthracycline-induced cellular and cardiotoxicity

Adapted from (Yang *et al.*, 2014) (Force and Wang, 2013).

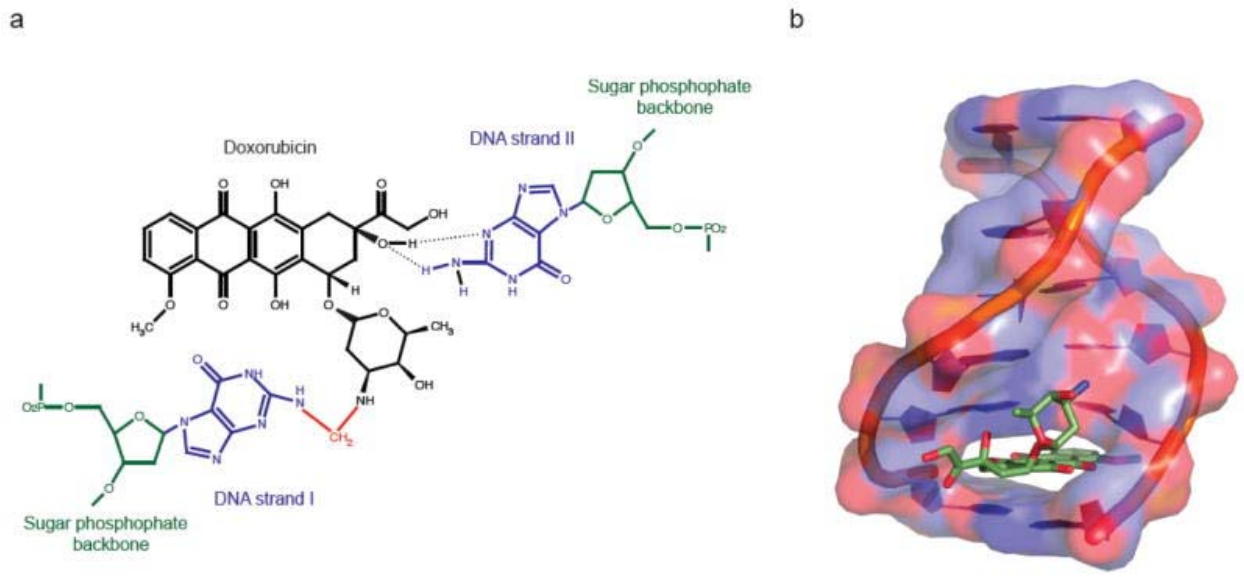


Figure 4. Structure of the doxorubicin-DNA complex

Adapted from (Yang *et al.*, 2014).

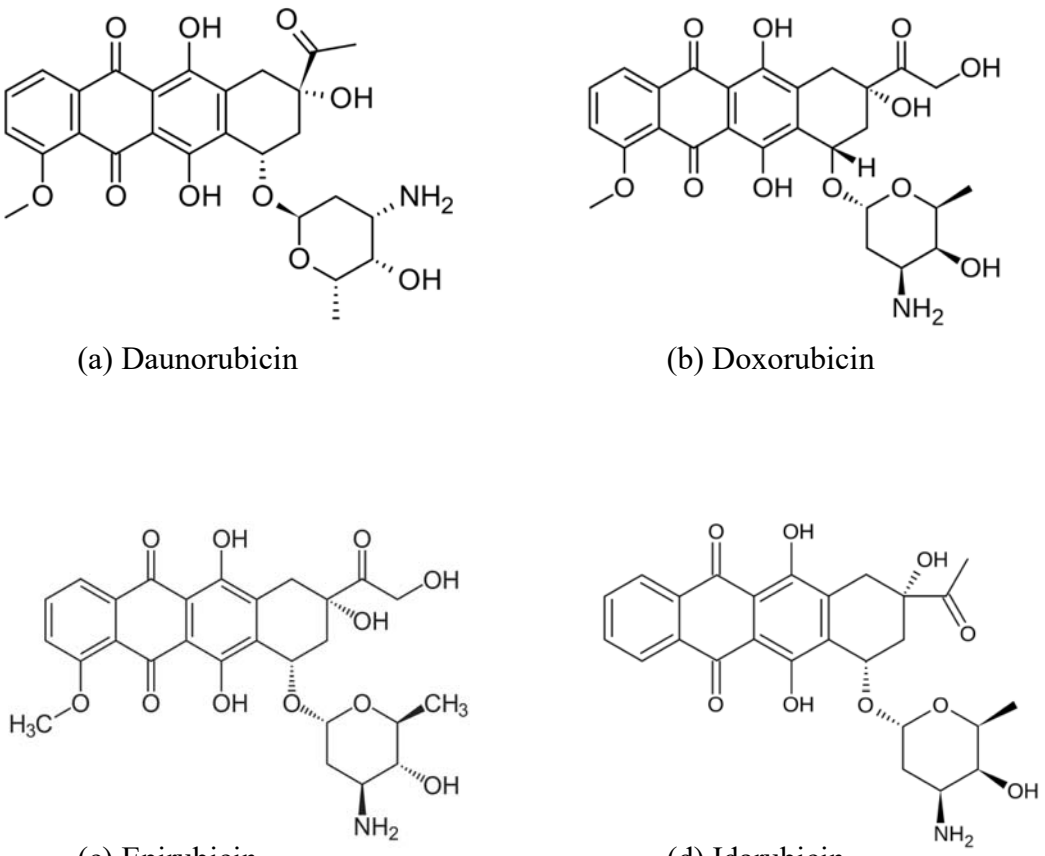


Figure 5. The four anthracycline derivatives

Taxanes are cytoskeletal drugs. The cell cycle is characterized by a faithful replication of DNA and generation of daughter cells. The eukaryotic cell cycle consists of four phases. These phases are the G1 phase, S phase (synthesis), G2 phase, and M phase (mitosis and cytokinesis). G1 phase, S phase, and G2 phase are known as interphase. M phase consists of two tightly coupled processes: mitosis, and cytokinesis. In mitosis, the nucleus divides, and during cytokinesis, the cytoplasm divides, forming two daughter cells. Activation of each phase relies on the proper progression and completion of the preceding one (Bavle, 2014; Zhou *et al.*, 2002).

When mitosis starts, the chromosomes condense and become visible. Besides, the mitotic spindle begins to form. This phase corresponds to the prophase. Follows the prophase is the prometaphase, in which the nuclear envelope breaks down into small vesicles, the nucleolus disappears, and microtubules, polymers composed of repeating subunits of α- and β-tubulin heterodimers, protrude from opposite ends of the cell and attach to the kinetochores. Next, during metaphase, the chromosomes organize centrally within the cell. After that, the microtubules shorten to pull the sister chromatids of each chromosome apart toward opposite ends of the cell and condense maximally in late anaphase to help chromosome segregation and the re-formation of the nucleus. Next, during telophase, the polar microtubules continue to lengthen, and the cell becomes elongated, and a new nuclear envelope forms around the separated daughter chromosomes. Next, the cell undergoes cytokinesis in which the cell membrane pinches inward between the two developing nuclei to produce two new cells (Bavle, 2014; Malumbres, 2020).

Paclitaxel is one of the tubulin targeting cytoskeletal drugs. When treated with paclitaxel, cells suffer from defects in mitotic spindle assembly, chromosome segregation,

and cell division. Contrary to other medications that prevent microtubular assembly such as colchicine, paclitaxel stabilizes and prevents the microtubule polymer from disassembly. This inhibits the microtubules from shortening. Chromosomes are thus unlikely to reach a spindle configuration of the metaphase. This inhibits the continuation of mitosis, and sustained activation of the mitotic checkpoint contributes to apoptosis or reversal of the cell cycle into the G0 phase without cell division (Stanton *et al.*, 2011). In spite of its remarkable mechanism of action, paclitaxel's initial development was slow due to its rarity and poor solubility. Hypersensitivity reactions were associated with its administration due to the Cremophor EL vehicle. This reaction required the premedication with corticosteroids and histamine blockers. To address the underlying shortage of paclitaxel, docetaxel, a semi-synthetic agent, was developed. It is a more potent microtubule inhibitor *in vitro* and slightly more water-soluble.

Other chemotherapeutic drugs used in breast cancer treatment include:

- **Cyclophosphamide**: Oral cyclophosphamide is a rapidly absorbed drug, and It is changed in the liver to active metabolites by cytochrome P450 system (Huttunen *et al.*, 2011; Vredenburg *et al.*, 2015). 4-Hydroxycyclophosphamide is the primary active metabolite that exists with its tautomer, aldophosphamide. Aldophosphamide is oxidized by aldehyde dehydrogenase (ALDH) enzyme to produce carboxycyclophosphamide. A small portion of aldophosphamide enters into cells. Inside the cells, aldophosphamide is broken down into two components, phosphoramide mustard and acrolein (Boddy and Yule, 2000). The main action of cyclophosphamide is attributed to its metabolite phosphoramide mustard. Phosphoramide mustard forms DNA crosslinks both between and inside DNA

strands at guanine N-7 positions (known as interstrand and intrastrand cross-linkages, respectively). This effect is irreversible and prompts cell apoptosis (Hall and Tilby, 1992).

- **Methotrexate** is an anti-folate treatment. It competitively inhibits dihydrofolate reductase (DHFR). Methotrexate has an affinity for DHFR that is about 1000-fold that of folate. DHFR catalyzes the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is necessary for the synthesis of the nucleoside thymidine, which is needed for DNA synthesis and also is essential for purine and pyrimidine base biosynthesis. Therefore, methotrexate inhibits the synthesis of DNA, RNA, thymidylates, and proteins (Rajagopalan *et al.*, 2002).
- **5-fluorouracil (5-FU)** is an analog of uracil, but at the C-5 position, a fluorine atom is present in place of hydrogen. It is an anti-metabolite drug that is commonly used in the treatment of colorectal and breast cancers. It works through the inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA (Longley *et al.*, 2003).

Adjuvant! categorizes adjuvant chemotherapy regimens as first, second, and third-generation. The first-generation regimen resulted in a 35 % reduction in breast cancer mortality compared with no adjuvant chemotherapy. The second-generation regimen achieved a 20 % reduction in breast cancer mortality compared with the first-generation regimen, and the third-generation regimen was more effective than the second generation causing a 20 % reduction in breast cancer mortality compared with the second-generation regimen (Anampa *et al.*, 2015). Chemotherapy regimens containing both anthracycline and taxane achieved the most risk reduction and remain the appropriate choice in high-risk

patients. Even though the use of adjuvant chemotherapy has contributed to reduced breast cancer mortality rates, metastatic breast cancer remains incurable in all affected patients.

1.4.5 **Endocrine therapy**

Endocrine treatment neutralizes estrogen-promoted tumor development. It is the essential systemic therapy for HR+/ERBB2− breast cancer. Conventional endocrine treatment comprises of oral anti-estrogen medication taken daily for five years. The choice of the anti-estrogen given to the patients depends on the menopausal status (Waks and Winer, 2019).

The main female sex hormone is estrogen. It is essential for the evolution and control of the female reproductive system and the secondary sex features. Females possess three major estrogens. These estrogens typically involve estrone (E1), estradiol (E2), and estriol (E3). All have an estrogenic hormonal function. The most important and predominant of these in premenopausal life is estradiol, whereas E1 plays a vital role after menopause, and E3 is the least potent estrogen and plays a bigger role during pregnancy (Cui *et al.*, 2013).

In females, estrogens are produced primarily by the ovaries. During pregnancy, it is produced by the placenta. Follicle-stimulating hormone (FSH) induces the ovaries to produce estrogens from the granulosa cells of the ovarian follicles and corpora lutea. Other tissues can make some estrogens. These tissues are the liver, pancreas, bone, adrenal glands, skin, brain, adipose tissue, and the breasts. Such secondary estrogen sources are particularly significant in postmenopausal women (Zhao, H. *et al.*, 2016).

Synthesis of estrogens takes place in the theca interna cells in the ovary. Cholesterol is converted to androstenedione which is a substance of mild androgenic activity (Zhao, H. *et al.*, 2016). It functions mainly as a precursor for stronger androgens such as testosterone and estrogen. Androstenedione passes through the basal membrane into the granulosa cells.

In the granulosa cells, it is processed into estrone, or into testosterone. In a further step, estradiol is made. 17β-hydroxysteroid dehydrogenase (17β-HSD) catalyzes the conversion of androstenedione to testosterone. However, the conversion of androstenedione and testosterone into estrone and estradiol, respectively, is mediated by aromatase enzyme (Zhao, H. *et al.*, 2016) (Figure 6).

Estradiol binds to the estrogen receptors, estrogen receptor alpha (ERα), or estrogen receptor beta (ERβ) with a high affinity and specificity (Fox *et al.*, 2009). Once estrogen binds the ER, the heat shock proteins dissociate, and receptor dimerization and nuclear localization follow. Once translocated to the nucleus, estrogen: estrogen receptor (E2: ER) complex binds the estrogen response elements (EREs) (Levin, 2009). E2: ER complex acts as a transcription factor. The transcriptional function is mediated through two regions of the ER named activation functions AF1 and AF2, which call up transcriptional co-activators or co-repressors to the complex and lead to changes in the rate of transcription of estrogen-regulated genes.

Direct and indirect mechanisms promote the contribution of estrogen to breast cancer initiation and progression. Estradiol and other estrogens, to a lesser degree, enhance the proliferation of the breast epithelium and stroma and thus raise the chances of mutation in the rapidly proliferating epithelium (Yaghjyan and Colditz, 2011). Anti-estrogen therapy for HR-positive breast cancer helps to lower estrogen levels in the body or stop estrogen from inducing breast cancer cells growth.

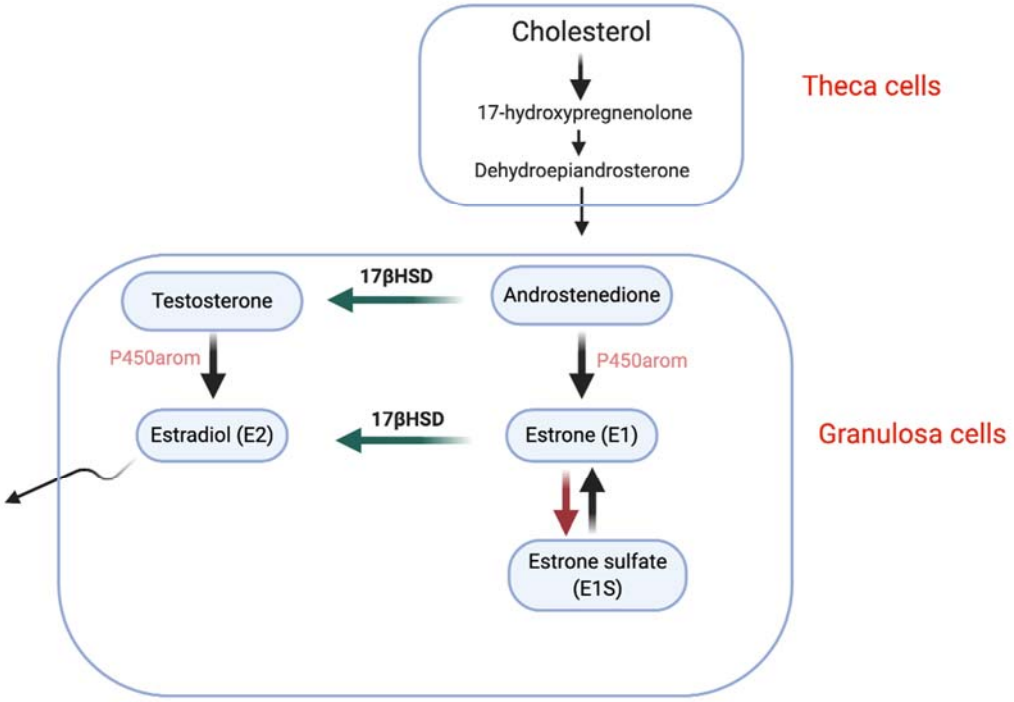


Figure 6. Estrogen biosynthesis (created by me using Biorender)

1.4.5.1 Types of hormone treatment

- *Luteinizing hormone-releasing hormone (LHRH) agonist*: This treatment reduces ovarian estrogen formation. More estrogen is also required to promote the development of ER-positive breast cancer. This is the most popular treatment in early-stage ER-positive breast cancer females that have not yet entered menopause. These medications may induce transient menopause symptoms, such as hot flashes, vaginal dryness, and changes in mood (PDQ Adult Treatment Editorial Board, 2019).
- *Aromatase inhibitors*: Aromatase inhibitors inhibit aromatase enzyme and reduce the amount of estrogen produced in the body. Therefore, less estrogen is available in the body to encourage ER-positive breast cancer cells to grow. Aromatase inhibitors are only used in females who have already gone through menopause. These drugs target tissues where the body produces estrogen after menopause. They target the adrenal gland and fat tissue, but they do not prevent the ovaries from producing estrogen. Aromatase inhibitors include anastrozole (Arimidex), exemestane (Aromasin), and letrozole (Femara). Side effects of these drugs comprise of hot flashes, muscle pain, pain or stiffness in the joints, osteoporosis (PDQ Adult Treatment Editorial Board, 2019; Waks and Winer, 2019).
- *Selective estrogen receptor response modulators*: Selective estrogen-receptor response modulators (SERMs) function as estrogen receptor blockers on the breast cells. Therefore, they prevent estrogen from sending the signal to the breast cancer cells to grow. SERMs include tamoxifen (Nolvadex, Soltamox) and toremifene (Fareston). Adverse side effects of these treatments include mood swings, hot

flashes, vaginal dryness, or discharge. Less common side effects include uterine cancer, blood clots, deep vein thrombosis, pulmonary embolism, and stroke (Waks and Winer, 2019).

- *Selective estrogen receptor down regulators*: selective estrogen receptor down regulators (SERDs) also inhibit the estrogen effects. They block estrogen receptors on the breast cells; therefore, fewer estrogen receptors will be available for estrogen to bind. One example of a SERD is fulvestrant (Faslodex). It is described for postmenopausal females with advanced ER-positive breast cancer when tamoxifen is not working. Prolonged use of these drugs can cause osteoporosis (PDQ Adult Treatment Editorial Board, 2019).

1.4.6 Targeted therapy

20% of women with breast cancer have HER2-positive breast tumors. These tumors have increased HER2 expression and are characterized by aggressive growth and spread. A number of drugs are available to target this protein as described below. Unless otherwise noted, their description and usage are referenced in (Targeted Therapy for Breast Cancer).

Trastuzumab (Herceptin) is a monoclonal antibody against HER2/neu that is used in combination with chemotherapy or may be used alone. Trastuzumab is used to treat early and late-stage breast cancer. In the early stages of breast cancer, this medicine is prescribed for a duration between 6 months to one year. For advanced breast cancer, treatment is often administered for the patients as long as the treatment is effective. Another type of trastuzumab therapy is available called trastuzumab and hyaluronidase-oysk (Herceptin Hylecta), which is given subcutaneously.

Pertuzumab (Perjeta) is a monoclonal antibody that can be used with trastuzumab and chemotherapy. It can be given to treat early-stage breast cancer or to treat advanced breast cancer.

Ado-trastuzumab emtansine is an antibody-drug conjugate in which an anti-HER2 monoclonal antibody is attached to a chemotherapy drug. This drug is administered intravenously (IV) and is currently used to treat women with advanced breast cancer who have failed trastuzumab and chemotherapy.

Lapatinib (Tykerb) is a kinase inhibitor that is taken daily as a pill to treat advanced breast cancer. It may also be used in combination with certain chemotherapy drugs, trastuzumab, or hormone therapy drugs.

Neratinib (Nerlynx) is also a kinase inhibitor that is taken daily as a pill. It is prescribed to treat early-stage breast cancer after the completion of one year of trastuzumab treatment. It is usually given for one year. Some clinical trials indicate that it may also be useful in advanced breast cancer.

Targeted therapy for HER2-positive breast cancer has many unwanted secondary effects. They are mild, but some can be life-threatening. Treatment with trastuzumab, pertuzumab, or ado-trastuzumab emtansine can cause cardiac diseases. This condition improves when the treatment is stopped. The risk of developing congestive heart failure is increased when these drugs are combined with doxorubicin (Adriamycin) and epirubicin (Ellence).

All the previously mentioned treatments, with the exception of aromatase inhibitors, target tumor cells directly. However, there is ample evidence indicating that the tumor microenvironment (TME) in which tumor cells grow plays a role in breast cancer

development, progression, and response to therapy. The TME is composed of a variety of different cell types (including endothelial, immune cells, fibroblasts, and adipocytes), various secretory factors (cytokines, chemokines, growth factors, and exosomes), and structural components that include extracellular matrix (ECM). As breast cancer progresses, there is an increase in the stromal cell populations within the TME (Cheung and Ewald, 2014).

These cells have the ability to influence cancer development through crosstalk initiated by secretory factors such as cytokines, chemokines, growth factors, and exosomes. Recent data indicate that during disease progression, major gene expression and epigenetic alterations take place in the cells residing in the tumor microenvironment. TME is directly linked to tumor growth and metastasis. Thus, developing novel therapies targeting the TME is a necessity (Place *et al.*, 2011).

One major target in the TME involved in breast cancer progression and development is angiogenesis. Cancer is defined by a number of hallmarks that describe the distinctive and complementary capabilities that enable tumor growth and metastatic dissemination. The six primary hallmarks of cancer include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Recently, more cancer hallmarks have been proposed, including the deregulation of cellular energetics, evading immunological destruction by T and B lymphocytes, macrophages, and natural killer cells, genomic instability, and tumor-promoting inflammation (Hanahan and Weinberg, 2011).

Tumors need to build up a blood supply similar to healthy organs in order to fulfill their demand for oxygen and nutrients and perform other metabolic functions. Tumor blood supply is accomplished mainly via angiogenesis, the mechanism by which new blood vessels from a pre-existing vasculature. It was proposed by Judah Folkman in 1971 that tumors have a very limited capacity to grow without vascular support, and the maximum values of 1 to 2 mm were recognized as the limit for neoplastic expansion without new blood vessels formation (Folkman *et al.*, 1971). Consistently, several pre-clinical trials have shown that the targeting of angiogenic factors can achieve successful inhibition of tumor development. Nonetheless, the clinical outcomes of anti-angiogenic treatment are very disappointing, as anti-angiogenic medications increase life by just a few months (Ellis and Fidler, 2010; Stockmann *et al.*, 2014).

The net angiogenic activity relies upon the equilibrium between the pro and anti-angiogenic factors. These modulators firmly regulate the action of various molecules, including cellular junction proteins, extracellular matrix-degrading enzymes, and cell adhesion receptors. The high level of pro-angiogenic factors at the tumor site renders the endothelial cells more invasive and migratory (Ribatti *et al.*, 2007).

Under physiological condition, the angiogenesis activators such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF), angiogenin, transforming growth factor TGF-α, TGF-β, tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor, interleukin-8 (IL-8), hepatocyte growth factor, and epidermal growth factor promote vessel sprouting through the stimulation of matrix protease production, inhibition of endothelial cell apoptosis, and increased endothelial cell mobility (Rajabi and Mousa, 2017). Angiogenesis

inhibitors (Table 2) negate the activity of angiogenesis activators and keep new blood vessel growth under control. The mechanisms of action of angiogenesis inhibitors comprise interfering with signal transduction stimulated by angiogenesis inducers, inhibiting the proteases that generate inducers, inhibiting endothelial cell proliferation, migration, and tubule formation, and initiating endothelial apoptosis through stimulation of the CD36 receptor and Fas-L signaling (Pollina *et al.*, 2008).

The dominance of the angiogenic inhibitors in healthy tissues keeps the vasculature quiescent (Stockmann *et al.*, 2014). On the other hand, in tumors, angiogenesis depends on the downregulation of negative regulators and shifting the balance toward the increase of angiogenesis stimulators, which are released by neoplastic cells and inflammatory cells, causing the growth of the tumor blood vessels (Stockmann *et al.*, 2014).

In spite of the increase in blood vessel density in tumor tissues, these vessels are structurally and functionally abnormal. The vasculature in the body normally is well-differentiated and structured in a hierarchy of equally spaced, arteries, arterioles, capillaries, venules, and veins. In contrast, the blood vessels of the tumors are irregular and disorganized. Tumor vasculature typically has a twisty route, branch abnormally, and develop arteriovenous shunts (Rajabi and Mousa, 2017). In addition, tumor blood vessels are characterized by excessive and unusual bulging, blind ends, discontinuous endothelial cells, and abnormal basement membrane and pericyte distribution (De Palma *et al.*, 2017). These aberrant features of tumor vasculature render them inefficient (Stockmann *et al.*, 2014), resulting in hypoxic tumor cells.

Table 2. Angiogenic inhibitors and their mechanism of action.

Endogenous Angiogenesis Inhibitors	Mechanisms
Soluble VEGF-1	Decoy receptors for VEGF-B
Angiostatin	Suppress EC adhesion, migration, proliferation
Thrombospondin-1 and -2	Suppress EC adhesion, migration, proliferation
Angiopoietin-2	Oppose Angiopoietin 1
Platelet Factor-4	Inhibit bFGF (FGF2) and VEGF binding
Endostatin	Suppress EC adhesion, migration, proliferation
Anti-thrombin III Fragment	Suppress EC adhesion, migration, proliferation
Osteopontin	Serve as ligand for integrin binding
Collagen	Substrate for MMPs
Kininogen Domains	Suppress EC adhesion, migration, proliferation
Tissue Factor Pathways Inhibitor	Antagonist for Tissue Factor
Vasostatin	Suppress EC adhesion
Calreticulin	Suppress EC adhesion
TIMPs	Suppress EC adhesion
A cartilage-derived angiogenesis inhibitor	Suppress EC adhesion
Meth-1 and Meth-2	Suppress EC adhesion
Maspin	Inhibits proteases
Laminin 511	Suppresses metastases
CCN3	Suppresses EC adhesion
Endorepellin	Suppresses EC adhesion
MULTIMERIN2 (Endoglyx-1)	Suppresses EC migration

Adapted from (Rajabi and Mousa, 2017).

Hypoxia is a significant promoter of tumor blood vessel formation. Hypoxic cancer cells release vascular endothelial growth factor A (VEGF-A), which triggers blood vessel formation by interacting with VEGF receptor-2 (VEGFR-2) expressed on adjacent blood vessels endothelial cells (ECs). VEGF-A gradients activates the development of tip cells, motile ECs. The tip cells degrade the adjacent extracellular matrix (ECM) and guide the expansion of the blood vessel sprouts toward VEGF-A. This process relies on the involvement of specific signaling molecules like Delta ligand-like 4(DLL4), which regulates the phenotype of tip cells and angiopoietin 2 (ANGPT2), which disrupts junctions between the ECs (De Palma *et al.*, 2017). Although the cancer cells can be an important source of VEGF-A, many signals that emerge from various tumor-associated stromal cells (TASCs) and the ECM sustain angiogenesis after the angiogenic switch. Therefore, a proper understanding of the extrinsic regulation of angiogenesis by tumor microenvironment (TME) may be instrumental in developing more effective anticancer therapies targeting angiogenesis.

1.5 Regulation of angiogenesis by TASCs

TASCs vary considerably in their composition between tumors. TASCs can be subdivided into tumor-infiltrating cells of hematopoietic origin recruited from the bone marrow, tumor-infiltrating cells of non-hematopoietic origin also recruited from the bone marrow, and tissue-resident cells (De Palma *et al.*, 2017).

Macrophages: These cells are specialized phagocytes that can ingest microbes and release different cytokines. They have the unique ability to change their phenotype according to changes in their microenvironments. Two macrophage phenotypes are distinguished, M1 (classically activated) or M2 (alternatively activated) macrophages. The

M1 phenotype is proinflammatory and is associated with a T-helper-1 response. They secrete bactericidal factors in response to lipopolysaccharide and interferon γ (IFNγ) exposure. M2 macrophages are immunosuppressive and display a T-helper-2 cytokine expression pattern (Mantovani and Sica, 2010).

In vitro studies have shown that macrophages extracted from normal or inflamed tissues are capable of lysing tumor cells, presenting tumor-associated antigens to T cells, and releasing immunostimulatory cytokines to promote T cell and natural killer (NK) cell proliferation and antitumor functions. Macrophages derived from experimental or human tumors, by comparison, display substantially lower rates of these behaviors, likely owing to their sensitivity to tumor-derived molecules such as IL-4 and IL-10, which may cause tumor associated macrophages (TAMs) to evolve into M2 macrophages. The antigen presentation of these cells is weak. They also secrete mediators that inhibit the growth and activity of T-cells. In contrast to M1 macrophages phenotype, these cells function mainly to scavenge debris, promote angiogenesis, and repair and remodel damaged tissues (Lewis and Pollard, 2006).

In solid tumors, hypoxia frequently happens as a result of rapid tumor cell proliferation and the inefficiency of tumor-associated blood vessels (TABVs). The secretion of tumor-derived chemoattractants including colony-stimulating factor-1 (CSF-1 also known as M-CSF), the CC chemokines, CCL2, CCL3, CCL4, CCL5, and CCL8, and vascular endothelial growth factor (VEGF) attracts TAM to the hypoxic areas of the tumor site (Lewis and Pollard, 2006). Once attracted to the hypoxic areas, the microenvironment promotes TAMs to adapt to hypoxia by upregulating hypoxia-inducible factors (HIF)-1,

HIF-2, and VEGF (Stockmann *et al.*, 2014). VEGF-A serves as a robust mitogen for endothelial cells by binding to VEGFR-1 and VEGFR-2 (Squadrito and De Palma, 2011). Moreover, TAMs have the capacity to release a number of other angiogenesis activators. For example, they can release basic fibroblast growth factor (b-FGF), platelet-activating factor (PAF), M-CSF, platelet-derived growth factor (PDGF), heparin-binding epidermal growth factor (HB-EGF), macrophage-inhibitory factor (MIF), and TGF-β. TAM can secrete cytokines such as IL-1, IL-8, tumor necrosis factor-alpha (TNF-α), and MCP-1 (Dirkx *et al.*, 2006; Stockmann *et al.*, 2014). TAMs also promote angiogenesis by releasing prostaglandin E2 (PGE2) as a result of stimulation by apoptotic tumor cells (Brecht *et al.*, 2011).

Beside producing pro-angiogenic factors in hypoxic conditions, TAMs downregulate the expression of angiogenesis inhibitors, such as vasohibin-2, and express several angiogenesis-modulating enzymes, such as COX-2, iNOS, and various matrix metalloproteinases to promote angiogenesis (Stockmann *et al.*, 2014). Several pieces of evidence support the theory that TAMs are influenced by tumor microenvironment and tumor cells to support tumor growth and metastasis, and "re-education" of TAMs is an innovative new cancer therapy approach through the inhibition of angiogenesis and normalization of tumor vessels.

Neutrophils: These cells are the largest population of blood leukocytes and are critical for the initial defense against invading microbes. Neutrophils are abundant in triple-negative breast cancer (Soto-Perez-de-Celis *et al.*, 2017). Neutrophils can be recruited to the tumor microenvironment by CXC chemokines. CXCL8 (IL-8) controls neutrophil migration and degranulation via CXCR1 and CXCR2 signaling. Tumor cells and myeloid

cells secrete IL-8 and attract neutrophils into the tumor microenvironment (Nagarsheth *et al.*, 2017).

While traveling toward the source of IL-8, neutrophils release a series of enzymes that are indispensable in remodeling the ECM and neovascularization of the involved tissue. The matrix metalloproteinases (MMPs) and their inhibitors are essential moderators of ECM remodeling. Neutrophils secrete high levels of MMP-9/gelatinase B and release a soluble factor in response to IL-8, which activates latent MMP-2 released by other cells (De Larco *et al.*, 2004). The expression of MMP-9 fosters angiogenesis by releasing angiogenic factors from the extracellular matrix (Bergers *et al.*, 2000).

In addition, neutrophils express a certain sulfatase and heparanase that are essential for releasing ECM growth factors. Such transformation helps neutrophils to disperse and speeds up the mobilization of other immune cells. The modified matrix is less resilient to cells escaping the tumor; thus, it allows tumor cells to be dissociated from the primary tumor mass. The growth factors secreted during this remodeling process, such as basic fibroblast growth factor, will serve as both a chemoattractant and a growth stimulator for these tumor cells (De Larco *et al.*, 2004).

Mast cells (MCs): These cells are best known for their function in allergy and anaphylaxis. They are also important in angiogenesis, immune tolerance, wound healing, defense against pathogens, and blood-brain barrier activity (Polyzoidis *et al.*, 2015). These cells are derived from CD34+, CD117+ (KIT) pluripotent hematopoietic stem cells in the bone marrow (Kirshenbaum *et al.*, 1999). Under physiological conditions, MCs are particularly abundant in the close proximity of capillaries and lymphatic vessels. In inflammatory disorders that are characterized by profound vascular remodeling, the

infiltrate displays numerous MCs that exhibit the structural features of degranulating elements (Crivellato *et al.*, 2009).

In neoplastic diseases, mast cell infiltration has been observed in almost all solid tumors, and in hematological malignancies (Sammarco *et al.*, 2019; Stockmann *et al.*, 2014). For multiple tumor types, there is a strong association between the existence of mast cells, neovascularization, and tumor progression. This phenomenon has been seen in plasmacytoma, breast cancer, colon cancer, and cervical cancer. In experimentally induced tumors, mast cell infiltration takes place before the angiogenic switch and the development of carcinomas from dysplastic cells (Stockmann *et al.*, 2014). The attraction of the MCs to the tumor site depends on the secretion of tumor-cell derived soluble factors. These factors include stem cell factor (SCF), VEGF, platelet-derived endothelial cell growth factor (PD-ECGF), FGF-2, RANTES, monocyte chemotactic protein (MCP)-1, and adenosine (Stockmann *et al.*, 2014).

Thus, in various tumor models, MCs facilitate angiogenesis by releasing pro-angiogenic factors, such as FGF2, VEGF-A, TNF, and CXCL8. Tumor-infiltrating mast cells were also identified as important sources of MMP-9, tryptase, and chymase that activate pro-MMPs and remodel the tumor microenvironment (De Palma *et al.*, 2017; Stockmann *et al.*, 2014).

Eosinophils: These granulocytic cells are specialized in protecting the body from parasites by releasing extremely cationic proteins from their granules and play a vital role in allergies (Varricchi *et al.*, 2018). Eosinophil accumulation has been reported in different kinds of tumors, including nasopharyngeal and oral squamous cell carcinomas, gastrointestinal tract tumors, and lymphomas (Stockmann et al., 2014). Recruitment of

eosinophils into the tumor relies on the extremely selective chemokine CCL11 for this type of cell (De Palma *et al*., 2017). Eosinophils function as an important source for CCL11 within the tumor microenvironment (Stockmann *et al*., 2014).

Regarding their angiogenic function, it has been suggested that IL-5 mediated release of VEGF from eosinophils (De Palma *et al*., 2017) stimulates proliferation and migration of endothelial cells. Furthermore, eosinophil granules comprise a variety of molecules promoting angiogenesis, including b-FGF, IL6, CXCL8, GM-CSF, PDGF, TGF-β (Munitz and Levi-Schaffer, 2004), and MMP-9 (Varricchi *et al.*, 2018). These molecules are released when eosinophils are stimulated by TNF-α and CCL11 (De Palma *et al*., 2017). Eosinophils preferably penetrate the tumor's hypoxic areas; thus, eosinophils degranulation and angiogenic factors secretion in the tumor microenvironment may provide the angiogenic signal to the tumor's hypoxic regions specifically (Stockmann *et al*., 2014).

The tumor microenvironment is extensively infiltrated with naive myeloid cells, such as deactivated dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs). There are two populations of myeloid-derived suppressor cells (MDSCs), monocytic (M-MDSC) and granulocytic (G-MDSC). STAT3 in MDSCs is activated by tumor-derived factors, such as CSF-3, IL-1β, and IL-6. The activation of STAT3 in these cells promotes their expansion, inhibits their maturation into macrophages or neutrophils, and enhances their pro-angiogenic functions in the TME (De Palma *et al*., 2017). MDSCs and macrophages were defined in cancer as both immunosuppressive and pro-angiogenic. Under the impact of VEGF-A and FGF2 derived from myeloid cells, TABV ECs down-regulate expression and abrogate intercellular adhesion molecule 1 (ICAM1) and vascular

cell adhesion molecule 1 (VCAM1), thereby restricting T cell adhesion and extravasation (De Palma *et al*., 2017).

Lymphocytes: B cells and T cells may indirectly regulate tumor angiogenesis by modulating myeloid cell activation (De Palma *et al*., 2017). There is growing evidence that B-cells can directly as well as indirectly increase tumor angiogenesis. B cells can promote angiogenesis in tumors through the use of different pro-angiogenic mediators, including VEGF-A, FGF2, and MMP-9, in STAT3 dependent manner (Yang, C. *et al.*, 2013). Indirectly, B cells can stimulate tumor angiogenesis by polarizing macrophages through the activation of IgG receptors expressed on these cells (Andreu *et al.*, 2010).

T cells, directly and indirectly, can modify angiogenesis in the tumor area. CD4+ T helper 1 (TH1) cells elicited by immunotherapy can suppress the angiogenesis of the tumor by promoting TABV maturation and/or quiescence, directly (Tian *et al.*, 2017). IFNγ involvement is possible in this process. IFNγ restricts EC proliferation and may trigger the regression of immature blood vessels if overexpressed experimentally. In addition, T cells can indirectly affect tumor angiogenesis. CD4+ TH2 cells release IL-4 and activate the STAT6-dependent activation of TAMs. IL-4 activation of TAM confers immunoinhibitory, tissue-remodeling, and pro-angiogenic functions of TAMs. Contrarily, the secretion of IFNγ by CD4+ TH1 cells or CD8+ cytotoxic T lymphocytes (CTLs) can activate TAMs to secrete angiostatic cytokines including CXCL9, CXCL10, and CXCL11. In comparison to CTLs and TH1 cells, the regulatory T (Treg) cells can positively enhance tumor angiogenesis. Treg cells can indirectly foster angiogenesis by inhibiting effector TH1 cells producing IFNγ. In addition, hypoxia-induced CCL28 attracts Treg cells expressing VEGF-A. The depletion of Treg cells reduces VEGF-A and angiogenesis levels

in TME. This indicates that Treg cells can decrease antitumor immunity and also function to stimulate tumor angiogenesis (Motz and Coukos, 2011).

Platelets: These cells have the capacity to produce angiogenesis stimulators and inhibitors which are contained in separate α-granules. The release of the angiogenesis stimulators and inhibitors is promoted by differential activation of protease-activated receptors (PAR)-1 and PAR-4 receptors. The stimulation of PAR-1 causes the secretion of VEGF, and the activation of PAR-4 results in the release of anti-angiogenic endostatin. Pinedo et al. (Pinedo *et al.*, 1998) suggested that platelets were engaged in the angiogenesis process more than 20 years ago. In the early and advanced phases of angiogenesis, platelets were involved in stabilizing newly formed vessels. Platelets stimulate EC proliferation. This effect relies on platelet adherence to the ECs via their surface adhesion molecules. The activated platelets stimulate expression of the tissue factor on ECs to induce coagulation by the interactions between CD154 present on platelet and ECs CD40. CD40 ligation promotes the adhesion molecules expression, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), that increase the adhesion of cells to the ECs (Wojtukiewicz *et al.*, 2017).

Platelets are important reservoir and delivery means for pro-angiogenic and anti-angiogenic growth factors, cytokines, chemokines, and bioactive lipids. It was documented that platelets are necessary for bone marrow-derived cells (BMDCs) recruitment into ischemia-induced vasculature. Platelets were shown to induce BMDC homing in a VAMP-8-dependent manner. For BMDC homing and subsequent angiogenesis, the secretion of platelet α-granules is essential (Wojtukiewicz *et al.*, 2017). Surprisingly, human platelets carry cytokines secreted by luminal breast cancer cells and transmit them to indolent

metastatic tumor foci, enhancing tumor progression by activating the production of new blood vessels (Wojtukiewicz *et al.*, 2017).

Platelet-derived microparticles (PMPs) and microRNAs (miRNAs) have crucial functions in promoting angiogenesis. Platelet-derived microparticles stimulate *in vitro* and *in vivo* new blood vessels formation to a degree similar to that of whole platelets. Moreover, PMPs increase vascular permeability. Platelets release PMPs upon activation. The membranes of these PMPs are loaded with tissue factor and have negatively charged surfaces where clotting factors can be assembled. The assembly of clotting factor complexes significantly increases blood coagulation and tumor angiogenesis. PMPs can present and transfer active receptors from platelet membranes to various types of cells, thereby promoting the engraftment of hematopoietic stem/progenitor cells. In addition, PMPs have the capacity to intracellularly transmit angiogenic factors. Plus, they can activate pro-angiogenic genes via direct interaction with endothelial cells (ECs), or through fusion with tumor cells or ECs (Wojtukiewicz *et al.*, 2017). PMPs are the circulation's most concentrated microvesicles. They enclose a significant amount of miRNAs that control angiogenesis, including miR-19, miR-21, miR-126, miR-133, miR-146, and miR-223 (Wojtukiewicz *et al.*, 2017). These findings demonstrate the positive effect of the platelets on tumor blood vessel formation.

1.6 The extracellular matrix

The ECM is a complex system of fibrous proteins, glycoasaminoglycans, and matricellular proteins. ECM gives structural assistance and biochemical and biomechanical signals for the growth of tumor cells. The basement membrane (BM) is a specialized ECM that is formed by vascular ECs and mural cells. The basement membrane maintains the integrity and function of the blood vessels. Sprouting angiogenesis encompasses breakdown of the basement membrane by MMPs produced by activated ECs and myeloid cells recruited to the tumor area. The temporary ECM supports the proliferation and migration of ECs. The intact and mature basement membrane contributes to EC quiescence and vascularity in non-pathological angiogenesis. Sustained pro-angiogenic signaling in tumors has a detrimental effect on successive stages of vascular development, especially the formation of a quiescent endothelial cell phenotype and the formation of an unimpaired and selectively permeable vascular barrier.

Tumor blood vessels have many structural abnormalities. Commonly, the vascular endothelial cells and the pericytes are usually loosely connected to the discontinuous vascular basement membrane. These abnormalities result in the advancement of vascular leakiness and the enhancement of cancer cell intravasation and metastasis. The ECM possesses pro-angiogenic and vascular-stabilizing functions. It acts as an arsenal for several angiogenesis stimulating growth factors, including VEGF-A, FGFs, PDGF-B, and TGF-β. The proteolytic processing of the ECM by plasmin and proteases such as MMPs releases pro-angiogenic growth factors in their bioactive forms. The breakdown of the ECM can produce chemoattractants, which will attract cells that have tumor growth-enhancing effects. On the opposite, there may be angiostatic features of some ECM proteins, such as

THBS1, osteonectin, and proteoglycan decorine. Continuous ECM remodeling in tumors can produce Collagen Type IV and XVIII fragments that restrict the blood vessels formation through the competition with intact collagen fibers over communication with ECs integrins. The aberrant mechanical and biophysical features of tumor ECM affect tumor blood vessel formation in direct and indirect ways. The aberrant design of tumor ECM fibers enhances tumor blood vessel formation by promoting the immigration of ECs and TAMs, which occurs extra quickly on linearized collagen fibers that are abundant in tumors compared to normal and healthy tissues.

As mentioned above, the tumor microenvironment (TME) encompasses various types of cells, extracellular matrix (ECM), proteolytic enzymes and their inhibitors, growth factors, and signaling molecules (Quail and Joyce, 2013). The basic function of TME is to dynamically interact with malignant cells, which influences tumor growth, metastasis, and, ultimately, prognosis. Malignant cell interactions with the adjacent tumor microenvironment are dependent upon complex systemic pathways. In addition to direct cell-to-cell interaction, released factors exert a critical function in intercellular communication. Exosomes are the key elements of extracellular vesicles (EVs), ranging in size from 30-150 nm (Jeppesen *et al.*, 2019; Kalluri, 2016).

1.7 Extracellular vesicles (EVs)

EVs are membrane-bound vesicles derived from the cells. EVs are composed of a lipid bilayer membrane and a tiny amount of cytosol free from organelles. Extracellular vesicles encompass three main forms initially categorized based on their size and method of production: exosomes, apoptotic bodies, and microvesicles. In tumors, exosomes and microvesicles are commonly active in the regulation of a suitable microenvironment for

cancer cell growth and metastasis. Exosomes are smaller than microvesicles. The accumulated information indicates that for cancer development and metastasis, exosomes are more biologically essential (Kikuchi *et al.*, 2019). After fusion of the multivesicular body (MVB) with the plasma membrane, exosomes are released from the cells. This releases intraluminal vesicles (ILVs) into the extracellular environment. What we know as exosomes are the intraluminal vesicles (ILVs) (Figure 7) (Edgar, 2016; Jeppesen *et al.*, 2019).

Intraluminal vesicles (ILV) (and thus exosomes) are formed by invagination of the endosomal membrane by either ESCRT-dependent (ESCRT stands for endosomal sorting complexes required for transport) or ESCRT-independent mechanisms (Edgar, 2016; Jeppesen *et al.*, 2019). The ESCRT system comprises of a series of cytosolic protein complexes attracted to the endosome by membrane proteins marked with ubiquitin on their cytosolic regions. The ubiquitin label is identified by ESCRT-0, the first of the ESCRT complexes. Afterward, ESCRT-0 is attracted to the endosomal membrane. Next, Tsg101 component of ESCRT-I recognizes ubiquitin tag on ESCRT-0 which results in the transfer of the ubiquitinated cargo to ESCRT-I. The attraction of the ESCRT machinery functions both to assemble the ubiquitinated cargo proteins on the endosome and to stimulate the incurvation of the endosomal membrane to create ILVs (Figure 8). Nonetheless, even in the absence of ESCRTs, ILVs are still generated which indicates the existence of other means of producing ILVs (Edgar, 2016).

Mature endosomes gather ILVs within their lumen. MVBs have distinct outcomes, may fuse with lysosomes, or may fuse with the plasma membrane where released ILVs are now called ‘exosomes’ (Edgar, 2016). Some studies show that some molecules act as a

regulatory network and is responsible for the formation and secretion of exosomes. For example, it was reported that Rab27a and Rab27b were associated with exosome secretion. They target multivesicular endosomes (MVEs) to the plasma membrane. The size of MVEs was significantly affected by Rab27a and Rab27b silencing. Knocking down of Rab27 or its effectors SYTL4 and EXPH5 inhibits the secretion of exosomes in HeLa cells (Sun, Z. *et al.*, 2018).

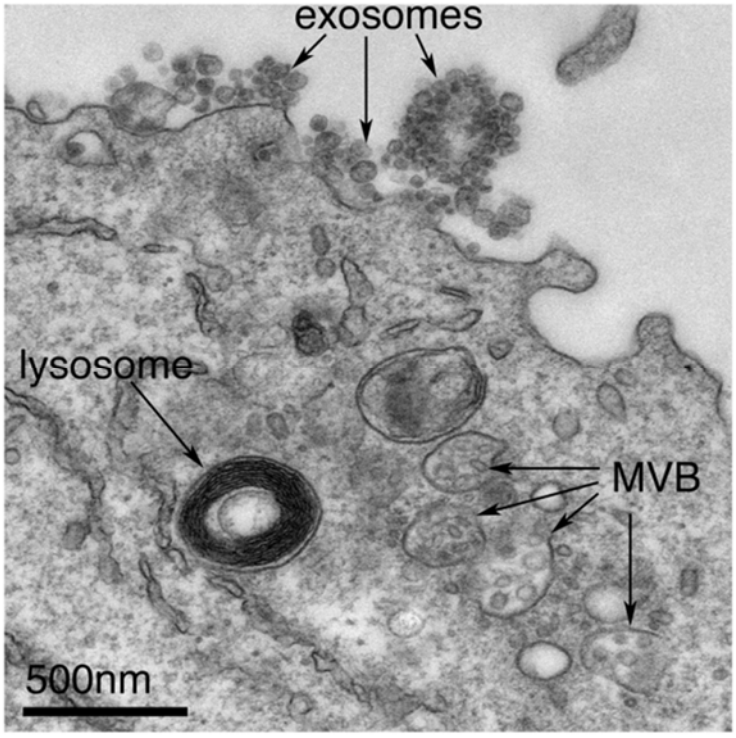


Figure 7. Exosomes are intraluminal vesicles of multivesicular bodies

Adapted from (Edgar, 2016).

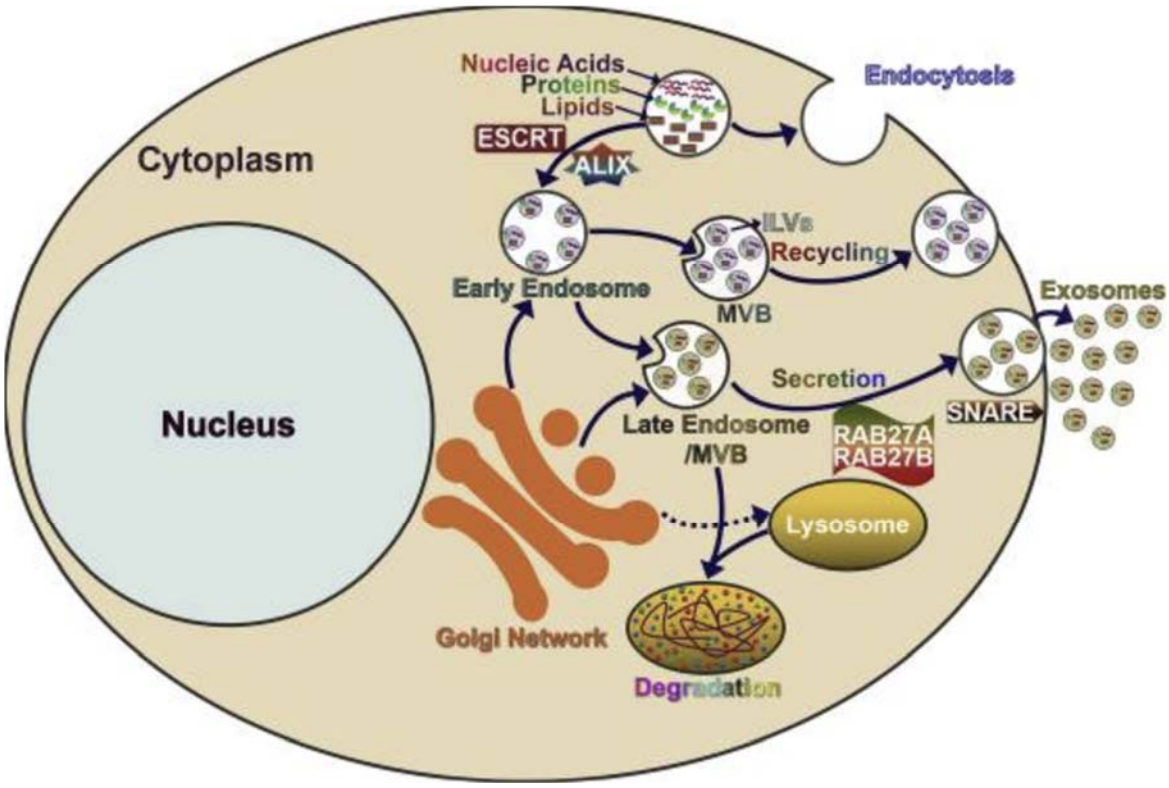


Figure 8. Exosome biogenesis

Adapted from (Wang *et al.*, 2019)

Furthermore, both the tumor repressor protein p53 and its downstream effector TSAP6 increase exosome production. Moreover, syndecan heparan sulfate proteoglycans and their cytoplasmic adaptor syntenin-1 regulate the formation of exosomes (Sun, Z. *et al.*, 2018). Syntenin-1 interacts directly with ALIX14 through three Leu-Tyr-Pro-X(n)-Leu (LYPXnL) motifs located in its N-terminus and with the conserved cytoplasmic domains of the syndecans, via its PDZ domains. ALIX14 is a component of the ESCRT machinery. Because ALIX bonds various ESCRT proteins, syntenin-ALIX adjusts syndecans and syndecan cargo to the MVB's ESCRT budding machinery. For this process of exosome production, heparan sulfate is required. Syndecan cargo, attached to syndecan's heparan sulfate, is thought to group syndecans and create syndecan clusters that attract syntenin-1 and ALIX, and then cause the membrane to bud. Such a mechanism enables heparan sulfate-binding cargo, like FGFR1, to be recruited, and can decide which element is directed to exosomes. These studies indicate that a group of molecules is crucial for the formation and secretion of exosomes in the cells (Roucourt *et al.*, 2015). The levels of cholesterol on MVBs also seem to play a part in regulating their destiny. Cholesterol-rich MVBs are usually targeted to the plasma membrane for exosome release, whereas cholesterol-poor MVBs are directed to the lysosome (Edgar, 2016).

Exosomes are crucial elements for intercellular communication. They have various physiological functions, such as maintaining cellular homeostasis via the release of harmful intracellular components and activating immune responses via the delivery of antigens or activating ligands. Emerging evidence indicates the critical role of exosomes in tumor development. Exosomes can be transported between tumor cells or between tumor cells and the surrounding microenvironment. Cancer-associated exosomes can encourage tumor

growth and expansion. They help establish a tumor-promoting niche by enhancing angiogenesis, renovating the extracellular matrix, and inhibiting the function of immune cells. Moreover, exosomes can convey drug-resistance properties among tumor cells (Wang *et al.*, 2019).

In order to induce a response from recipient cells, exosomes might either fuse with the plasma membrane, be taken up entirely via endocytosis, or may attach to the surface of recipient cells to stimulate a signaling response. Follicular dendritic cells carry on their cell surface exosomes that carry MHC–peptide complexes and other proteins that they do not express and are thus able to activate immune cells with which they engage (Edgar, 2016).

A great variety of molecules are incorporated into exosomes, including protein, lipids, mRNA, and microRNA, and can be transferred into acceptor cells (Edgar, 2016; Pegtel and Gould, 2019). Exosomal proteins encompass a wide array of transmembrane proteins, lipid-anchored membrane proteins, peripherally associated membrane proteins, and soluble proteins of exosome lumen (Pegtel and Gould, 2019) CD81, CD63, CD37, CD82 are tetraspanin protiens that are highly enriched in exosomes but not in the generic plasma and lysosomal membranes. CD81, CD63, and CD9 are among the most commonly used exosomal marker proteins. Tetraspanin-associated partner proteins in exosomes, including major histocompatibility complex II proteins, immunoglobulin superfamily member 8 (IGSF8), ICAM-1, syndecans, and integrins, support the tetraspanins in mediating the exosomal inclusion of other proteins (Pegtel and Gould, 2019). Exosomes also carry an array of lipid-anchored proteins including ectonucleotidases CD39 and CD73, the complement-inhibiting proteins CD55 and CD59, glypican-1, and and both the cellular prion protein (PrPC) and its amyloidogenic conformer, PrPSC. The exosomal surface is

also rich in proteins that are involved in signaling. The inner membrane of the exosomes carries acylated, lipid-anchored proteins including small GTPases (Rabs, Ras, Rho, etc.), myristoylated signaling kinases (for example, Src), and palmitoylated membrane proteins (Pegtel and Gould, 2019). Exosomes are heterogeneous in composition and among the bioactive molecules incorporated in the exosomes are the miRNAs. miRNAs have attracted great attention due to their regulatory function in gene expression.

MicroRNAs (miRNAs), a class of short non-coding RNAs (ncRNAs) of approximately 19–24 nt in length, regulate the expression of target genes at the post-transcriptional level, necessarily via binding to fully or partially complementary sites within the 3′ UTR of target mRNAs. miRNAs participate in a variety of cellular processes, including cell proliferation, differentiation, and death. Specifically, miRNAs have been found to have an essential role in the onset and progression of cancer, such as tumor growth, invasion, and metastasis. miRNAs are crucial exosomal constituents, and exosomal miRNAs are confirmed to involve in cancer occurrence and development (Wang *et al.*, 2019).

1.8 Biogenesis of miRNAs

miRNA biogenesis begins with the RNA polymerase II/III transcripts processing post- or co-transcriptionally. Approximately half of all known miRNAs are intragenic. They are mainly generated from introns and very few protein-coding exons. The rest are intergenic, transcribed separately from a host gene, and controlled by their own promoters. Occasionally, MiRNAs are transcribed as single lengthy transcript known as clusters. They possibly possess identical seed regions; therefore, considered as a family. The biogenesis of miRNA is classified into two pathways canonical and noncanonical (Figure 9).

The canonical pathway is the central pathway by which miRNAs are processed. In this pathway, pri-miRNAs are transcribed from their genes and then processed into pre-miRNAs by the microprocessor complex. This microprocessor complex consists of an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha (Denli *et al.*, 2004). DGCR8 identifies an N6-methyladenylated GGAC and other motifs within the pri-miRNA (Alarcón *et al.*, 2015). Drosha cuts the pri-miRNA duplex at the base of the characteristic hairpin structure of pri-miRNA. This results in the formation of a 2 nt 3′ overhang on pre-miRNA (Han *et al.*, 2004). Once pre-miRNAs are generated, they are exported to the cytoplasm by an exportin5/RanGTP complex. Pre-miRNAs are then processed by the RNase III endonuclease Dicer resulting in a mature miRNA duplex (Zhang, H. *et al.*, 2004).

The 5p strand originates from the 5′ end of the pre-miRNA hairpin, while the 3p strand arises from the 3′ end. Both 5p and 3p strands originating from the mature miRNA duplex can be loaded in an ATP-dependent manner to the Argonaute (AGO) protein family (AGO1-4 in humans). The strand with lower 5′ stability or 5′uracil is named the guide strand and is usually preferentially carried onto AGO. The unloaded strand is named the passenger strand and is loosened from the guide strand according to the degree of complementarity (Ha and Kim, 2014).

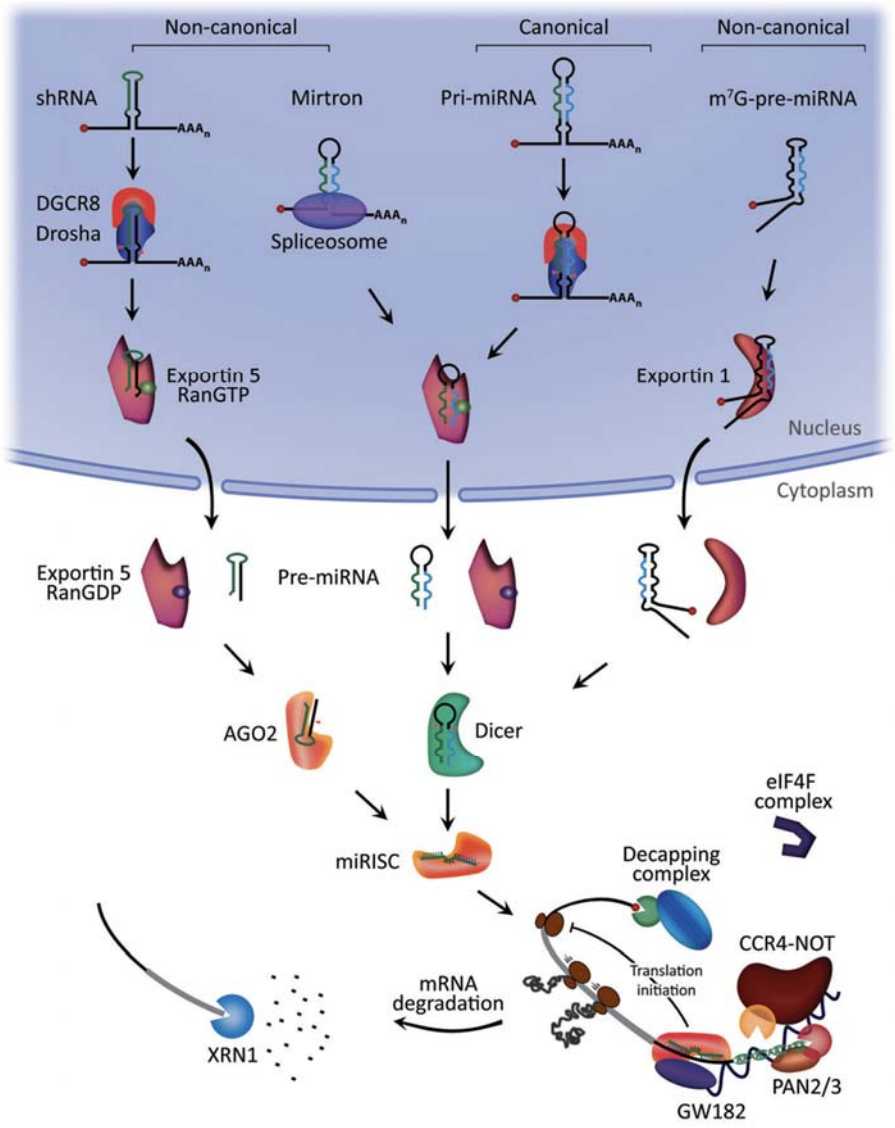


Figure 9. miRNA biogenesis

Adapted from (O'Brien *et al.*, 2018).

Non-canonical pathways use different assortments of the proteins involved in the canonical pathway, mainly Drosha, Dicer, exportin 5, and AGO2. Generally, the non-canonical miRNA biogenesis can be classified into Drosha/DGCR8-independent and Dicer-independent pathways. 7-methylguanosine (m7G)-capped and mirtrons are examples of pre-miRNAs produced by the Drosha/DGCR8- independent pathway that resembles Dicer substrates. Using exportin 1, these RNAs are transported directly to the cytoplasm without requiring Drosha cleavage. Most likely there is a strong 3p strand preference because of the m7 G cap which blocks 5p strand loading into Argonaute (Xie *et al.*, 2013). On the other side, Dicer-independent pathways use endogenous short hairpin RNA (shRNA) transcripts, which are processed by Drosha. AGO2 is needed to complete the maturation of these pre-miRNAs within the cytoplasm because they are not long enough to be Dicer-substrates (Yang, J. *et al.*, 2010). This induces the loading of the entire pre-miRNA into AGO2 and AGO2-dependent slicing of the 3p strand. The 3′ -5′ trimming of the 5p strand causes their maturation (Cheloufi *et al.*, 2010).

The guide strand and AGO form the miRNA-induced silencing complex (miRISC). miRISC interacts with complementary sequences on target mRNA called miRNA response elements (MREs) and fully complementary miRNA: MRE interaction elicits AGO2 endonuclease activity and targets mRNA cleavage (Jo *et al.*, 2015). However, the majority of miRNA: MRE interactions are not fully complementary due to central mismatches in the MRE to their guide miRNA preventing AGO2 endonuclease activity. Therefore, AGO2 will function as a mediator of RNA interference (O'Brien *et al.*, 2018).

The formation of a silencing miRISC complex starts with the recruitment of the GW182 family of proteins by miRISC following miRNA:target mRNA interaction.

GW182 gives the scaffolding required to recruit other effector proteins (Behm-Ansmant *et al.*, 2006), such as the poly(A)-deadenylase complexes PAN2-PAN3 and CCR4-NOT (O'Brien *et al.*, 2018). Target mRNA poly(A)-deadenylation is started by PAN2/3 and completed by the CCR4-NOT complex. Effective deadenylation is achieved via the association of tryptophan (W)-repeats of GW182 and poly(A)-binding protein C (PABPC) (Jonas and Izaurralde, 2015). Once the deadenylation is accomplished, decapping ensues promoted by decapping protein 2 (DCP2) and related proteins (Behm-Ansmant *et al.*, 2006). Finally, RNA is degraded by the exoribonuclease 1 enzyme activity (O'Brien *et al.*, 2018).

Most studies found that miRNAs bind to a specific sequence at the 3′ UTR of their target mRNAs. This binding results in translational repression and mRNA deadenylation and decapping. However, miRNA binding to other mRNA regions, including 5′ UTR, coding sequence, and within promoter regions, has also been detected. The binding of miRNAs to 5′ UTR and coding regions has been reported to have silencing effects on gene expression. On the other hand, miRNA interaction with the promoter region induces transcription (O'Brien *et al.*, 2018). AGO2 and FXR1 are involved in the miRNA-mediated activation of translation instead of GW182. Certain examples of miRNAs gene activation include binding to the 5′ UTR of mRNAs coding ribosomal proteins during amino acid exhaustion; therefore, it suggests that miRNA-mediated gene expression upregulation happens under particular circumstances (O'Brien *et al.*, 2018).

It is established now that miRNAs play crucial roles in different cancer biological processes, including tumor growth, metastasis, angiogenesis, and drug resistance. MiRNAs can alter the expression of pro- angiogenic or anti-angiogenic factors in tumor cells,

thereby modulating the proliferation and migration of endothelial cells in a paracrine fashion. Some miRNAs have anti-angiogenic and anti-metastatic effects, and others are pro-angiogenic and pro-metastatic. Some of these anti-angiogenic miRNAs target VEFG signaling. MiR-29b, miR-93, MiR-20, miR-126, miR-195, miR-200, miR-190, miR-203, miR-497, miR-638, and miR-503 specifically target the 3'UTR section of VEGF-A mRNA in various cancer types leading to inhibition of VEGF/VEGFR-2 pro-angiogenic signaling in ECs (Lou *et al.*, 2017).

Hypoxia-inducible factor (HIF) is a transcriptional factor. It responds to low oxygen levels; therefore, it is crucial for the blood vessels formation in cancer. Some miRNAs regulate VEGF-dependent tumor angiogenesis by targeting the HIF pathway. miRNA-519c decreases endothelial cell angiogenic activity and suppresses angiogenesis and metastasis by lowering levels of HIF-1α. miRNA-107 suppresses the expression of HIF-1β, thereby inhibiting cancer angiogenesis, growth, and VEGF expression (Lou *et al.*, 2017).

Angiopoietin-2, a member of angiopoietins, is mainly produced by ECs. This promotes angiogenesis caused by VEGF in several cancers. Thus, inhibiting the angiopoietin-2-related pathway may inhibit cancer angiogenesis and metastasis. Through binding directly to its 3′UTR, miRNA-542-3p inhibits angiopoietin-2. In addition, miRNA-542-3p is a promising prognostic marker for tracking breast cancer development, as its expression correlates negatively with clinical progress (Lou *et al.*, 2017).

Matrix metalloproteinases (MMPs) are calcium-dependent endopeptidases that are important for cancer angiogenesis and metastasis-related tissue remodeling. miRNA-9 induction inhibited MMP-14 levels, leading to reduced angiogenesis, invasion, and

metastasis of both *in vitro* and *in vivo* neuroblastoma cells. miRNA-181-5p directly targeted MMP-14 in breast cancer cells, which resulted in attenuating breast cancer cell migration, invasion, and angiogenesis. Furthermore, miRNA-199a-3p attenuated HCC growth, invasion, migration, and angiogenesis by partially targeting MMP2 (Lou *et al.*, 2017).

Interleukin 6 (IL-6), a pro-inflammatory cytokine, exerts a crucial role in cancer metastasis by inhibiting the expression of E-cadherin. It promotes metastasis in breast cancer; therefore, high levels of IL-6 in breast cancer is indicative of poor prognosis. miRNA-26a suppressed *in vitro* HCC cell invasion, migration, and *in vivo* metastasis by downregulating IL-6 (Lou *et al.*, 2017).

Several miRNAs have been reported to promote angiogenesis. miR-21 and miR-210 are induced by hypoxia. MiR-21induces the tube-forming capacity of primary bovine retinal microvascular endothelial cells (RMECs). Moreover, it enhances tumor angiogenesis by enhancing the expression of VEGF. MiR-210 supports angiogenesis and the maturation of vasculature in post-ischemic brain tissue via increasing the expression of Notch, VEGF, and vascular endothelial growth factor receptor-2 (VEGFR-2) in HUVECs. miR-210 establishes angiogenesis by negatively regulating the target gene, ephrin A3, which is an important member of the ephrin angiogenesis regulatory gene family (Sun, L. L. *et al.*, 2018).

There has been substantial evidence of the participation of miRNAs in angiogenesis and metastasis of cancer. Therefore, the use of miRNA to block angiogenesis may represent an effective therapeutic strategy for metastatic cancer. Anti-angiogenesis medications have played a major role in the treatment of a number of metastatic cancers, such as metastatic

renal cell carcinoma. Nonetheless, due to adverse effects such as bleeding and resistance to anti-angiogenic treatment, the results are unsatisfactory. Therefore, new anti-angiogenic therapy alternatives are needed. However, tumors have different ways to develop vasculature. Among these ways are the **angiogenesis**, the sprouting of endothelial cells from neighboring blood vessels, and the **vasculogenesis**, the formation of blood vessels from progenitor cells. Angiogenesis was previously generally thought to be the only process involved in the creation of new blood vessels in postnatal life. This conventional notion of the development of blood vessels has been challenged after the discovery of the endothelial progenitor cells (EPCs), which can be recruited from the bone marrow (BM) to the site of the injured endothelium where they differentiate into mature ECs.

1.9 Endothelial progenitor cells (EPCs)

EPCs successfully migrate to the tumor site and integrate in the neovasculature with high specificity (Nolan *et al.*, 2007). Therefore, EPCs can be vital elements to tumor growth and metastasis. EPCs have become a significant target for anti-neoplastic therapies in a range of neoplastic diseases (Zhao, X. *et al.*, 2016). While EPCs' contribution to the formation of new vessels has been identified previously, the underlying mechanisms remain unclear and require further research. This can be a crucial target for therapeutic intervention by blocking the mechanism and signal pathways involved in tumor neo-vessel formation mediated by EPC.

Properties of EPCs

EPCs are defined as a population of BM-derived immature cells that circulate in the peripheral circulation and have the ability to home to the sites of damaged vessels to engage in the formation of new blood vessels under physio-pathological conditions

(Asahara *et al.*, 1997; Zhao, X. *et al.*, 2016). The biomarkers initially used to characterize EPCs include the hematopoietic stem cell marker (CD)34 and the EC marker vascular endothelial growth factor receptor-2 (VEGFR-2). However, these markers are also present on hematopoietic progenitor cells and mature ECs. Recently, multiple markers are used to characterize the EPCs. These biomarkers include CD34, CD133, CD31, VEGFR-2, von Willebrand factor (vWF), Tie2, CD117, CD62E and CD45 (Zhao, X. *et al.*, 2016). The CD133 protein is expressed on immature stem cells, but not on mature ECs. Therefore, it is considered as a more appropriate marker for immature progenitor cells (Peichev *et al.*, 2000; Zhao, X. *et al.*, 2016). CD133+/VEGFR-2+ cells represent immature progenitor cells located mainly in the BM, whereas CD133−/CD34+/VEGFR-2+ cells reflect more mature cells that have limited proliferative capacity (Khakoo and Finkel, 2005; Zhao, X. *et al.*, 2016). Based on the characters of the culture, early and late EPCs are two main types of EPCs that have been identified. Early EPCs appear after short-term culture of peripheral blood. These cells are similar to the colony-forming unit ECs and express CD31, CD133, CD34, and VEGFR-2 (Janic and Arbab, 2010). On the other hand, late EPCs or endothelial colony forming cells appear after long-term culture of mononuclear cells and express VE-Cadherin, vWF, CD31, CD133, CD34, and VEGFR-2 (Janic and Arbab, 2010; Zhao, X. *et al.*, 2016).

Both early and late EPCs participate in postnatal angiogenesis and vasculogenesis (Zhao, X. *et al.*, 2016). Therefore, EPCs have been considered as very crucial targets in the treatment of solid tumors.

1.10 Tumor vascularization

The first step in EPC-mediated vasculogenesis is the mobilization of EPCs from the BM into circulation. Under physiological circumstances, the number of circulating EPCs is very low (Zhao, X. *et al.*, 2016) and most cells stay in the BM through the interaction between the integrins on EPCs and the stromal cells (Lapidot *et al.*, 2005; Zhao, X. *et al.*, 2016). Tumor vasculogenesis necessitates crosstalk between cancer cells and BM-located EPCs to induce EPCs to travel into the circulation, home to tumor areas and invade the growing tumors (Ferrara and Alitalo, 1999; Zhao, X. *et al.*, 2016).

The recruitment process involves many steps that are controlled by a broad range of cytokines and chemokines. VEGF has been well documented to play a key role in the mobilization of VEGFR-2+ EPCs from the BM. VEGF is secreted in the tumor at high levels, and the high levels of VEGF cause tumor vasculogenesis and expansion by releasing BM-resident EPCs into the peripheral circulation. This process encompasses a variety of enzymes and cytokines. VEGF induces BM nitric oxide (NO) synthase and stimulates NO production. Stem cell-active soluble kit ligand is released upon the interaction of NO with matrix metalloproteinase-9. Stem cell-active soluble kit ligand supports VEGFR-2+ EPC mobility and induces mobilization of the cells from the BM into the peripheral circulation (Heissig *et al.*, 2002). VEGF can increase the levels of G-CSF. G-CSF has a crucial role in EPC mobilization (Lévesque *et al.*, 2001; Zhao, X. *et al.*, 2016).

The role of stromal derived factor-1 (SDF-1) in EPC mobilization has been studied extensively. The growth of the tumor results in tissue hypoxia. Upon tissue hypoxia, hypoxia-inducible factor-1 (HIF-1) is upregulated. High levels of hypoxia-inducible factor-1 (HIF-1), increases the levels of VEGF and SDF-1, and induces the mobilization and

recruitment of EPCs from the BM into the peripheral circulation (Zhao, X. *et al.*, 2016). Tumors can also release CCL2 and CCL5. Both participate in EPC mobilization. In addition, cells in the tumor microenvironment produce other factors to mobilize and recruit EPCs to the tumor site. Adiponectin is a peptide hormone secreted by adipocytes. It promotes EPC mobilization and breast tumor expansion (Zhao, X. *et al.*, 2016). In our laboratory, it was found that 17β-estradiol directs the recruitment of the bone marrow-derived endothelial progenitor cells (BM-EPC) toward the tumor sites. Moreover, 17β-estradiol was found to intensify the pro-vasculogenic capacity of the BM-EPC and their release of the angiogenic growth factors within the tumor microenvironment (TME) (Suriano *et al.*, 2008). These findings emphasize the significance of estrogen in estrogen receptor-positive breast cancers as well as in estrogen receptor-negative breast cancers.

The adhesion of EPCs necessitates the expression of molecular targets by EPCs and by the vascular tissues they move to. P selectin glycoprotein ligand-1 (PSGL-1) is a major ligand of P-selectin and E-selectin expressed on ECs. PSGL-1 is expressed on EPCs and plays a major role in cell adhesion. The binding of PSGL-1 to P-selectin and E-selectin induces trans-endothelial migration of EPCs into the blood vessel wall where vascular repair is needed (Di Santo *et al.*, 2008). Moreover, the EPCs adhesion to the endothelial cells is facilitated by integrins. The interaction of integrins with ICAM-1and fibrinogen causes the adherence of the EPCs to active angiogenic areas and mediates the trans-endothelial cell migration (Chavakis *et al.*, 2005; Qin *et al.*, 2006; Zhao, X. *et al.*, 2016). At the tumor site, EPCs participate in the process of new vessel formation. Early EPCs induce angiogenesis by secreting growth factors and cytokines. However, late EPCs

provide structural support via differentiation into mature ECs (Hur *et al.*, 2004; Zhao, X. *et al.*, 2016).

Angiogenesis is a crucial element in metastasizing cancer. Blocking angiogenesis as a therapeutic approach for metastatic cancers has been examined. While anti-angiogenesis drugs played a primary role in the treatment of a number of metastatic cancers such as metastatic renal cell carcinoma, the breast cancer results were unsatisfactory. Therefore, novel alternatives are necessary. A multi-targeted approach might exert increased antitumor efficacy. Given the importance of EPCs and estrogen to new blood vessel formation around the tumor, and the irreplaceable role of miRNAs in blood vessel formation by regulating the proliferation, differentiation, apoptosis, migration and tubule formation of angiogenesis-related cells, and the indispensable role of exosomes in facilitating effective intercellular communication, this study was designed to understand the cellular and molecular mechanisms by which EPCs contribute to tumor growth which might provide a potentially effective therapeutic target for cancer treatment.

2 CHAPTER II: HYPOTHESIS AND SPECIFIC AIMS

Based on previous studies conducted in our lab (Suriano *et al.*, 2008), it was found that EPCs are attracted from the bone marrow to the site of the tumor under the effect of estrogen. These findings indicated the estrogen responsiveness of the EPCs. Moreover, it has been found that EPCs are present in high numbers in the TME of TNBC. TNBC is an aggressive subtype of breast cancer. It lacks the expression of ER, PR, and HER2. Currently, it is treated with chemotherapy only, which is effective only in 20% of the TNBC patients. No targeted therapies are available for the treatment of those patients. The disease affects young women and is characterized by poor prognosis and early metastasis. Once metastasis occurs, the overall survival is less than 18 months. The fact that EPCs are attracted to the TME under the effect of estrogen may open the venue for the use of anti-estrogen treatment for TNBC patients.

Given the important functions of EPCs, we hypothesize that **Precursor progenitor cells of angiogenesis, endothelial progenitor cells (EPCs), interact with breast cancer cells and modulate their phenotype.**

Based on our hypothesis, our aims in this study are:

- **Specific Aim: Characterize endothelial progenitor cells (EPCs) from human umbilical cord blood and validate their estrogen responsiveness**
- **Specific Aim 2: Evaluate the effect of an anti-estrogen on EPCs**
- **Specific Aim 3: Evaluate the interaction of EPCs-Breast cancer cells and identify the putative mediators**

3 CHAPTER III: MATERIALS AND METHODS

3.1 Cell culture

HUVECs (human umbilical vein endothelial cells) were obtained from Lonza and grown in EGM-2 medium (Lonza) supplemented with EGM-2 SingleQouts (Lonza). Cells were shifted to phenol red free EBM (Lonza) supplemented with EGM-2 SingleQouts (Lonza) 24 hours (hours) before any experiment.

MCF-7 and MDA-MB-231 (human breast cancer cell lines) were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% fetal bovine serum (FBS) (BioTC), penicillin 10,000 IU/ml, streptomycin 10,000 µg/ml (Corning) and 2 mM L-glutamine (Corning).

3.2 Isolation and *in vitro* culture of EPCs

Progenitor cells positive for $CD133^{+}$marker ($AC133^{+}$) were isolated from the human umbilical cord blood obtained as pathological discards from clinical neonatal units at Phelp Memorial hospital through a collaboration with Dr. Austin Guo. The cord blood mononuclear cell (MNC) population was separated by centrifugation through a histopaque-10771 density gradient (Sigma) according to the manufacturer's protocol. CD133-positive mononuclear cells were isolated by means of magnetic beads coated with antibody to CD133 using the MidiMACS system (MiltenyiBiotec) similar to the protocol of the supplier. In Stemline II media (Sigma), newly isolated CD133 + cells were supplied with 40 ng / ml of FLT3, 40 ng / ml of stem cell factor (SCF), and 10 ng / ml of thrombopoietin (TPO) (all from Pepro Tech). The cells were cultivated as a suspension culture and kept in a concentration between 5×10^{5}-1×10^{6} cells/ml. The cells were monitored daily for the

increase in cell numbers by inverted phase-contrast microscope. Trypan blue exclusion assay was utilized to calculate the number of live cells. Fresh media was added to the cells after the cell numbers are determined. Cells always kept in a concentration between 5×10^5-1×10^6 cells/ml. Cells were used for experiments between day 7-14 of primary culture.

3.3 Cell cultures and co-cultivation

EPCs cells and the breast cancer cell lines, MDA-MB-231 and MCF-7, were co-cultured in 24-well Boyden chambers with 0.4 μM inserts. Breast cancer cells were seeded in the upper chambers (0.4 μM inserts), which are permeable to supernatant but not to cellular components**.** EPCs were seeded on the lower chamber. EPCs were cultured in stem cell expansion media and breast cancer cells were cultured in starvation media.

3.4 Flow cytometry

Cells cultivated in suspension culture for expansion or cells cultivated under differentiation conditions were collected, washed in ice-cold 1x PBS, and incubated with the corresponding fluorescently labeled antibodies in the dark for 10 minutes on ice. A minimum of 10,000 events were analyzed for each sample using flow cytometer of MACSQuant (Miltenyi Biotec). Live cells used for the study were gated depending on the characteristics of forward angle light scatter (FSC) and side angle light scatters (SSC) and analyzed by using the MACSQuantify program 2.8 (Miltenyi Biotec). Specific antibodies that were used in flow cytometric experiments to analyze the expression of cell surface markers were: mouse anti-human CD133 IgG1, mouse anti-human CD34 IgG2a, and mouse anti-human VEGFR2 IgG1 (all from Miltenyi Biotec). The recommended antibody

dilution (for the three antibodies used) for labeling of cells and subsequent analysis by flow cytometry is 1:50 for up to 10^6 cells/100 µL.

3.5 Immunofluorescence analysis

EPCs were seeded in duplicates in 8-well chamber slides (Falcon) at $15x10^3$ per well and allowed to differentiate for 2 weeks. After 2 weeks, cells were washed with PBS (1x) 2-3 times, followed by permeabilization by 0.2% triton X-100 in 1X PBS for 10 minutes at room temperature (RT). Cells were washed 2-3 times with 1X PBS and then blocked (0.1% Triton x-100, 10% goat serum, 1% BSA) for 30 minutes at RT. Blocking buffer was then removed and primary antibodies were added to cells at a concentration of 1:250 in 1% BSA/PBS and incubated overnight at 4°C. Cells were washed 2-3 times with 1X PBS and then incubated with fluorescent secondary antibodies in 1% BSA/PBS at a concentration of 1:250 in the dark for 45 minutes at RT. Cells were washed 2-3 times with 1X PBS, and then DAPI with vectashield was added for 3 minutes followed by sealing the slide. Micrographs were taken using Axiovision Rel 4.8 on an Axiovert 200M microscope at 20x and 100x magnification.

3.6 Incorporation of DiI-Ac-LDL

As mentioned, cells (EPCs) were differentiated for two weeks at day 7 of the *in vitro* primary culture. After differentiation, 10 mg / ml of acetylated low-density lipoprotein, labeled with 1,19-dioctadecyl–3,3,39,39-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, Inc.) was incubated with the cells. Cells were washed with medium, fixed in 3% paraformaldehyde, and examined by fluorescent microscopy after 4 hours of incubation at 37°C and 5% CO2.

3.7 Tubule formation

Using 24-well cell culture plates (BD-Discovery Labware), wells were coated with phenol red-free growth factor reduced Matrigel matrix (Corning), and plates were allowed to solidify at 37°C for 1 hr. Cells were trypsinized, and 5 x 10^4 cells were added per well in 500 µl of the medium. Cell viability was determined by Trypan blue exclusion stain before seeding. Tubule formation was observed periodically overtime under a phase-contrast microscope and representative micrographs were taken at 24 hours.

3.8 XTT cell proliferation/viability assay

For this assay, we loaded 4×10^3 cells suspended in 200 µl medium into each well of 96-well plates. We incubated the cells at 37°C in the 5% CO_2 incubator overnight to allow them to adhere. The media was discarded the next day, and treatments added in a total volume of 200 µl and incubated for 24 hours. The media then was removed, and fresh media was added without treatment. Following that, 50 µl XTT, a compound that identifies living cells, was added. The optical density (OD) was read after 4 hours in a microplate reader at 450 nm and reference at 630 nm. The mean optical density values were determined for each treatment and were used to compare the treatment and control groups.

3.9 Invasion assay

Approximately, 5×10^3 cells, resuspended in 500 µl DMEM containing 0.1% BSA supplemented with treatment or untreated, were plated in the upper insert of Boyden matrigel invasion chambers. In the lower well of the Boyden chamber, the chemoattraction used was 750 µl of DMEM, which contained 5% FBS. After 18 hours of incubation, the non-invading cells had been eliminated from the upper insert surface with a cotton swab.

The invading cells were then fixed using paraformaldehyde, permeablized using methanol, and stained using 1% toluidine blue 1% borax stain. After each stage, three washings were carried out with distilled water. Following 18 hours of incubation, the number of the invading cells was determined by the number of the cells that penetrated the Matrigel relative to the number of the cells that migrated through the control membrane. Data is represented as number of invading cells for each sample well (four 10x micrographs per well were counted) relative to the migration through the control membrane.

3.10 Western blot analysis

Trypsin (0.25%) (corning) was used to detach the cells from the flasks, and then lysates of whole cell were prepared using the RIPA buffer combined with Halt protease/phosphatase inhibitor (Thermofisher). The samples were then kept at 4°C for 40 minutes with periodic vortexing. Next, the lysates were centrifuged at 4°C for 20 minutes at 14,000 rpm. The supernatant was collected after. Twenty micrograms of protein were then resolved on 10% SDS gels under reducing conditions (presence of β-mercaptoethanol) and transferred to Immobilon-P membranes (Millipore) using Bio-Rad wet electroblotting systems at 220 mA for 2 hours. Then, the Membranes were blocked for 2 hours with 5% dry milk in TBST on a shaker at RT. Next, the membranes were incubated with the primary antibodies or GAPDH (Cell Signaling Technology) at a 1:1000 dilution (unless indicated otherwise) in 2% bovine serum albumin in TBST (1X) overnight at 4°C. After overnight incubation, membranes were washed three times with 1X TBST. Membranes were then incubated with the respective horseradish peroxidase (HRP) conjugated secondary antibody at a 1:20,000 dilution in 2% dried milk in 1X TBST for 2 hours at RT on a shaker. After four washes with 1X TBST, the membranes were developed using Pierce ECL

western blotting detection substrate (Thermofisher) and detected on CL-Xposure Film (Thermofisher). After scanning the films, the band intensity was calculated using Image J Software.

3.11 Human angiogenesis antibody array

Human Anioggenesis Array Kit (RayBiotech, Inc) was used to evaluate levels of angiogenic factors in conditioned media obtained from cultured cells. The angiogenesis antibody membrane was placed in the 4-well multi-dish. Next, 2 ml of array blocking buffer was added onto each membrane for 30 minutes at RT. After the incubation, the blocking buffer was removed, and 1 ml of the conditioned medium collected was placed on the membrane. The membrane was incubated overnight at 4°C on an orbital shaker. The membrane was then washed with 2 ml of 1X wash buffer for 5 minutes at RT for a total of 3 washes followed by three washes with 2 ml of 1x wash buffer II for 5 minutes each at RT. The membrane was then incubated with 1 ml of biotinylated antibody cocktail overnight at 4°C. A second wash was done in a similar way to the first wash using 1 x wash buffer I and II. After that, 2 ml of 1x streptavidin-HRP solution was applied to the membrane and incubated on an orbital shaker for 2 hours at RT. Three washings of the membrane were then done with a 1X wash buffer. Then, the membrane was incubated for 2 minutes at RT with the detection buffer mixture. The membrane was then developed using Pierce ECL detection substrate (Thermofisher) and detected on CL-Xposure Film (Thermofisher). Films were scanned and intensity analyzed using the Image J Software.

3.12 Scratch wound assay

The migratory ability of cells was determined by the scratch wound assay. Four thousand cells were seeded in a 96-well plate and allowed to grow until a 70% confluency was reached. Consequently, three vertical lines were created per well using a 200 µl sterile pipette tip. Cells were then incubated in complete fresh media with treatment or no treatment (NT) for 24 hours. Micrographs were taken in the bright field phase contrast at 10X, and migration distance was measured using the scale on the microscope.

3.13 Isolation of exosomes

Exosomes were isolated from $CD133^+/CD34^+$ EPCs culture media by exosome precipitation technique using total exosome isolation reagent (Invitrogen by ThermoFisher scientific). This technique uses water-excluding polymers such as polyethylene glycol (PEG) to bind water molecules and force less soluble components out of solution. Samples were incubated with total exosome isolation reagent (0.5 volumes of the total exosome isolation reagent), and the solution was incubated overnight at 2°C to 8°C. The precipitated exosomes were recovered by centrifugation at 10,000 x g for 60 minutes. The pellet was then resuspended in PBS or similar buffer for downstream analysis.

3.14 miRNA isolation and cDNA synthesis

miRNA was isolated from $CD133^+/CD34^+$ EPCs exosomes using miRNeasy Mini-kit (Qiagen). QIAzol lysis Reagent (0.7 ml) was added to each sample and incubated at RT for 5 minutes. Next, 0.14 ml of chloroform was added to each sample. The homogenates were shaken for 15 seconds, and incubated at RT for 3 minutes, and centrifuged for 15 minutes, at 12,000 x g at 4℃. The upper aqueous phase was collected, and 1.5x volumes

of 100% ethanol were added and mixed for miRNA precipitation. The purification of miRNA was performed using buffers contained in the kit following the manufacturer's manual. miRNA was eluted from the column by adding 40 uL of Rnase-free water. cDNA synthesis was performed on isolated $CD133^+/CD34^+$ EPCs exosome miRNA using a miRNA miScript II reverse transcription kit (Qiagen). Reverse transcription reaction components include 5X miScript Hispec buffer, 10X miScript Nuclear Mix, RNase-free water, miScript RT Mix, and template $CD133^+/CD34^+$ EPCs exosome RNA. RT-PCR was performed using an MJ Research PTC-200 Thermal Cycler.

3.15 miRNA expression profile

miScript miRNA PCR Array (Qiagen) was used to determine the miRNA expression profile of $CD133^+/CD34^+$ EPCs exosomes. Applied Biosystem 7900 HT (standard 96-well plates setting) was used to analyze miRNA expression profile. miScript miRNA PCR Array Data Analysis (Qiagen) was used for data analysis.

3.16 Transmission electron microscopy (TEM)

Standard TEM was performed on samples submitted to the Department of Pathology in the Westchester Medical Center, Valhalla, NY to monitor the ultrastructure of endothelial progenitor cells. Cells were fixed and embedded in 4% paraformaldehyde and 2.5% glutaraldehyde. Thin sections were cut and examined at 200 kV using an H-600 transmission electron microscope.

3.17 Quantitative human angiogenesis array

Quantibody® Human Angiogenesis Array Kit (RayBiotech) was used to detect and quantify 30 human angiogenic factors in the conditioned media of breast cancer cells

according to the manufacturer's protocol. The antibody array is a glass-chip-based multiplexed sandwich ELISA system. There are 16 wells in each chip (6 for Standards and 10 for Samples). One hundred twenty spots in quadruplicate in each well, which represent 30 cytokines. In short, samples and standards were applied to chip array wells and incubated at RT for 2 hours. Buffers included in the kit were used after the incubation to wash the wells. Washes were followed by the addition of primary antibody and HRP-conjugated streptavidin to the wells. Then, the chip was scanned using a Genepix scanner and quantified using Quantibody Analyzer software (Ray Biotech Inc).

3.18 Statistical calculation

The results shown in this study represent three independent experiments with statistical significance determined using a paired Student's t-test with a probability ('p' value) ≤ 0.05 used to reject the null hypothesis.

4 CHAPTER IV: RESULTS

Rationale: Recent evidence has illustrated the significance of bone marrow-derived endothelial progenitor cells (EPCs) in postnatal physiological and pathological neovascularization, as well as in tumor growth and angiogenesis (Balaji *et al.*, 2013; Zhao, X. *et al.*, 2016). These cells are attracted undifferentiated, in response to systemic or chemoattractive signals. They settle in the tumor or lesion tissue and differentiate into endothelial cells in response to local stimuli and cell-cell interactions (Botelho and Alves, 2016). Estrogen has an essential role in the genesis and progression of breast cancers, whether they are positive or negative for the estrogen receptor (ER). As previously reported,17β-estradiol has been detected as one of the attractants that is crucial for migration and vascular incorporation of BM-derived EPCs in the tumor vasculature (Suriano *et al.*, 2008). Therefore, the estrogen responsiveness of EPCs must be validated.

Specific Aim1: Characterize endothelial progenitor cells (EPCs) from human umbilical cord blood and validate their estrogen responsiveness

EPCs perform a vital function in postnatal endothelial repair and vasculogenesis in adults in conditions such as myocardial ischemia and infarction, unstable angina, stroke, limb ischemia, wound healing, atherosclerosis, diabetic microvasculopathies, ischemic retinopathies, pulmonary arterial hypertension and tumor vascularization (Janic and Arbab, 2012; Khakoo and Finkel, 2005; Rafii and Lyden, 2003; Sekiguchi *et al.*, 2009; Tateishi-Yuyama *et al.*, 2002; Ward *et al.*, 2007). EPCs are one of the key targets of regenerative medicine and cancer research because of their powerful therapeutic potential. Many studies have been launched to explicitly describe the origin, phenotype, and differentiation

hierarchy of the putative EPCs. Currently, EPCs are known to express CD133, CD34, and VEGFR-2 markers. In addition, these cells are also positive for the endothelial cell-specific antigens such as platelet EC adhesion molecule 1 (PECAM-1 or CD31), VE-Cadherin, and E-selectin. CD34 and CD133 expression is downregulated during differentiation into mature ECs. On the other hand, the expression of mature EC markers increases. Several studies, on the other hand, suggested that CD45 positive cells had angiogenic capacity and all the key elements of typical EPCs. Despite the extraordinary effort to classify the phenotypic and functional characteristics of EPCs, there is still similarity between EPCs, HSCs, and ECs, and it remains the source of debate. It remains uncertain whether the therapeutic potential of the EPCs depends on their differentiation / maturation status (Janic and Arbab, 2012). Our research focuses on EPCs, which we describe as a subpopulation of CD34-positive cells that is positive for CD133 (CD34+/CD133+).

EPCs can be detected in the peripheral blood (PB), bone marrow (BM), and umbilical cord blood (UCB). UCB contains a significantly higher number of EPCs than PB or BM (Hristov *et al.*, 2003). We decided to use umbilical cord blood in our research since it is an excellent reservoir of stem and progenitor cells. Progenitors derived from cord blood have many features over the progenitors produced from adult bone marrow. They are more likely to form colonies, proliferate longer and faster, and have longer telomeres. Furthermore, cord blood collection does not involve invasive procedures.Transplanted cord blood-derived EPCs efficiently enhance postnatal neovascularization *in vivo* (Murohara *et al.*, 2000). In addition, our experiments utilized EPCs expanded in suspension cultures since the *in vitro* expansion of the attached EPCs phenotype was associated with an increase in senescence and inferior proliferation capacity (Janic and Arbab, 2012).

4.1 EPCs are highly proliferative

First, we examined the growth potential of EPCs and the expression of endothelial progenitor phenotype suggestive cell surface markers. The cord blood mononuclear cell (MNCs) population was recovered by Histopaque-1077 gradient centrifugation. MNCs were enriched for CD133+ cells by immunomagnetic positive selection. In Stemline II media supplied with 40 ng/ml of stem cell factor (SCF), 40 ng/ml of FLT3, and 10 ng/ml of thrombopoietin (TPO), freshly isolated CD133-positive cells were suspended. Under these conditions, the cells were expanded as a suspension culture, and the concentration of the cells maintained around about 10^6 cells/ml and monitored daily for the increase in cell numbers by inverted phase-contrast microscopy. Trypan blue exclusion assay was used to determine the number of live cells, and the results were used to create the growth curve, as shown in (Figure 10). After 3 days of cell expansion, a substantial increase in cell numbers was observed. This potential increase in cell numbers, compared to the cell numbers at day 0, was sustained throughout the culture, and at day 11 of culture, cells achieved a 79-fold increase.

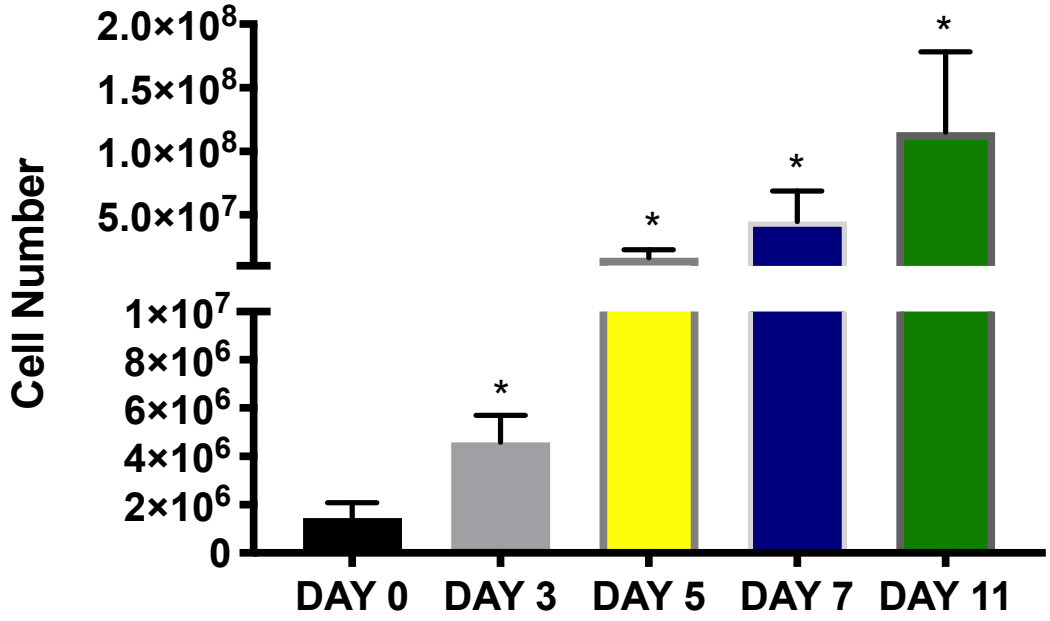


Figure 10. Growth kinetics of EPCs

For 30 days, EPCs were cultivated with 40 ng/ml of FLT3, 40 ng/ml of stem cell factor (SCF), and 10 ng/ml of thrombopoietin (TPO). At the time points shown on the graph, cell numbers were calculated by the Trypan blue exclusion assay. * $p<0.05$. Results are compared to day 0. Error bars represent ±SEM.

4.2 EPCs express CD133 and CD34

Next, as markers for endothelial progenitors, we examined the expression of CD133, CD34, and VEGFR-2 (KDR) using flow cytometry. Upon isolation on immunoaffinity magnetic columns, over 90% of cells expressed CD133 and CD34. Approximately, 91.8% ± 0.4 of the isolated cells were positive for CD133, and 99.2% ± 0.27 of cells positive for CD133 also expressed CD34. However, these cells were negative for the VEGFR-2 (KDR) (Figure 11A). Results were also confirmed using immunofluorescence (Figure 11B).

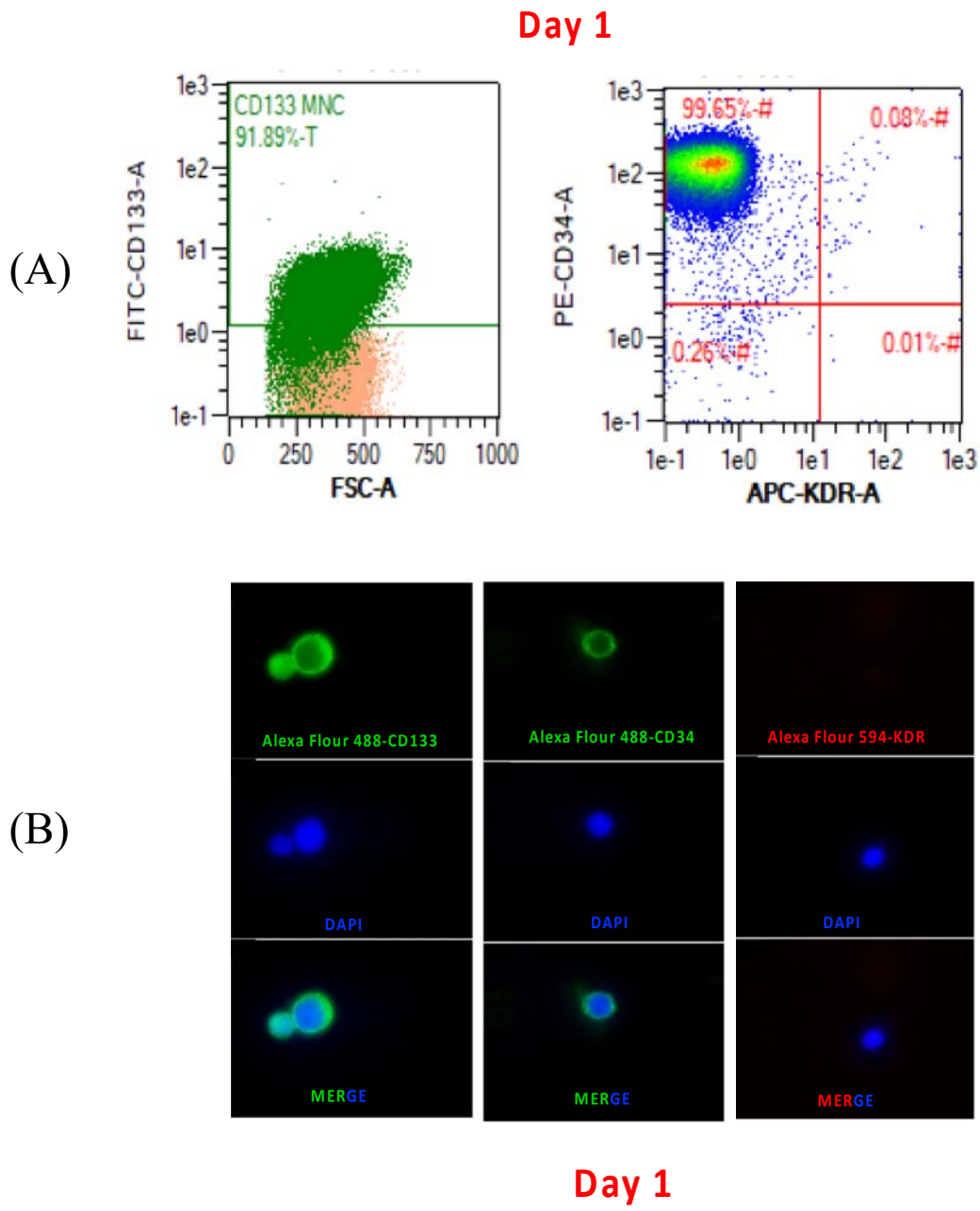


Figure 11. Expression of cell surface markers in EPCs during *in vitro* culture

The levels of CD133, CD34, and KDR protein expression in EPCs at day 1 of *in vitro* culture are depicted by flow cytometry (A) flow cytometry and immunofluorescence (B). At least 10,000 live gated cells were analyzed by flow cytometer for FITC, PE, or APC

expression. Isotype controls are shown as orange dots. Gates were set to exclude 95% of the negative control events.

4.3 EPCs can differentiate into functional ECs

To characterize the potential of EPCs to differentiate into functional ECs, EPCs were seeded in the presence of 2% FBS and 50 ng/ml of VEGF at day 7 of primary culture. Cells were differentiated for 2 weeks and analyzed for the changes in expression of EPC markers and morphology compared to undifferentiated cells. A very small proportion of cells in undifferentiated cultures were positive for CD133 (0.51%±0.04 of total) and only 6.14%±0.79 of cells were positive for the three markers (Figure 12). In contrast, 46.51±4.2% of the total differentiated cells continued to express CD133. Gated on CD133, 42.09±1.39% of the cells were also positive for CD34 and KDR. The induction of differentiation induced the cells to upregulate the expression of KDR (Figure 12), and VE-Cadherin (Figure 13). The fact that these endothelial cell markers were upregulated during the induction of differentiation indicates that EPCs can differentiate toward the endothelial lineage. In addition, it indicates that the isolated cells (CD133+/CD34+ cells) represent EPCs.

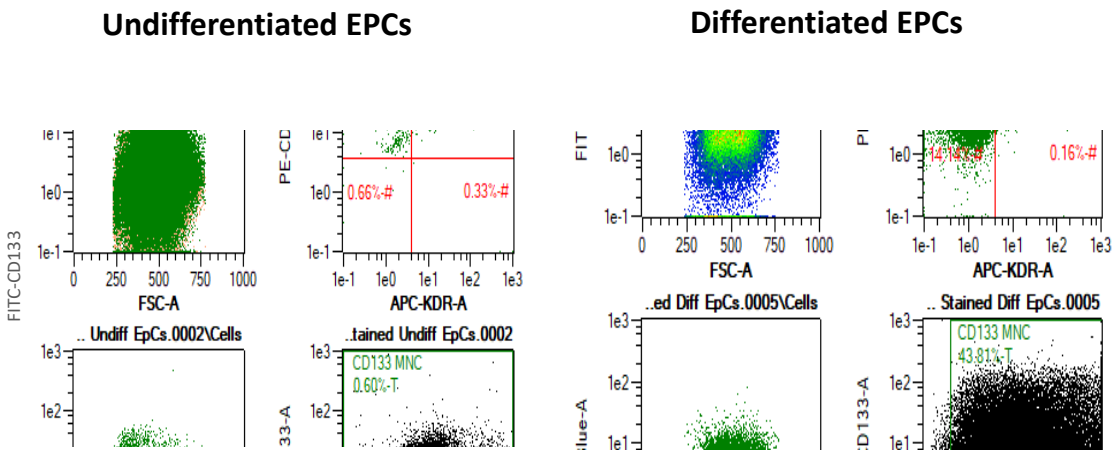


Figure 12. Expression of CD133, CD34, and KDR in differentiated and undifferentiated EPCs

Cells were induced to differentiate for 2 weeks at day 7 of primary culture. Flow cytometric analysis showed that 2 weeks of differentiation induced the cells to upregulate KDR and maintain CD133 and CD34. At least 10,000 live gated cells were analyzed for FITC, PE or APC expression. Isotype controls are shown as orange dots. Gates were set to exclude 95% of the negative control events.

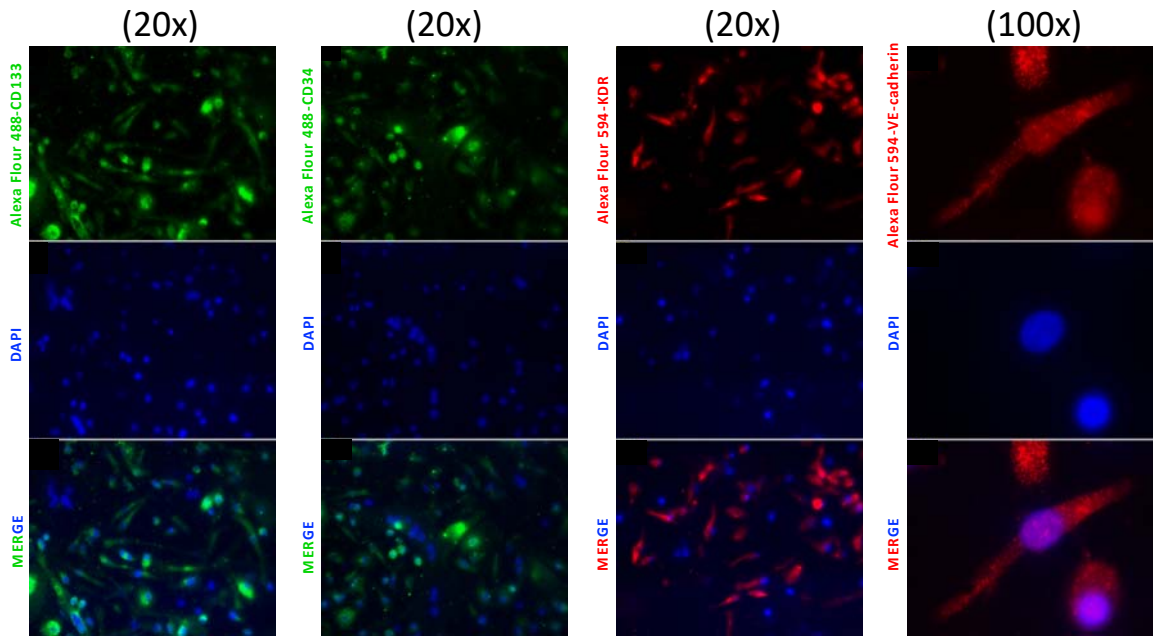


Figure 13. Expression of CD133, CD34, KDR, and VE-Cadherin in differentiated EPCs

Cells were induced to differentiate for 2 weeks at day 7 of primary culture. Cells were probed with CD133, CD34, KDR, VE-Cadherin primary antibodies. Flourescent secondary antibodies in addition to DAPI (nuclear stain) were used for immunofluorescence. Images were taken by Axiovision Rel 4.8 at 20x and 100x.

Besides the changes in the expression of cell surface markers that were marked by the upregulation of ECs markers, the differentiated cells exhibited morphological changes indicative of the capacity of the EPCs to differentiate into competent ECs. As revealed by light microscopy, morphological changes were observed in differentiated cells 1 day after differentiation induction (Figure 14B). At about day 3 of differentiation, clusters of round cells centrally and sprouts of spindle-shaped cells at the periphery started to appear. These clustered structures are similar to reminiscent of the blood island-like clusters normally found in the developing embryonic yolk sac (Figure 14C). Around day 7 of differentiation, most of the cells were spindle-shaped (Figure 14D) in an attempt to organize into linear, tube-like structures, which were observed when differentiated cells were plated on Matrigel (Figure 14E).

To confirm whether differentiated EPCs displayed functional characteristics of physiologically competent ECs, uptake of acetylated low-density lipoprotein (Ac-LDL) was tested. Endothelial cells take up the Acetylated–LDL through the scavenger pathway of LDL metabolism. In the endothelial cells, this metabolism occurs at an accelerated rate relative to other types of cells (Stein and Stein, 1980; Voyta *et al.*, 1984). Uptake of Ac-LDL was very often used to identify the EC population and to assess ECs' functional integrity (Ma *et al.*, 2008; Werner *et al.*, 2008). Therefore, we analyzed the uptake of DiI-Ac-LDL in EPCs that were differentiated for two weeks after 7 days of primary culture. As shown in (Figure 15), the differentiated cells exhibited uptake of DiI-Ac-LDL that was displayed as uniform, perinuclear red fluorescence. This pattern is typical of mature ECs and indicates the presence of a functional EC population.

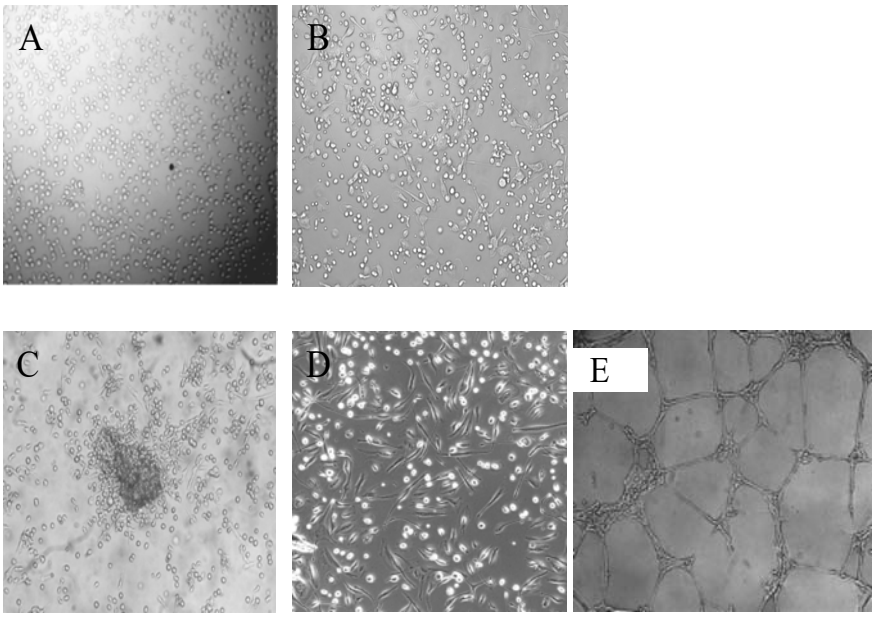


Figure 14. Attachment, cluster formation, and capillary network development by EPCs *in vitro*

(A) EPCs before differentiation. (B) Morphological changes were observed in differentiated cells 1 day after differentiation induction. (C) Cluster formation was observed after 3 days of differentiation followed by (D) Spindle shaped attaching cells throughout the culture 7 days after differentiation induction. (E) Tube-like structures are formed on Matrigel by differentiated cells. Phase contrast photomicrographs from 3 representative experiments. Magnifications used: 10x.

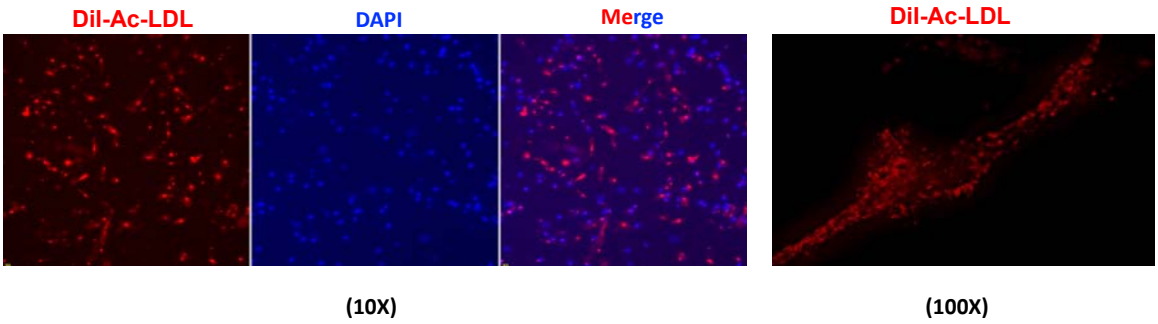


Figure 15. Differentiated EPCs exhibited the uptake of DiI-Ac-LDL (red)

The adherent cells in culture were incubated with 10 mg/mL DiI-acetylated low-density lipo-protein for 4 hours at 37C and then washed with PBS. After fixation with 3% formaldehyde, the cells were examined by fluorescence microscopy. Magnifications used: 10x (right) and 100x (left).

4.4 EPCs are estrogen responsive

Next, we wanted to determine the estrogen responsiveness of EPCs. Estrogens are considered to play a crucial role in breast cancer by promoting the proliferation of both the normal and the neoplastic breast epithelium. Multiple mechanisms are proposed to be involved in their carcinogenic effects, including stimulation of cellular proliferation through their receptor-mediated hormonal activity, direct genotoxic effects by increasing mutation rates through a cytochrome P450-mediated metabolic activation, and induction of aneuploidy (Russo and Russo, 2006). Our group previously discovered a new role of estradiol in breast cancer. E2 induces the mobilization of BM-EPCs into the circulation and homing to tumor tissues. Therefore, in the next set of experiments, we wanted to determine the effects of estradiol on EPCs.

To determine whether EPCs are estrogen-responsive or not, we treated the cells with different concentrations of 17 β-E2 (the most abundant form of estrogen found in the human body). Figure 16 shows the effect of 17 β-E2 on EPCs when cells were cultured under starvation conditions (phenol-red-free medium, no serum) for 24 hours and then stimulated with different concentrations of 17 β-E2 for another 24 hours. Cell proliferation was measured by XTT, a colorimetric assay system based on the reduction of a yellow XTT to an orange formazan dye by metabolically active cells and used as an indicator of cell viability, proliferation, and cytotoxicity. We observed that 7 nM and 10 nM concentrations of 17 β-E2 resulted in a significant increase in the EPCs' proliferation, suggesting that EPCs are estrogen-responsive and might be responsive to the growth regulatory cellular effects of the E2–ER interaction.

Using 10 nM of 17 β-E2, the invasive potential of EPCs following E2 exposure was assayed by the invasion assay. We observed that cell invasion increased in the presence of E2 (Figure 17). Compared with control cells, this increase in invasion was approximately 20.2% for E2-treated cells. This result indicates that estrogen significantly enhances the invasive capacity of EPCs.

Moreover, estrogen treatment of EPCs enhanced the capacity of these cells to secrete angiogenic factors. The treated EPCs with estrogen secreted significantly higher amounts of angiogenin, GRO, IL-8, TIMP-2, and EGF (Table 3). All these factors act as pro-angiogenic factors enhancing the growth of tumor vasculature and some of these factors (GRO and IL-8) are chemoattractants. Estrogen not only mobilizes EPCs from the bone marrow, but also induces these cells to secrete factors that attract inflammatory cells making the TME more conducive for tumor growth.

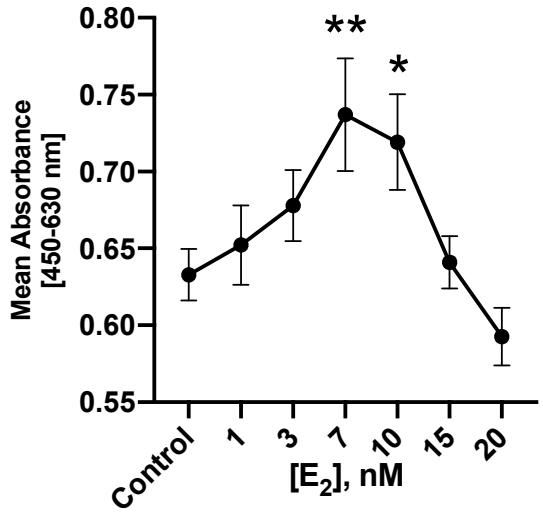


Figure 16. Estrogen stimulates proliferation of EPCs

Effect of E2 on proliferation of EPCs was determined by XTT assay. Cells were starved overnight with the starvation medium (phenol red free- serum free) for 24 hours and then seeded at a density of 1×10^8 cells per well treated with different concentrations of E_2 for 24 hours. The colorimetric Cell Proliferation Kit XTT based method was utilized to assess cell proliferation. XTT reagent was applied to each well, and absorbance (wavelength 450 nm and reference 690 nm) was determined after another 4 hours of incubation. The graph depicts three independent experiments (n=3). The asterisk denotes statistically significant increase * $P<0.05$ ** $P<0.01$ in the experimental groups compared with controls. Results are compared to untreated control (UT). Error bars represent ±SEM.

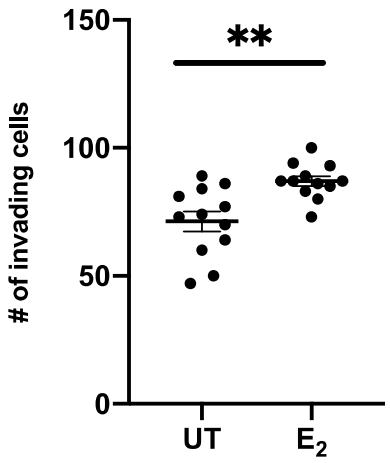


Figure 17. Estrogen enhances invasive capacity of EPCs

5×10^3 cells, suspended in 500 µl DMEM containing 0.1% BSA supplemented with 10 nM E_2 or left untreated, were seeded in the upper insert of growth factor reduced Matrigel Boyden invasion chambers. In the lower well of the chamber, the chemoattractant used was 750 µl of DMEM, which contained 5% FBS. Data is represented as number of invading cells for each sample well (four 10x micrographs per well were counted) relative to the migration through the control membrane. Results are representative of at least three independent experiments. ** indicates significant difference from control at a level of $P<0.01$.

Table 3. Angiogenic factors secreted by EPCs after estrogen treatment

Angiogenic protein	Fold increase after estradiol treatment	P-value
ANG	**1.821**	**0.001**
GRO	**1.858**	**0.001**
IL-8	**1.606**	**0.001**
TIMP-2	**4.818**	**0.008**
EGF	**0.809**	**0.011**

4.5 EPCs express estrogen receptor alpha

EPCs are not known to act as traditional estrogen-responsive cells. To determine the biochemical rationale for the estrogen responsiveness of these cells, Western blot analysis on whole cell lysates was performed. We observed that EPCs expressed only ER-α but not ER-β (Figure 18). MCF-7 was used as a positive control for the detection of ER-α and ER-β, and MDA-MB-231 was used as a negative control for ER-α. This suggests that these cells are presumably responsive to the E_2–ER-mediated growth signaling pathway.

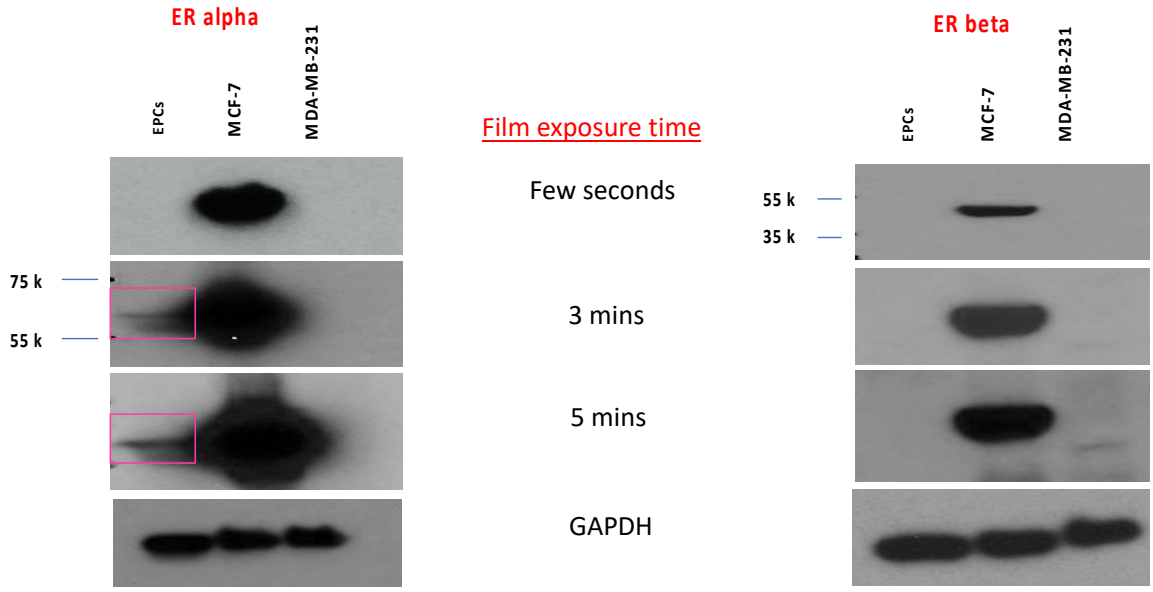


Figure 18. Endothelial progenitor cells express estrogen receptor ER-α

15 µg of whole cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blot analysis, for ER-α (45 KDa) and ER-β (60 KDa), was performed. MCF-7 lysates were used as positive controls for ER-α and ER-β expression, and MDA-MB-231 lysates were used as negative controls.

4.6 Summary of specific aim 1

- Endothelial progenitor cells (EPCs) phenotypically characterized as CD133+/CD34+ are highly proliferative and can differentiate to functional endothelial cells (ECs)
- EPCs express ER alpha
- Estrogen treatment enhances proliferation, invasion of EPCs, and inflammatory cytokine production

Specific Aim 2: Evaluate the effect of an anti-estrogen on EPCs

Rationale: Previously, estrogen was implicated in modulating angiogenesis, both under physiological and pathological conditions. However, data generated in our laboratory indicated that estrogen is also implicated in modulating vasculogenesis**.** Our results indicated that estrogen mobilizes endothelial progenitor cells from the bone marrow to the tumor site, where they differentiate into ECs supporting tumor growth and metastasis. EPCs are estrogen-responsive owing to their expression of ER-α. 3,3′-Diindolylmethane (DIM) is an indole-3-carbinol (I3C) major acid condensation product. I3C and DIM are naturally occurring phytochemicals found in cruciferous vegetables such as broccoli, kale, cauliflower, and cabbage that stimulate cellular responses incompatible with tumor growth in a number of human cancer cells (Chang *et al.*, 2005; Rajoria *et al.*, 2011). DIM has anticarcinogenic and anti-estrogenic effects. It inhibits the proliferation of a wide range of cancer cell types such as prostate (Chang *et al.*, 2005), breast (Rahman and Sarkar, 2005), and colon (Kim *et al.*, 2007) cancer cells through induction of cell cycle arrest and apoptosis. Data from our laboratory indicated that DIM exerts anti-estrogenic effects and can modify estrogen metabolism in patients with proliferative thyroid diseases resulting in an increase in the ratio of 2-hydroxy estrone to 16α-hydroxy estrone (Rajoria *et al.*, 2011) favoring the antiproliferative effect of DIM. Similar effects of DIM have been reported in postmenopausal women with a history of breast cancer (Dalessandri *et al.*, 2004), and in estrogen-sensitive prostate cancer cells (Smith *et al.*, 2008). Therefore, to confirm that EPC functions are E_2 dependent, the effects of this anti-estrogen on EPCs was tested. The first step was to determine the effects of DIM on HUVECs as a well-established model of *in*

vitro angiogenesis, and to explore the possibility of abrogating the pro-angiogenic effects of estrogen using this treatment.

DIM significantly reduces *in vitro* tubule formation of HUVECs and abolishes the pro-angiogenic effects of E2

DIM has been noticed to have anti-proliferative properties in a wide variety of cell lines. An XTT assay was performed on HUVECs treated for 24 hours with different DIM concentrations in order to determine the effect of DIM on the proliferative capacity of HUVECs. A dose-dependent inhibition in cell proliferation was observed during 24 hours of treatment. At a concentration of around 47 μM DIM, DIM decreased the proliferation of HUVECs by 50% (Figure 19). Even though 47 μM DIM concentration achieved a 50% inhibition of HUVECs proliferation, we decided to use the 25 μM DIM concentration in the subsequent experiments to characterize the effect of DIM on HUVECs further.

A tubule formation assay was performed by plating HUVECs on a layer of basement membrane matrix (matrigel). The cells attached, migrated, and formed tubule-like structures with a lumen within 24 hours of incubation. In order to explore the possible anti-angiogenesis and anti-estrogenic effects of DIM, HUVECs were treated with estrogen, DIM, or a combination of both and tested by the *in vitro* tubule formation assay. As expected, the pro-angiogenic estradiol did significantly enhance the tubule formation by HUVECs while DIM treatment, at a concentration of approximately 25 μM, resulted in significant inhibition of tubule formation ($p<0.01$) (Figure 20). DIM treatment also inhibited estradiol-induced tubule formation as shown by the 69.5% and 68.4% decreases in tubule formation, respectively, for DIM and DIM+E_2 treated HUVECs (Figure 20).

These results indicate that DIM acted directly on HUVECs and exhibited a strong anti-angiogenic effect and abrogated estradiol-induced effects.

Based on the fact that angiogenesis is a multi-step process involving enzymatic degradation of basement membrane, endothelial cells (ECs) activation by pro-angiogenic growth factors, ECs proliferation, directed ECs migration, and tubule formation (Cook and Figg, 2010), further analysis of the effect of this treatment on each step of *in vitro* angiogenesis was done to confirm the observations in Figure 20 and to determine DIM's mechanism of action.

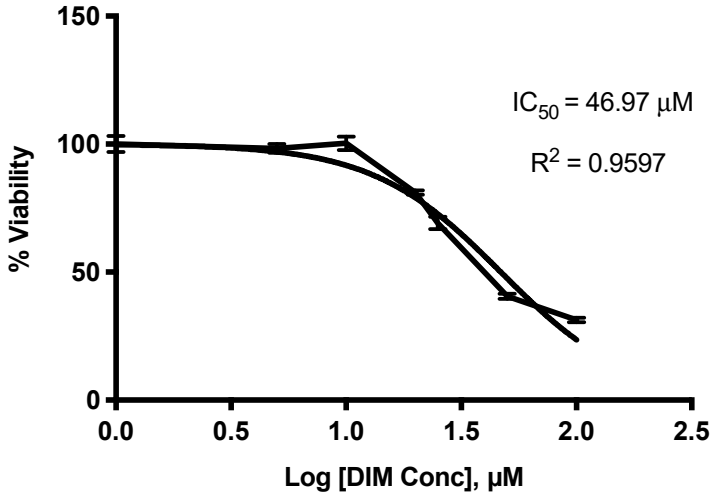


Figure 19. DIM causes dose dependent inhibition in cell viability of HUVECs

HUVECs were seeded in 96-well plates at density of 1×10^3 cells per well. Cells were allowed to adhere overnight. HUVECs were then treated with different concentrations of DIM for 24 hours. Next, XTT assay was performed, and colorimetric measures (OD 450 – OD 630 nm) were taken after 4 hours. Data are means ± SEM of at least three independent experiments (n = 3).

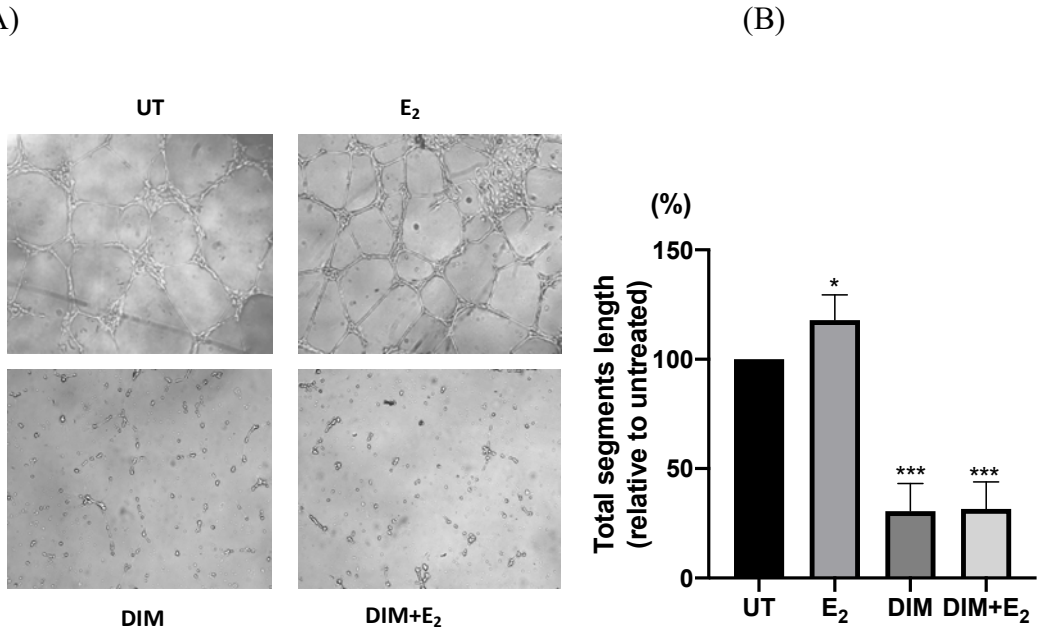


Figure 20. DIM significantly reduces *in vitro* tubule formation of HUVECs and abolishes the pro-angiogenic effects of E2

Endothelial cells were trypsinized and 5 x 10^4 were seeded per well in 500 ul medium in wells of a 24-well plate coated with phenol red-free growth factor reduced matrigel. Cells were then treated with 25 μM DIM, 25 μM DIM + 10 nM E_2, 10 nM E_2 only, or left untreated (UT) for 24 hours. Tubule formation was observed periodically, and representative pictures were taken at 24 hours. (A) Phase contrast light micrographs show the appearance of tubules formed by HUVECs with each treatment after 24 hours. (B) Comparison of mean length of tubules formed after each treatment. Results are representative of at least three independent experiments. Five areas were photographed in each well and measured to obtain the segment lengths. Significant differences from

untreated (UT) controls are indicated as * at a level of $P<0.05$ and ** at a level of $P<0.01$. Numbers in (B) were calculated based on untreated cells which were set as 100%. Error bars represent ±SEM.

4.7 DIM significantly reduces the invasion of HUVECs

The first basic step of sprouting angiogenesis includes the degradation of the capillary basement membrane (Cook and Figg, 2010). A cell invasion assay was conducted to examine the effect of DIM on HUVECs movement through a matrigel matrix. HUVECs were loaded in a trans well chamber with 25 μM DIM ± 10 nM E_2, 10 nM E_2 only, or no treatment and were allowed to invade through the Matrigel during 18 hours of incubation. As shown in figure 21, the HUVECs were able to infiltrate via the Matrigel. Upon treating the cells with estradiol, their invasion capacity significantly improved by 91.5%. By using DIM in combination with estradiol, this substantial increase in cell invasion was entirely nullified. The invasive ability of HUVECs was significantly reduced by 79% (compared to untreated) when cells were treated by DIM alone or in combination with an estrogen (Figure 21). These data indicate that DIM can abolish estrogen induced cellular invasion, thus targeting a significant phase in angiogenesis.

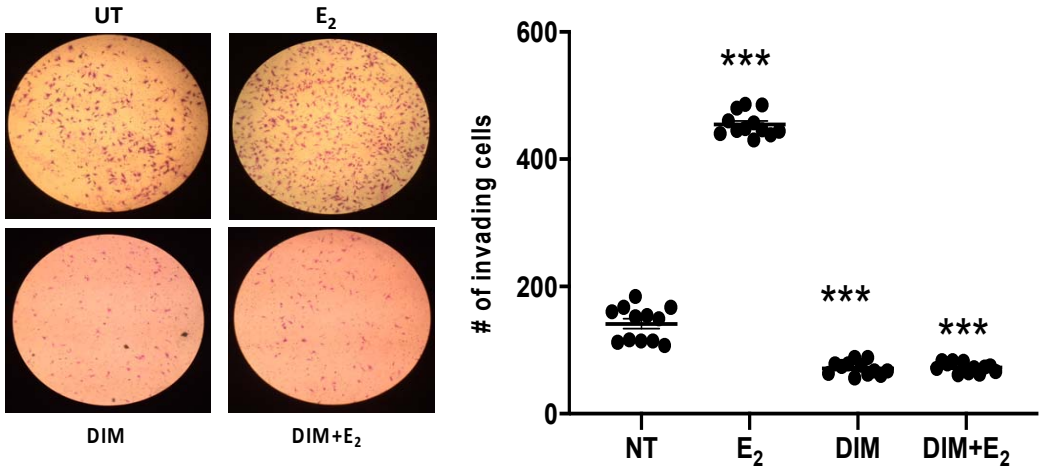


Figure 21. DIM significantly reduces the invasion of HUVECs

5×10^3 HUVECs, suspended in 500 µl DMEM containing 0.1% BSA supplemented with 25 µM DIM ± 10 nM E_2, 10 nM E_2 only, or left untreated, were seeded in the upper chamber of growth factor reduced matrigel invasion chambers. In the lower well of the chamber, the chemoattractant used was 750 µl of DMEM, which contained 5% FBS. Data is represented as number of invading cells for each sample well (four 10x micrographs per well were counted) relative to the migration through the control membrane. Results are representative of at least three independent experiments. *** indicates significant difference from untreated control at a level of $P<0.001$. Numbers were calculated based on untreated cells which were set as 100%. Error bars represent ±SEM.

4.8 DIM reduces the pro-angiogenic cytokines secretion by HUVEC

The next step was to investigate the nature and levels of angiogenic molecules present in HUVEC conditioned media and their modification after DIM ± estradiol treatment. Angiogenesis protein array was performed which showed that the conditioned medium from HUVECs contains a number of potent angiogenic factors that can be induced by estradiol treatment (Figure 22A). Adding DIM to the estradiol treated HUVECs inhibited the production of some of the potent pro-angiogenic cytokines (Figure 22B). The same inhibition pattern was observed when DIM treatment used alone without estradiol (Figure 22C). Among the cytokines that were markedly inhibited by DIM are IFNγ, leptin, MCP-1, PDGF-B1, PLGF, VEGF, and VEGF-D. These cytokines have different roles in mediating angiogenesis, and the ability of DIM to significantly inhibit multiple angiogenic molecules other than VEGF makes it a compelling candidate to target angiogenesis at multiple levels.

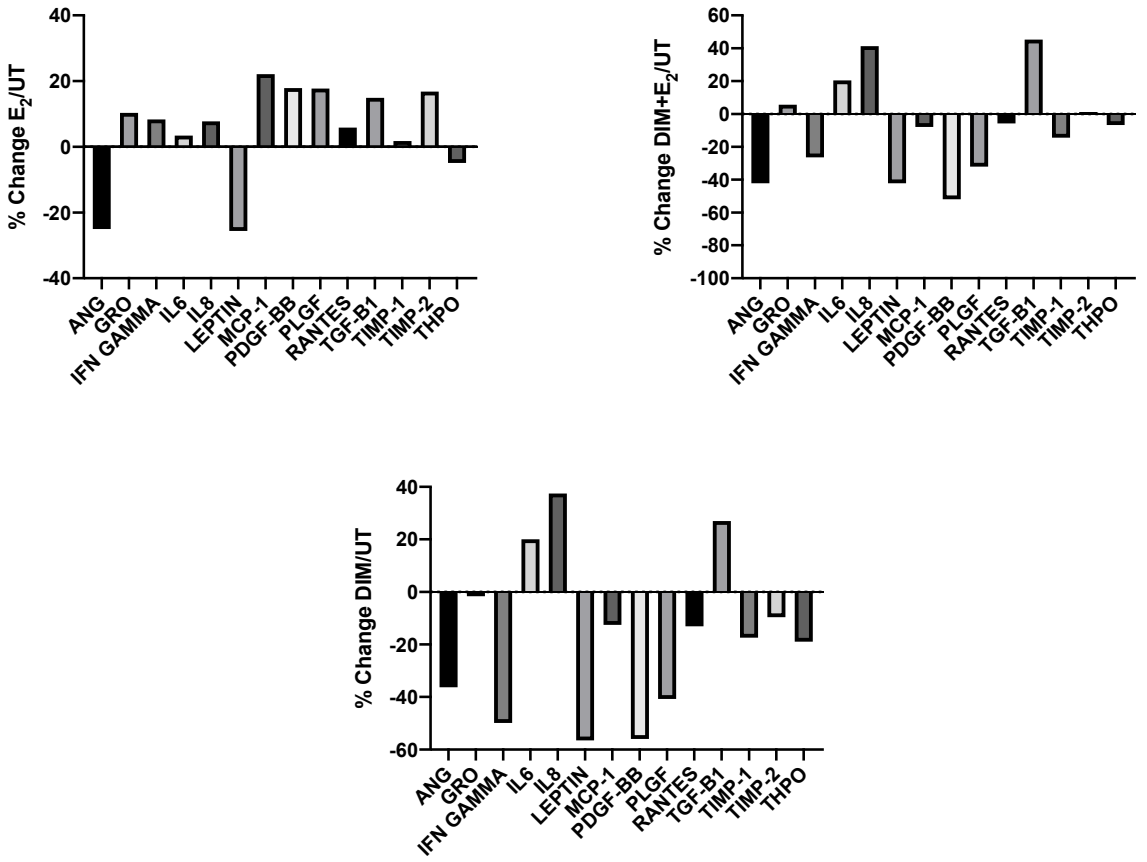


Figure 22. DIM reduces the pro-angiogenic cytokines secretion by HUVECs

HUVECs were suspended in 500 µl of phenol red free EBM media with 25 µM DIM ± 10 nM E_2, 10 nM E_2 only, or untreated and seeded in wells of a 24-well plate coated with phenol red free growth factor reduced matrigel. After 24 hours, supernatant was collected, and angiogenesis arrays were performed. Array spots were taken and analyzed by Image J software. Expression levels were presented as a mean pixel density normalized by the positive spot references, and then results are normalized to the untreated control.

4.9 DIM reduces the proliferation of HUVECs

In sprouting angiogenesis, ECs proliferation occurs after enzymatic degradation of the basement membrane and endothelial cells (ECs) activation by pro-angiogenic growth factors. Data from Figure 20 demonstrates the anti-proliferative effect of DIM on HUVECs. To confirm the effect of this anti-estrogen and estradiol on HUVEC proliferation, HUVECs were suspended in phenol red-free EBM medium, seeded in 6-well plates, and then treated with either 10 nM E_2 only, 25 μM DIM + 10 nM E_2, 25 μM DIM, or left untreated. Then, the cells were allowed to grow for 24 hours. HUVECs grown in a medium treated by estradiol showed a significant 24% increase in the proliferation rate compared to the untreated cells while HUVECs treated with 25 μM DIM showed a significant 48.9% decrease (Normalized to 100% and $p<0.05$) in proliferation rate (Figure 23). Adding 25 μM DIM to estradiol also inhibited EC proliferation by 50.4% suggesting that DIM may prevent estrogen enhanced proliferation and can potentially act as an effective anti-estrogen, anti-angiogenic agent.

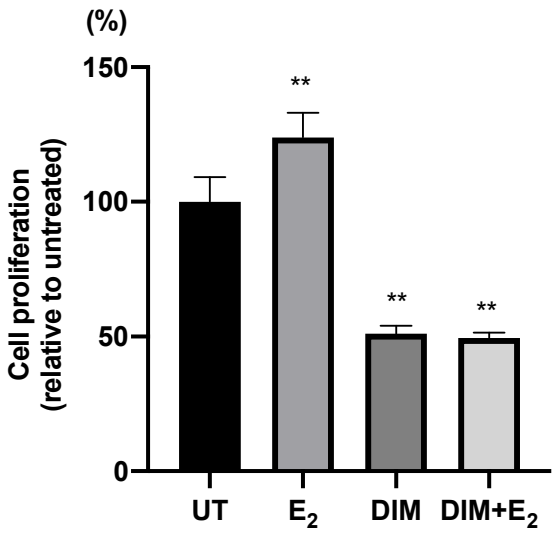


Figure 23. DIM significantly reduces proliferation of HUVECs

8 x 10^4 HUVECs were seeded per well in 6-well plates and allowed to grow for 48 hours. The cells were then treated with 25 μM DIM, 25 μM DIM + 10 nM E_2, 10 nM E_2 only, or left untreated for an additional 48 hours. Cell numbers were assessed at various time points by trypsinization and counting with a Coulter Z1 cell counter. Results are compared to (UT) untreated control cells. ** indicates significant difference from untreated (UT) control at a level of $P<0.01$. Numbers were calculated based on untreated cells which were set as 100%. Error bars represent ±SEM.

4.10 DIM significantly reduces migration of HUVECs

A trans-well migration assay was performed as described in the Material and Methods to study the migratory response of HUVECs to estrogen and anti-estrogen DIM. In concordance with previously reported results, DIM exhibited a strong anti-estrogenic/anti-migratory effect (Figure 24A&B). It significantly inhibited all the steps required for angiogenesis and abrogated estrogen-induced effects suggesting it may be a successful anti-angiogenic and anti-estrogenic treatment.

DIM is significantly active against BC exosome stimulation of HUVEC cell differentiation

As mentioned before, angiogenesis is one of the hallmarks of breast cancer. Breast cancer cells release exosomes to help to maintain a supportive tumor microenvironment. Exosomes were isolated from 1 mL conditioned media of MDA-MB-231 and MCF-7 human breast cancer cells and evaluated for their effects on HUVECs plated on growth factors reduced Matrigel in starvation medium. The addition of breast cancer exosomes caused significantly more tubule network formation and HUVEC proliferation (Figure 25). To confirm the effectiveness of DIM in inhibiting angiogenesis, HUVECs were incubated on Matrigel with 50 uM DIM and MDA-MB-231 or MCF-7 exosomes. Drug treatment at this concentration significantly reduced tubule formation by a 50.8% and a 53.9% in HUVECs treated with exosomes from MDA-MB-231 and MCF-7, respectively (Figure 25). These data indicate that breast cancer exosomes have a crucial role in the formation of blood vessels around the tumor as pro-angiogenic stimulators and also indicates the effectiveness of DIM in blocking this effect.

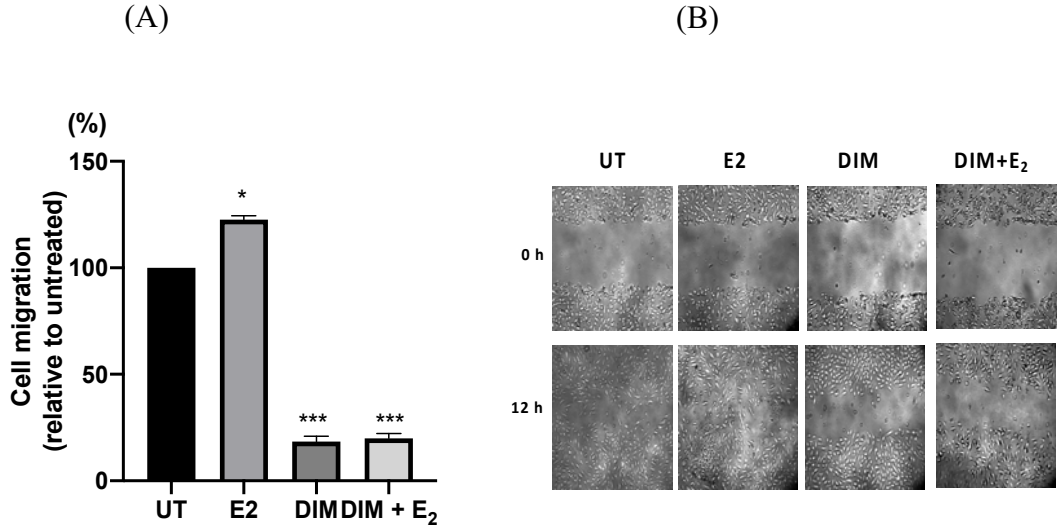


Figure 24. DIM reduces migration of HUVECs in invasion chambers and scratch wound assasys

(A) 5×10^3 cells, suspended in 500 µl DMEM containing 0.1% BSA supplemented with 25 µM DIM ± 10 nM E_2, 10 nM E_2 only, or left untreated, were seeded in the upper insert of migration chambers. In the lower well of the chamber, the chemoattractant used was 750 µl of DMEM, which contained 5% FBS. After 12 hours, the migrated cells were fixed, permeabilized, stained, and counted under the 10X objective. Data is presented as numbers of migrated cells for each sample and normalized to the untreated control which were set as 100%. Significant differences from untreated (UT) controls are indicated as * at a level of $P<0.05$ and *** at a level of $P<0.001$. (B) Representative photomicrographs from scratch wound assays of DIM and E_2 treated HUVECs performed as described in the materials and methods. Error bars represent ±SEM.

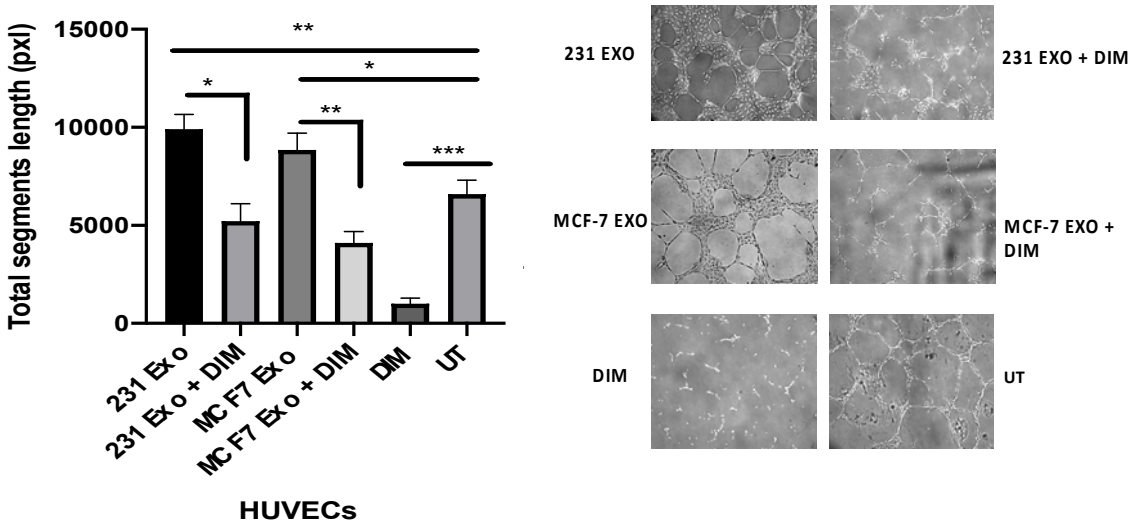


Figure 25. DIM is significantly active against BC exosome stimulation of HUVEC cell differentiation

HUVECs were trypsinized and seeded at 5 x 10^4 cells per well in 500 ul medium in 24-well plates coated with phenol red-free growth factor reduced matrigel. MDA-MB-231 and MCF-7 human breast cancer cells, seeded at 2 x 10^5 cells per well in 1 ml clear DMEM (no supplements added) in 6-well plates for 24 hours, were used to generate conditioned media. Exosomes were isolated from 1 mL conditioned media of MDA-MB-231 and MCF-7. HUVECs were then treated with MDA-MB-231 exosomes, 231 exosomes + 50 μM DIM, MCF-7 exosomes, MCF-7 exosomes + 50 μM DIM, 50 μM DIM, or left untreated for 24 hours. Tubule formation was observed periodically, and representative pictures were taken at 24 hours. Phase contrast light micrographs showing the appearance of tubules formed by HUVECs with each treatment after 24 hours (right). Comparison of mean length of tubules formed after each treatment (left). Results are representative of at least three independent experiments. Significant differences from untreated (UT) controls are

indicated as * at a level of $P<0.05$, ** at a level of $P<0.01$, and *** at a level of $P<0.001$. Error bars represent ±SEM.

4.11 HUVECs express ER-β and DIM enhances ERβ expression

The female sex hormone, estrogen has a pro-angiogenic activity associated with the physiological cyclic changes in the female reproductive tract (Hyder and Stancel, 1999; Johns *et al.*, 1996; Losordo and Isner, 2001; Seo *et al.*, 2004; Yasuda *et al.*, 1998), and associated with some pathological conditions as endometriosis (McLaren *et al.*, 1996), endometrial cancer (Fujimoto *et al.*, 1998), and breast cancer (Losordo and Isner, 2001; Seo *et al.*, 2004). Binding of estrogen to the ERs in target organs mediates the biochemical activity of estrogen. ERα and ERβ have distinct biological functions (Lee *et al.*, 2012). ERβ exerts an inhibitory effect on cellular proliferation and induces apoptosis, and ERα has pro-proliferative and anti-apoptotic effects.

Based on our previous results, DIM treatment of HUVECs exhibited a strong antagonism to the pro-angiogenic effects of estrogen; therefore, the effect of DIM on estrogen receptors (ER) was evaluated and compared to the effect of the synthetic anti-estrogen "fulvestrant". As shown in Figure 26, exposure to DIM for 24 hours upregulated the expression of ERβ protein levels in HUVECs similar to the effect exerted by fulvestrant. These results further prove that DIM is an anti-estrogen and support ERβ as a candidate mediating the antiangiogenic actions of DIM.

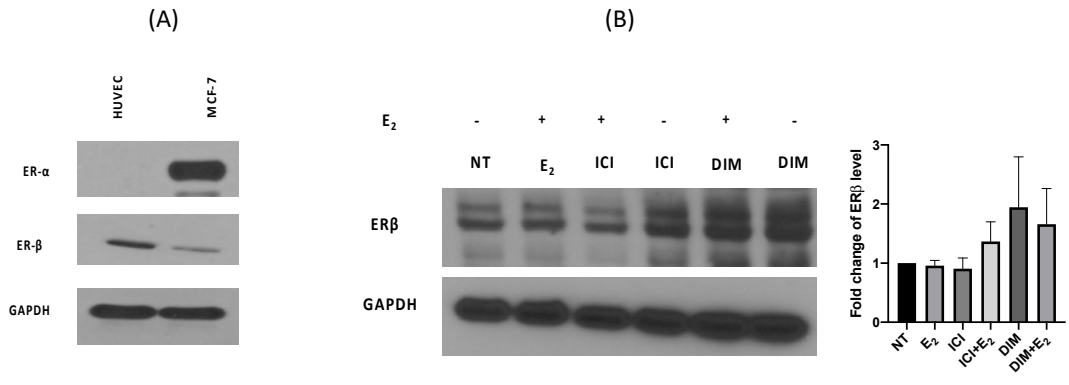


Figure 26. HUVECs express ERβ and DIM enhances ERβ expression

15 μg of whole cell HUVEC lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to Western blot analysis for ER-α (45 KDa) and ER-β (60 KDa) expression. (A) ER-β but not ER-α is expressed in HUVECs. MCF-7, ER-positive cells were used as a positive control. (B) Western blot analysis for ER-β protein level in HUVECs after treating the cells with 25 μM DIM, 25 μM DIM + 10 nM E_2, 1 uM fulvestrant, 1uM fulvestrant + 10 nM E_2, 10 nM E_2 only, or No treatment (NT) for 24 hours. Quantitative measurements of ERβ/GAPDH are shown to the right. Error bars represent ±SEM.

4.12 ERβ blocker (PHTPP) reverses DIM induced inhibition of cell proliferation and G2/M cell cycle arrest

It is known that DIM inhibits the proliferation of a wide range of cell types through induction of cell cycle arrest. Therefore, we used PHTPP, an ERβ selective antagonist, to further elucidate the mechanism of action of DIM on proliferation and cell cycle of HUVECs. PHTPP treatment alone did not alter any cellular phenotype pertaining to cell proliferation or cell cycle progression. Abolishment of ERβ signaling via PHTPP resulted in the loss of DIM mediated inhibition of cell proliferation and cell cycle progression of HUVECs. These results suggest that ERβ is involved in DIM-induced inhibition of *in vitro* angiogenesis (Figure 27). All previous results indicate the effectiveness of DIM in inhibiting angiogenesis; therefore, its effect on vasculogenesis was the next variable to be investigated.

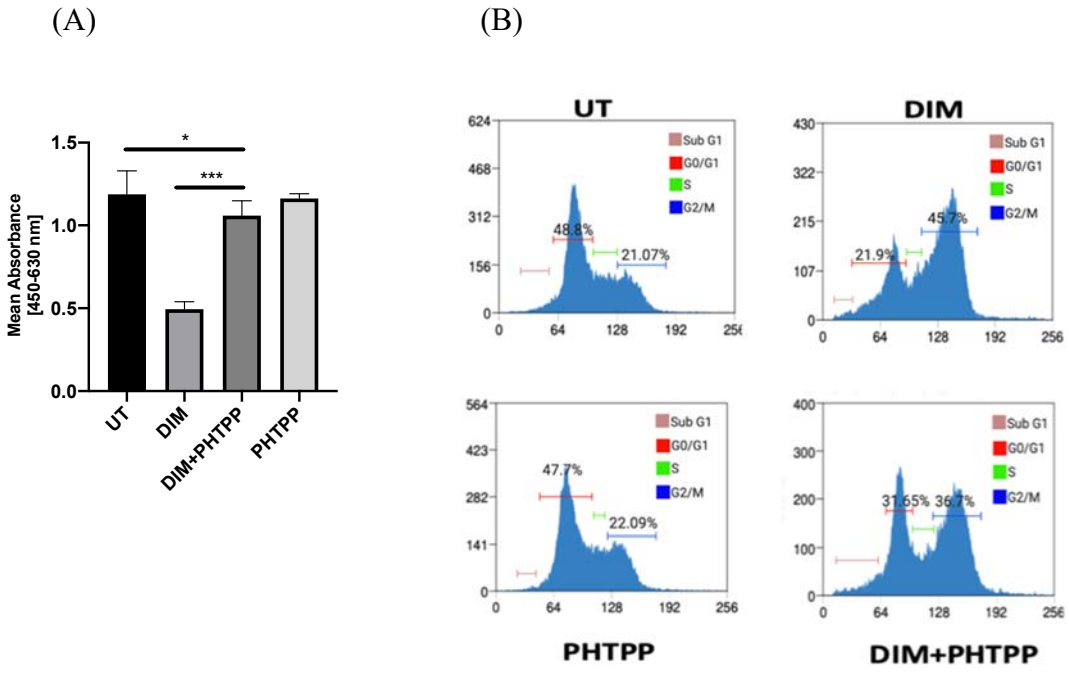


Figure 27. The ERβ blocker PHTPP reverses DIM induced inhibition of cell proliferation and G2/M cell cycle arrest

HUVECs were seeded at a density of 4000 cells per well in 96-well plates and incubated overnight. Cells then treated with 25 uM DIM, 0.1 uM PHTPP, DIM+PHTPP, or left untreated for 24 hours. **(A)** Effect of treatments on proliferation of was determined by XTT assay. XTT reagent was added and absorbance was measured (wavelength of 450 nm and reference of 630 nm) after a further 4 hours of incubation. The graph shows three representative experiment. Significant differences from untreated (UT) controls are indicated as * at a level of $P<0.05$ and *** at a level of $P<0.001$. Error bars represent ±SEM. **(B)** Effect of treatments on cell cycle was determined by flow cytometry.

4.13 DIM reduces secretion of pro-angiogenic cytokines by EPCs

EPCs are critical contributors to a tumor's new vessel formation during its development. Besides the structural support to the newly formed vessels, these cells can also regulate the angiogenic process by secreting several pro-angiogenic cytokines, thus playing a significant role in tumor neovascularization and development. Inhibition of EPC-mediated neovascularization may be a promising therapeutic approach in tumor treatment. EPC-mediated neovascularization is a complex process that requires a series of cytokines; therefore, we investigated the efficacy of DIM in blocking the secretion of pro-angiogenic cytokines by EPCs and antagonizing the enhancement effect of estrogen on EPC secretion of angiogenic factors. DIM markedly inhibited the secretion of a number of cytokines from EPCs, which include the potent pro-angiogenic cytokines angiogenin, GRO, IFN GAMMA, LEPTIN, MCP-1, PDGF-BB, PLGF, RANTES, TIMP-1, TIMP-2, and thrombopoietin (Figure 28). This inhibition was stronger when DIM treatment used alone without estradiol. One of the prominent features of the tumor microenvironment is the continued dominance of angiogenic stimulators, which throws the balance off, causing the growth of blood vessels. The fact that DIM has the capacity to bring down the levels of multiple angiogenic stimulators and eliminate the estrogen's enhancing effect on angiogenic stimulators secretion by EPCs and HUVECs supports investigating it as an antiangiogenic/anti-vasculogenic/anti-estrogenic treatment that may block tumor progression.

Similar to its effects on HUVECs, DIM exhibited strong anti-proliferative effects (Figure 29) and anti-invasive effects on EPCs at 50uM concentration (Figure 30). In HUVECs, we found that DIM is possibly acting through the ERβ pathway. In spite of the

fact that EPCs don't express ERβ, DIM exerted potent effects suggesting that this compound might work via different functional pathways.

The blockage of the angiogenesis and neovasculogenesis by DIM may reduce new vessel formation in the tumors, which in turn may reduce the tumor growth, invasion, and metastasis. Thus, a comprehensive understanding of DIM's mechanism of action may provide potential targets in cancer treatment through inhibition of tumor neovascularization and neoangiogenesis.

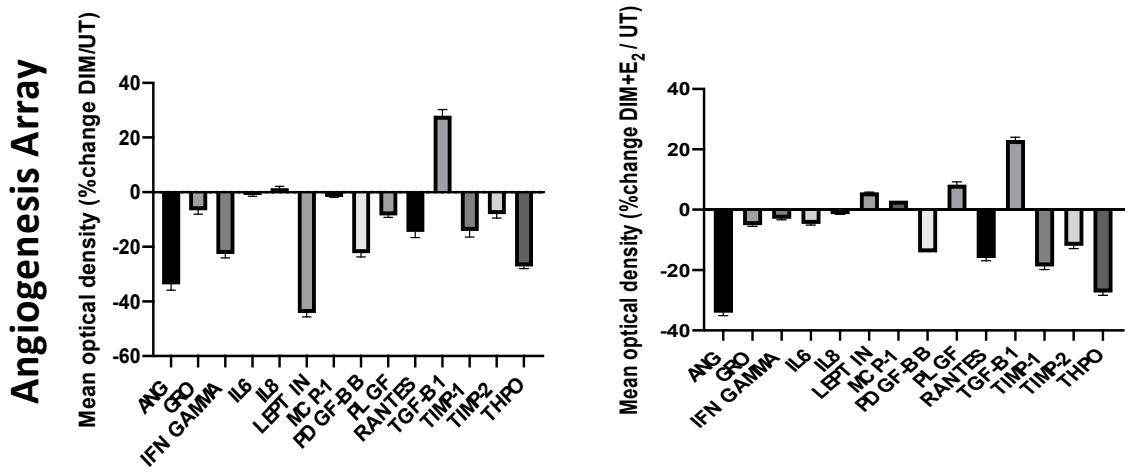


Figure 28. DIM reduces secretion of a number of pro-angiogenic cytokines by EPCs

EPCs were seeded at a density of $1x10^6$ cells per well in 24-well plates suspended in 1 ml of starvation media with 50 μM DIM ± 10 nM E_2 or left untreated. After 24 hours, media was collected, and secretion of cytokines was assayed with an angiogenesis array. Array spots were imaged and analyzed by Image J software. Expression levels were presented as a mean pixel density normalized by the positive spot references and then expressed as the % change in mean optical density compared to the untreated control.

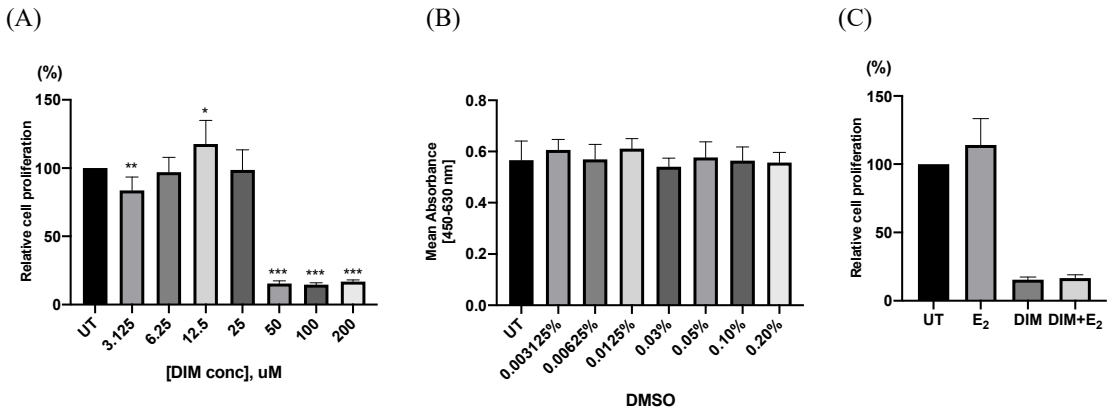


Figure 29. DIM causes dose dependent inhibition in cell viability of EPCs

EPCs were seeded at a density of 1×10^8 cells per well in 96-well plates and treated with either different concentrations of DIM for 24 hours (A), cells treated with vehicle (DMSO) (B), or treated with 50 µM DIM, 50 µM DIM + 10 nM E_2, 10 nM E_2 only, or left untreated for 24 hours (C). The colorimetric Cell Proliferation Kit XTT based method was utilized to assess cell proliferation. XTT reagent was applied to each well, and absorbance (wavelength 450 nm and reference 690 nm) was determined after another 4 hours of incubation. The graph shows three independent experiments (n=3). Significant differences from untreated (UT) controls are indicated as * at a level of $P<0.05$, ** at a level of $P<0.01$, and *** at a level of $P<0.001$. Numbers were calculated based on comparison to untreated controls which were set as 100%. Error bars represent ±SEM.

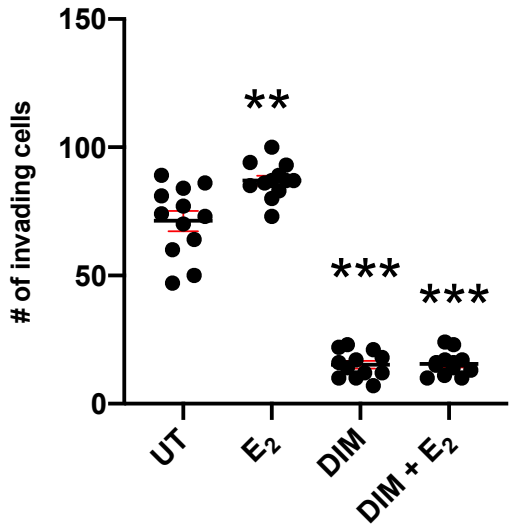


Figure 30. DIM significantly reduces the invasion of EPCs

5×10^3 EPCs, suspended in 500 µl DMEM containing 0.1% BSA supplemented with 50 µM DIM ± 10 nM E2, 10 nM E2 only, or left untreated, were plated in the upper insert of Boyden invasion chambers. In the lower well of the chamber, the chemoattractant used was 750 µl of DMEM, which contained 5% FBS. Data is represented as number of invading cells for each sample well (four 10x micrographs per well were counted) relative to the migration through the control membrane. Results are representative of three independent experiments. Significant differences from untreated (UT) controls are indicated as **at a level of $P<0.01$ and *** at a level of $P<0.001$. Error bars represent ±SEM.

4.14 Summary of specific aim 2

- **DIM exhibits anti-estrogen effects**
- **DIM successfully inhibits the estrogen mediated increase in *in vitro* tubule formation invasion, pro-angiogenic factor secretion, proliferation, and migration of HUVECs**
- **DIM treatment enhances ER beta expression by HUVECs, and ER beta blocker reverses DIM induced effects**
- **Estradiol mediated enhancement of EPC function is abrogated by DIM**
- **DIM is an effective antiangiogenic and anti-proliferative agent**

Specific Aim 3: Evaluate the interaction of EPCs with breast cancer cells and identify the putative mediators

Endothelial progenitor cells circulate in the peripheral circulation and home to the tumor bed to participate in new blood vessel formation. Our previous results indicate that these cells don't only provide structural support but also might be a very crucial component of a tumor supportive tumor microenvironment by paracrine secretion of a plethora of pro-angiogenic growth factors and cytokines, thus playing a crucial role in tumor development.

4.15 Conditioned media (CM) derived from endothelial progenitor cells induces proliferation of breast cancer cells

In order to evaluate the effect of EPC on breast cancer cells, we performed a simple proliferation (XTT) assay using conditioned medium (CM) from EPCs and tested its effect on MCF-7 and MDA-MB-231 proliferation. As expected, CM significantly induced the proliferation of breast cancer cells compared to untreated controls (Figure 31). This effect was in part due to the high expression of a repertoire of proinflammatory and pro-angiogenic cytokines in the CM of the EPC. As mentioned before, CM of EPCs is positive for the 20 tested pro-angiogenic growth factors and cytokines. Further, Recent evidence demonstrated that exosomes secreted by a variety of cells were implicated in tumor metastasis and chemotherapy resistance. We anticipated that EPCs might exert their effects on breast cancer cells by secreting exosomes into the culture medium. To verify, we isolated exosomes from EPC CM using a commercial reagent kit (Total exosome isolation reagent, ThermoFisher) and examined if exosomes, a component of paracrine secretion, are involved in the paracrine effect of the EPCs.

4.16 EPCs secrete exosomes

To establish whether exosomes are an active component of the EPC CM, we first examined if the EPCs produce and secrete exosomes. Electron micrographs identified several multivesicular bodies (MVBs) in the cytoplasm of EPCs. These MVBs contained exosome-like vesicles. These vesicles were composed of a lipid bilayer and were similar to the standard size of exosomes (30-100 nm) (Figure 32A). The MVB membrane invaginated inward, initiating the biogenesis of exosomes (Figure 32A). The MVBs carried these intraluminal vesicles to the cytoplasmic side of the plasma membrane, where the MVB fused and released the exosomes to the extracellular space near recipient cells (Figure 32A). Moreover, the exosomal surface marker proteins CD63 and CD81were positively expressed in these vesicles using western blot assay (Figure 33B).

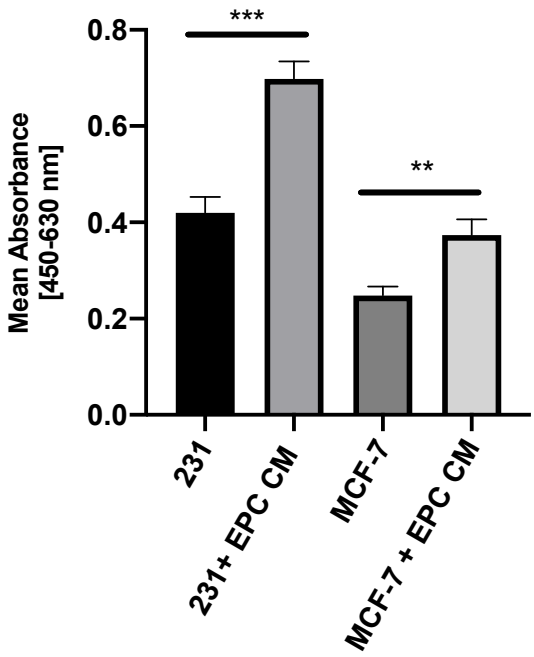
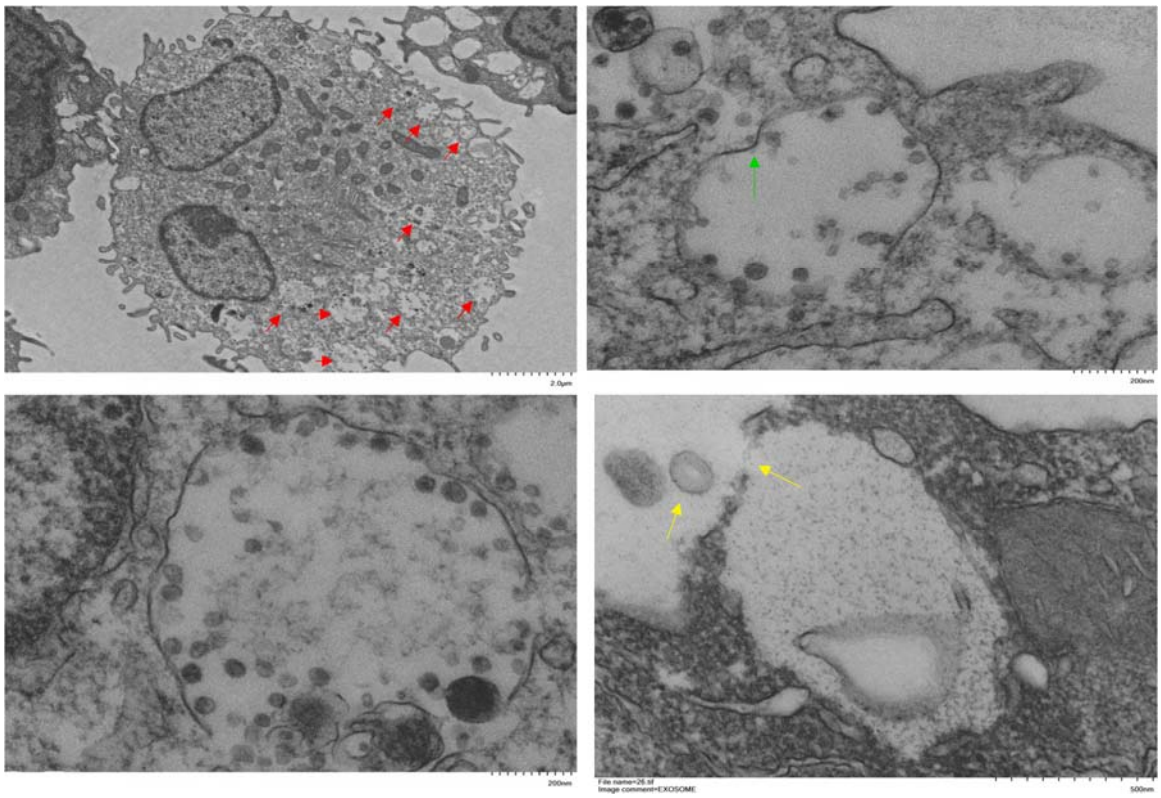


Figure 31. CM derived from endothelial progenitor cells induces proliferation of breast cancer cells

MDA-MB-231 or MCF-7 cells were seeded at a density of 4×10^3 cells per well in 96-well plate and were allowed to adhere overnight. The next day, the cells were starved overnight and treated the next day with either with DMEM or EPC CM for 24 hours. The colorimetric Cell Proliferation Kit XTT based method was utilized to assess cell proliferation. XTT reagent was applied to each well, and absorbance (wavelength 450 nm and reference 690 nm) was determined after another 4 hours of incubation. The graph depicts three independent experiments (n=3). ** indicates significant difference at a level of $P<0.01$. *** indicates significant difference at a level of $P<0.001$. Error bars represent ±SEM.

(A)



(B)

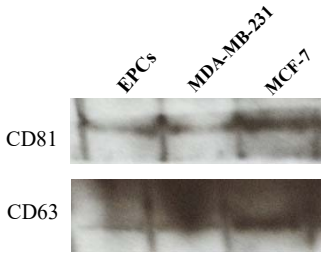


Figure 32. EPCs secrete exosomes

(A) Transmission electron micrographs of EPCs showing cytoplasm with MVBs (upper left micrograph, red arrows) enclosing numerous exosomes (lower left micrograph), inward invagination in the MVB membrane indicates the beginning of exosome biogenesis (upper right micrograph, green arrow), and fusion of MVB with cell membrane and exosomes secretion out from the cell (lower right micrograph, yellow arrows). (B)

Exosome markers CD81 and CD63 proteins were detected by Western blot assay in EPCs exosomes. 15 μg of exosome lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blot analysis, for exosomal markers CD81 and CD63, was performed. MDA-MB-23 and MCF-7 were used as positive controls.

Exosomes derived from EPCs significantly enhance proliferation, migration, and invasion of MCF-7 and MDA-MB-231

To examine if exosomes derived from EPCs can induce proliferation of breast cancer cells *in vitro*, cell proliferation was evaluated by XTT assay in MDA-MB-231 and MCF-7 that had been cultured for 24 hours with DMEM, EPC CM, or EPC exosomes in different concentrations (Figure 33). Proliferation was greater in MDA-MB-231 and MCF-7 when incubated with EPC CM or with EPC exosomes and was more pronounced at higher exosome concentration (Figure 33A). The efficacy of EPCs exosomes was equal to the EPC CM, indicating the potency of these secreted exosomes. Moreover, *in vitro* experiments were performed to evaluate the effect of EPC CM and EPC exosomes treatment on the migration and invasion of breast cancer MDA-MB-231 and MCF-7 cells. The scratch wound assays (Figure 33B) and Boyden matrigel invasion assays (Figure 33C) showed that both EPC CM and EPC exosomes significantly increased migration and invasion abilities of breast cancer cells compared to untreated control cells. Collectively, these results suggest that EPC secreted exosomes can promote metastasis, possibly by enhancing proliferation, migration, and invasion in breast cancer cells.

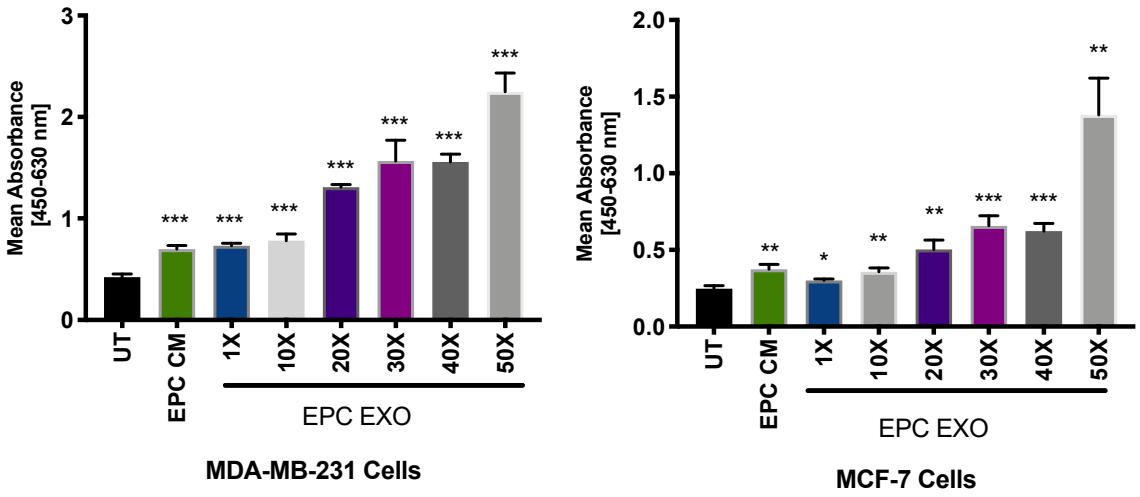
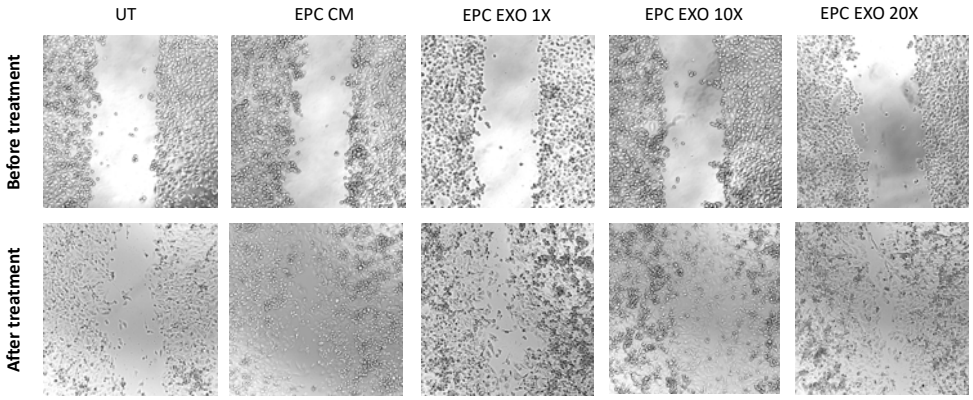
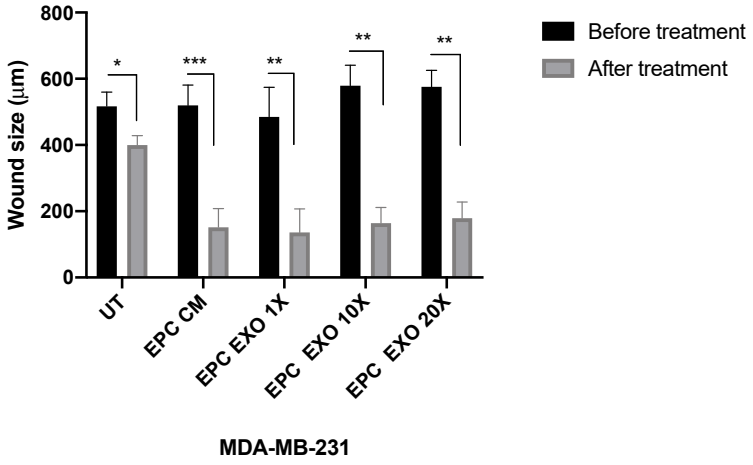


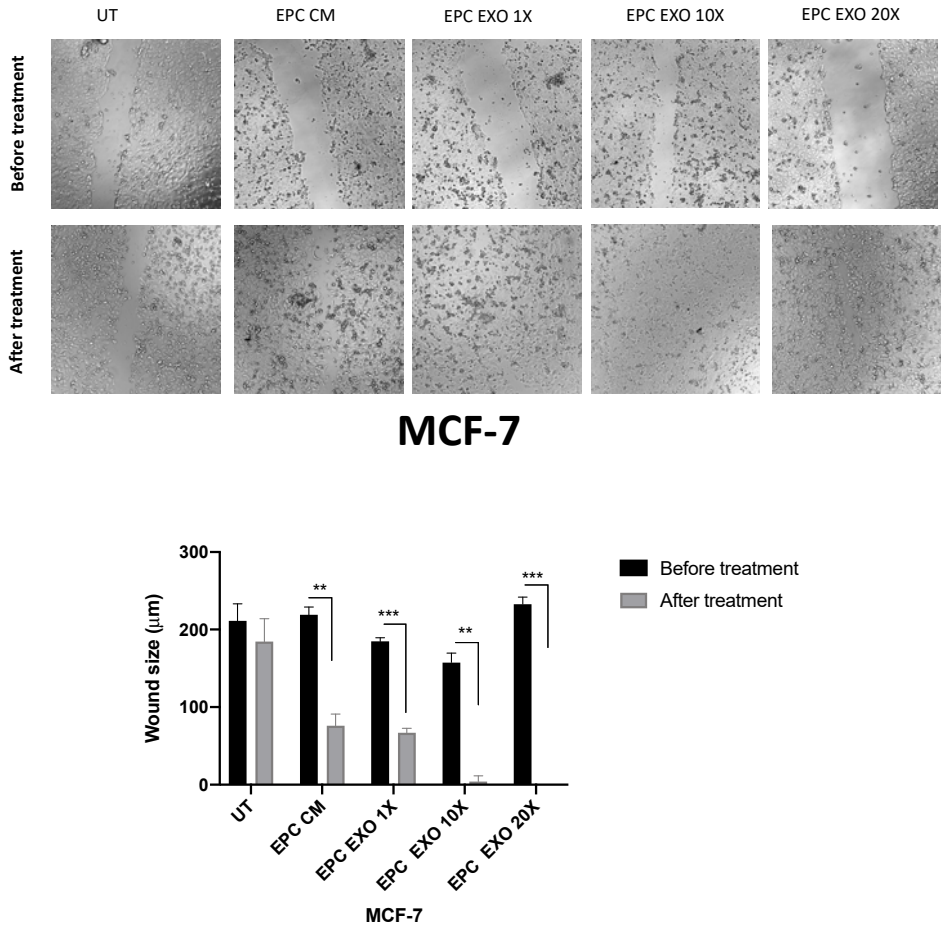
Figure 33. Exosomes derived from EPCs significantly enhance proliferation, migration, and invasion of MCF-7 and MDA-MB-231

(A) MDA-MB-231 or MCF-7 cells were seeded at a density of 4×10^3 cells per well in 96-well plate and were allowed to adhere overnight. The next day, the cells were starved overnight and treated the next day with either with DMEM, EPC CM, or different concentrations of EPC exosomes (1x concentration contains exosomes derived from 100 ul of EPC CM) for 24 hours (h). The colorimetric Cell Proliferation Kit XTT based method was utilized to assess cell proliferation. XTT reagent was applied to each well, and absorbance (wavelength 450 nm and reference 690 nm) was determined after another 4 hours of incubation. The graph depicts three independent experiments (n=3). Results are compared to (UT) untreated control cells. Significant differences from untreated (UT) controls are indicated as * at a level of $P<0.05$, ** at a level of $P<0.01$, and *** at a level of $P<0.001$. Error bars represent ±SEM.

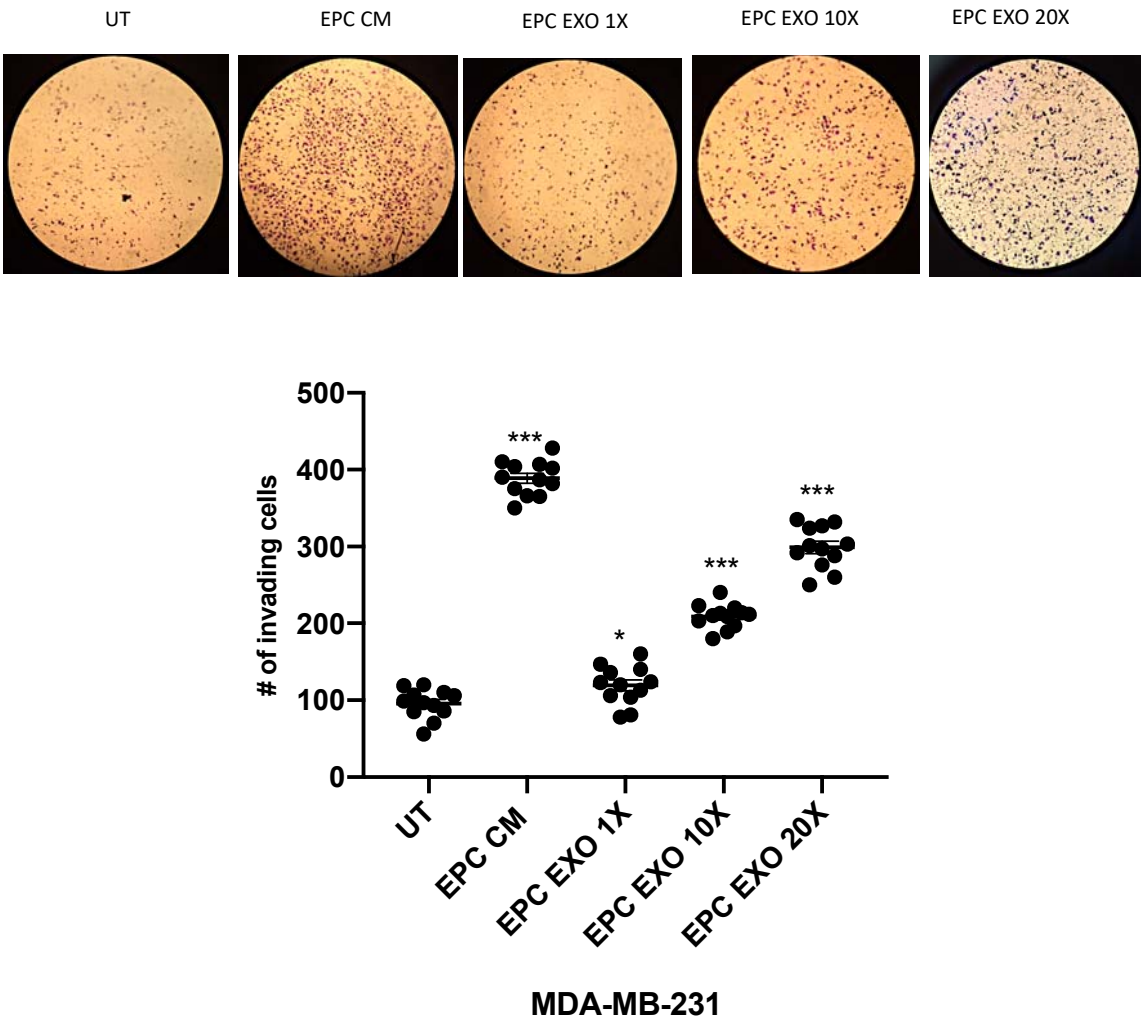

UT
EPC CM
EPC EXO 1X
EPC EXO 10X
EPC EXO 20X
Before treatment
After treatment

MDA-MB-231


Wound size (μm)
800
600
400
200
0
*
***
**
**
**
UT
EPC CM
EPC EXO 1X
EPC EXO 10X
EPC EXO 20X
Before treatment
After treatment
MDA-MB-231



(B) Migratory ability of MDA-MB-231 and MCF-7 cells was evaluated by a scratch wound assay. $4x10^3$ cells were seeded per well in a 96-well plate and allowed to grow till 60–70% confluency. Consequently, a vertical wound was made using a 200 µl sterile pipette tip. Next, cellular debris and detached cells were removed, and the wounded cells were then incubated in fresh complete media, EPC CM, or different EPC exosome concentrations. Images were taken using a light microscope at 10X magnification. The migration distance was then measured using a scale on the microscope. Significant differences are indicated as * at a level of $P<0.05$, ** at a level of $P<0.01$, and *** at a level of $P<0.001$. Error bars represent ±SEM.



(C) The invasive ability of MDA-MB-231 cells was assessed using Boyden invasion chambers. 5×10^3 cells of MDA-MB-231 were resuspended in 500 µl fresh media, EPC CM, or different concentrations of EPC exosomes and were plated in the upper insert of Boyden invasion chambers. In the lower well of the chamber, the chemoattractant used was 750 µl of DMEM, which contained 5% FBS. Images were taken using a light microscope at 10X magnification after 18 hours.

4.17 The exosomes of EPCs contain invasion potentiating miRNAs and oncomiRs

Exosomes can shuttle proteins and microRNAs (miRNAs) into other cells in the tumor microenvironment. An miScript miRNA PCR array (Qiagen) was used to determine the miRNA expression profile of exosomes derived from EPCs. As a comparison, exosomes derived from HUVECs were also analyzed for their content of miRNAs. As listed in (Table 4), exosomes derived from EPCs and HUVECs were enriched with miRNAs. Some of these miRNAs were present in both cell types. However, some miRNAs were specific for the cell type.

Exosomes derived from EPCs and HUVECs contained some miRNAs that are classified as oncomirs, according to the Oncomir Cancer Database. These miRNAs included hsa-miR-21-5p, hsa-miR-16-5p, hsa-miR-106b-5p, hsa-miR-181b-5p, hsa-miR-181a-5p, hsa-miR-17-5p, and hsa-miR-15b-5p. According to the OncomiR Cancer Database (OMCD) and the Cancer Genome Atlas (TCGA) database, miRNA level of these oncomirs are higher in invasive breast cancer tissue, compared with their levels in normal breast tissues. Among the differentially expressed miRNAs, the levels of has-mir-142-3p, one of the onco-miRNAs, was significantly increased in exosomes derived from EPCs compared to exosomes of HUVECs. This miRNA will be the focus of our future experiments since its role in the regulation of breast cancer cell aggressiveness and chemotherapy resistance remains to be characterized. Based on the foregoing data, we propose that endothelial progenitor cells can regulate the invasiveness of breast cancer cells through exosome-mediated delivery of oncogenic miRNAs. These results indicate that endothelial progenitor cells not only provide structural support but may also might be very

crucial mediators of a tumor supportive tumor microenvironment through paracrine secretion of a plethora of pro-angiogenic growth factors, cytokines, and exosomes.

Table 4. The exosomes of EPCs and HUVECs contain invasion potentiating miRNAs and oncomiRs

	2^(-Avg.(Delta(Ct))	
miRNA expressed in HUVEC and in EPC	HUVEC	EPC
hsa-miR-16-5p	81.4±0.9	42.2±1.2
hsa-miR-17-5p	57.4±4.2	22.4±8.7
hsa-miR-181a-5p	20.9±7.4	19.0±1.4
hsa-miR-181b-5p	26.1±4.4	24.0±4.3
hsa-miR-20a-5p	146.2±4.2	67.9±10.7
hsa-miR-21-5p	619.8±2.2	90.9±2.2
hsa-miR-106b-5p	51.5±0.8	26.8±0.1
hsa-miR-15b-5p	18.6±7.6	19.3±0.3
hsa-miR-221-3p	182.7±3.5	29.3±2.7
hsa-miR-222-3p	57.4±8.1	3.2±4.8
hsa-miR-22-3p	231.8±1.0	7.2±0.9
hsa-miR-25-3p	20.0±0.7	8.5±3.6
hsa-miR-409-3p	11.4±3.5	1.6±3.8
hsa-miR-423-5p	10.3±2.0	3.5±5.9
hsa-miR-151a-3p	13.6±0.6	1.7±4.6

miRNA expressed in HUVECs and in EPC	HUVEC	EPC
hsa-miR-451a	13.8±1.2	55.7±4.7
hsa-miR-146a-5p	3.6±4.1	8.3±3.9
hsa-miR-494-3p	8.1±1.19	12.5±5.6
hsa-let-7f-5p	3.5±1.65	11.3±1.09
hsa-let-7g-5p	7.7±3.6	10.6±4.6

miRNAs expressed in HUVEC but not EPC	HUVEC	EPC
hsa-let-7b-5p	14.2±1.3	0.7±0.1
hsa-miR-122-5p	5.1±1.34	0.0±0.007
hsa-miR-155-5p	2.4±4.32	0.8±0.04
hsa-miR-193a-3p	4.8±1.34	0.0±0.03
hsa-miR-29b-3p	6.8±2.9	0.6±0.021
hsa-miR-31-5p	29.6±3.1	0.0±0.01
hsa-miR-34a-5p	35.7±1.11	0.4±0.09
hsa-miR-365b-3p	20.9±2.4	0.02±0.02

miRNAs expressed in EPC but not HUVEC	HUVEC	EPC
hsa-miR-142-3p	0.9±0.05	61.0±6.8
hsa-miR-142-5p	0.1±0.23	2.4±1.0
hsa-miR-146b-5p	0.7±0.13	9.8±2.1
hsa-miR-223-3p	0.9±0.01	37.1±1.3
hsa-miR-199b-5p	0.1±0.12	3.2±4.2

The cutoff value of miRNA expression is **2^-ΔΔCt** >1.5.

4.18 Co-culture of EPCs with breast cancer cells induces differentiation of EPCs toward endothelial cells

Having demonstrated proliferation and invasion stimulating effects of EPCs CM and exosomes on breast cancer cells, the next set of experiments examined the effect of breast cancer cells on the EPCs. Breast cancer cells were co-cultivated with EPCs in a Boyden chamber, which prevents direct cell-cell contact. Breast cancer cells, either MDA-MB-231 or MCF-7, were seeded on the 0.4 μM membrane inserts, which are permeable to supernatants but not to cellular components. EPCs were seeded in the lower chambers and grown for three days. The results showed that breast cancer cells induced the EPCs to differentiate toward ECs (Figure 34A). Co-cultivating resulted in the upregulation of the endothelial cell marker VEGFR-2 in the EPCs, as revealed by flow cytometry (Figure 34A). Moreover, after the co-cultivation, the endothelial progenitor cells exhibited morphological changes and were also positive for the uptake of acetylated LDL (Figure 34B), which further proves that co-culturing the EPCs with breast cancer cells induced EPCs to differentiate toward the endothelial lineage. This effect may have a great impact on tumor growth since the differentiated EPCs are now capable of incorporating into the tumor vasculature, supporting tumor growth and metastasis.

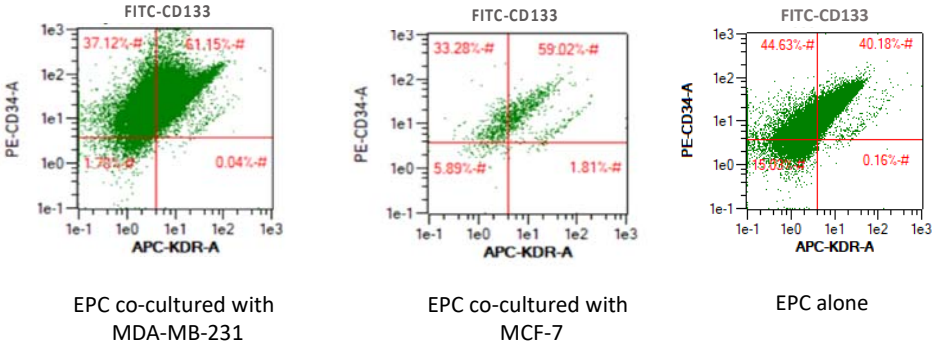
4.19 Exosomes derived from breast cancer increase viability of EPCs

Co-culturing EPCs and breast cancer cells resulted in significant induction of proliferation in the EPCs as measured by XTT assay (Figure 35). Analysis of breast cancer cells CM indicated that breast cancer cells also have the capacity to secrete exosome as revealed by Western blot results, and these exosomes significantly enhanced the proliferation of EPCs in a dose-dependent manner (Figure 36).

4.20 Triple-negative breast cancer secretes pro-angiogenic cytokines

Analysis of CM from breast cancer cells using quantitative angiogenesis array revealed important information in which we found that the CM of the more aggressive subtype of breast cancer (TNBC) is very rich in angiogenic factors compared to MCF-7 cells (Figure 37). These factors include TIMP-2, GRO, IL-6, IL-8, LIF, MCP-1, and IGF-1. This might explain the induction of proliferation seen during the co-culture, and it also might explain the aggressiveness of the TNBCs. These factors can be exploited clinically to target tumor growth.

(A)



(B)

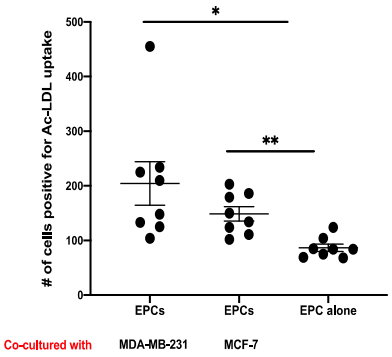


Figure 34. Co-culture of EPCs with breast cancer cells induces differentiation of EPCs toward endothelial cells

At day 7 of primary culture, EPCs were co-cultured with MDA-MB-231 or MCF-7 breast cancer cells in 24-well Boyden chambers for 3 days. Breast cancer cells were seeded in the upper chamber inserts containing 0.4 µm pores which are permeable to soluble molecules but not to cellular components. EPCs were seeded at a density of $1x10^6$ cell/ml in the lower chamber. (A) Expression of CD133/CD34/KDR were assessed using flow cytometry. Gates were set to exclude 95% of the negative control events. (B) Number of cells positive for the uptake of acetylated-LDL after co-culture. Significant differences are indicated as * at a level of $P<0.05$, ** at a level of $P<0.01$. Error bars represent ±SEM. N=4. Two images per well.

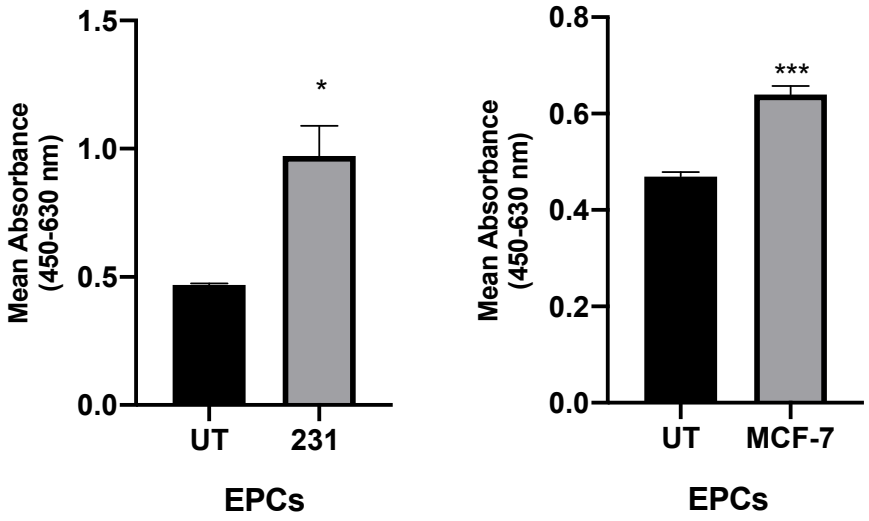


Figure 35. Breast cancer cells significantly enhance proliferation of EPCs

The proliferation of EPCs was measured after three days of co-culture with breast cancer cells. The colorimetric Cell Proliferation Kit XTT based method was utilized to assess cell proliferation. XTT reagent was applied to each well, and absorbance (wavelength 450 nm and reference 690 nm) was determined after another 4 hours of incubation. The graph depicts three independent experiments (n=3). Significant differences from the untreated control are indicated by * at a level of $P<0.05$ and *** at a level of $P<0.001$.

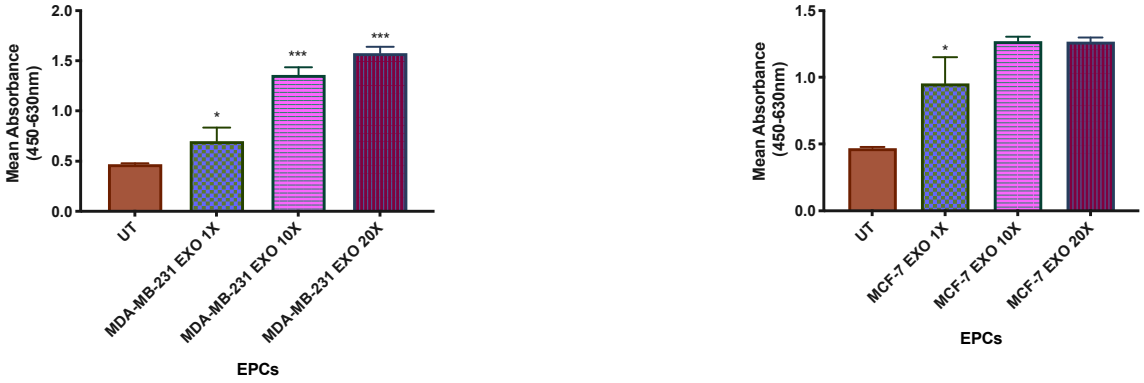


Figure 36. Exosomes derived from breast cancer increase proliferation of endothelial progenitor cells

EPCs were seeded at a density of 4×10^3 cells per well in 96-well. The cells were treated with either with fresh media or different concentrations of breast cancer cells exosomes for 24 hours. The colorimetric Cell Proliferation Kit XTT based method was utilized to assess cell proliferation. XTT reagent was applied to each well, and absorbance (wavelength 450 nm and reference 690 nm) was determined after another 4 hours of incubation. The graph depicts three independent experiments (n=3). Significant differences from the untreated control are indicated by * at a level of $P<0.05$ and *** at a level of $P<0.001$.

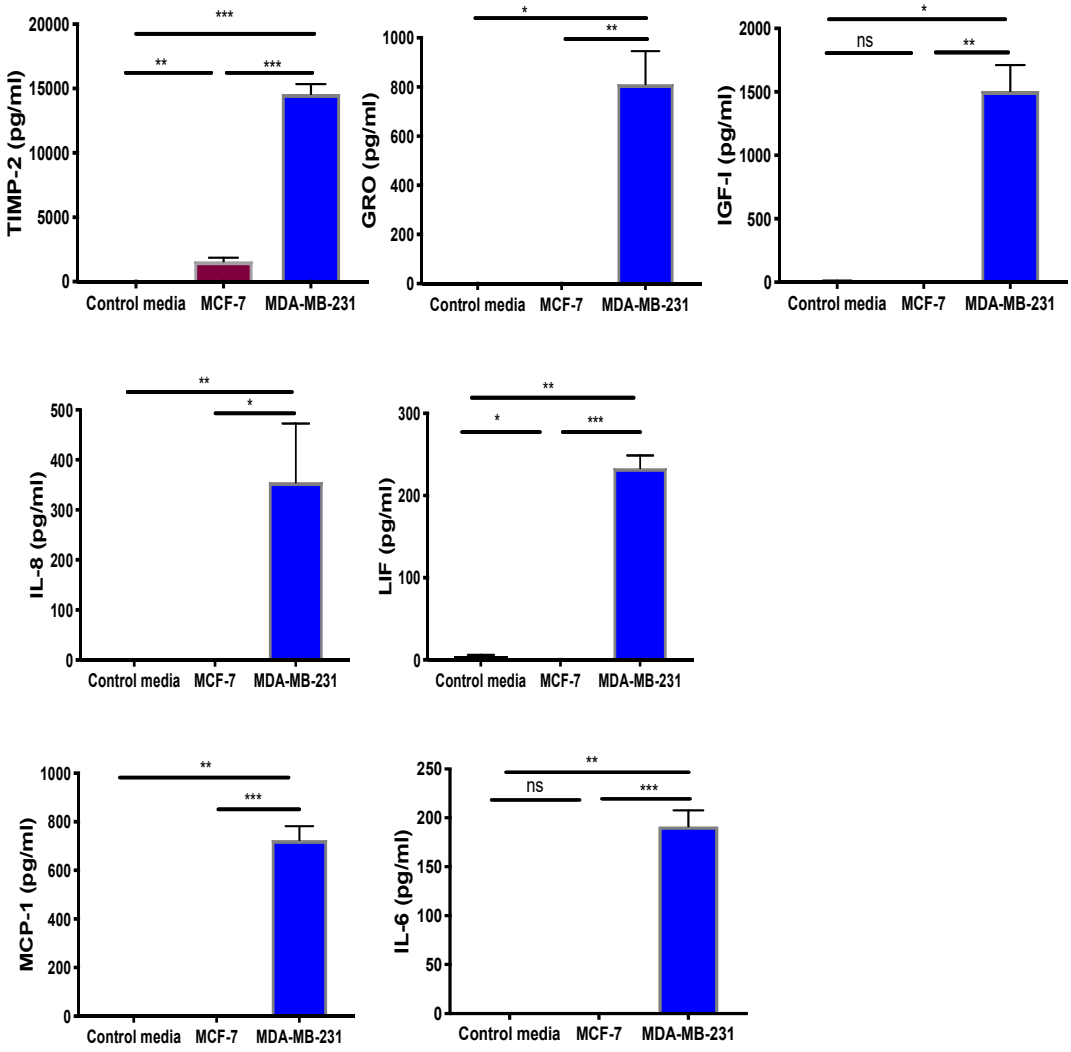


Figure 37. Triple-negative breast cancer cells secrete pro-angiogenic cytokines

Quantibody human quantitative angiogenesis array was performed on breast cancer cell lines MDA-MB-231 CM, MCF-7 CM, and control media to identify human angiogenic factors.

4.21 Summary of specific aim 3

- **CM of EPCs induces proliferation of breast cancer cells**
- **Exosomes of EPCs and secretory cytokines enhance breast cancer proliferation, migration, and invasion**
- **Distinct onco-miRNAs present in exosomes derived from EPCs are upregulated in invasive breast cancer**
- **These cell-cell interactive targets have clinical translatability**

Overall conclusions

Specific Aim 1: EPCs differentiate to functional endothelial cells, and are estrogen responsive

Specific Aim 2: DIM is an effective antiangiogenic and anti-proliferative agent and may be useful for TNBC patients if used in combination with standard therapy to inhibit breast cancer progression

Specific Aim 3: EPCs-Breast cancer cells is a two-way interaction mediated via exosomes containing discrete miRNAs which can be used as therapeutic targets

CHAPTER V: DISCUSSION

Breast cancer is the most common non-cutaneous cancer and the second most common cause of cancer-related deaths in U.S. women (PDQ Adult Treatment Editorial Board, 2019). Approximately 270,600 new cases of invasive breast cancer expected to be diagnosed in 2019 along with 63,000 new cases of non-invasive (in situ) breast cancer. Breast cancer is a heterogeneous disease and can be classified histopathologically into pre-invasive, invasive, and rare breast cancers. Approximately 20% of breast cancers are pre-invasive with DCIS and LCIS, representing 83% and 13% of the total in situ cases, respectively. However, these pre-invasive breast cancers can become invasive as a result of genetic alterations and multistep mutagenic processes. Between 80-85% of all invasive breast cancers are infiltrating ductal carcinoma and the remaining 10- 15% of cases are infiltrating lobular carcinoma (O'Sullivan, Ciara C. *et al.*, 2018). Landmark studies using gene expression profiling have classified breast cancer into at least four distinct intrinsic subtypes. These subtypes include two ER-positive (ER+; luminal A and luminal B) and two ER-negative (ER−; ERBB2 and basal subtypes).

Luminal A tumors show high expression of ER and PR related genes, GATA binding protein 3, low expression of proliferation-associated genes, and lack expression of HER2. Luminal B tumors show decreased expression of ER, overexpression of HER2, and a higher expression of proliferation-related genes. The ERBB2 tumor subtype represents high-grade tumors. It is characterized by the expression of HER2 and by lack of expression of ER and PR. The basal subtype (triple-negative) represents high-grade tumors displaying necrosis and prominent lymphocytic infiltration. Basal IDCs express cytokeratin and such

tumors are most commonly characterized by the lack of expression of ER, PR, and HER2 (Bombonati and Sgroi, 2011).

For nonmetastatic breast cancer, local and systemic therapies are available. Local treatment includes surgical resection and sampling or removal of axillary lymph nodes, with consideration of postoperative radiation. Systemic therapy, which can be given preoperative (neoadjuvant), postoperative (adjuvant), or both, is based on the breast cancer subtype. All HR+ tumors are treated with endocrine therapy and some patients also receive chemotherapy. All ERBB2+ tumors are treated with trastuzumab-based ERBB2-directed antibody therapy plus chemotherapy, and endocrine therapy is added if they are HR positive. Chemotherapy alone is used for triple-negative breast cancer (TNBC) (Waks and Winer, 2019).

TNBC, which affects younger women, typically carries a poor prognosis since it frequently metastasizes to the liver, lungs, and central nervous system early despite optimal adjuvant treatment. Patients with metastatic TNBC have poorer outcomes when compared with patients with other breast cancer subtypes. The median overall survival (OS) is 18 months or less even with vigorous treatment that typically includes a taxane or anthracycline combination. Therefore, novel treatment approaches are critical to improve survival outcomes.

Currently, treatments for breast cancer, except aromatase inhibitors, target the tumor cells directly. However, ample evidence indicates that the tumor microenvironment is a critical participant in each step of the multi-stage process of malignant progression. Breast cancer cells depend on different components of the microenvironment for their survival, dissemination, dormancy, and establishment of secondary sites to form

metastasis. The tumor microenvironment includes the cancer cells along with a variety of non-cancerous cells present in the tumor. These cells include fibroblasts, immune cells, endothelial cells, infiltrating inflammatory cells, adipocytes as well as signaling molecules, and extracellular matrix (ECM) components (Mittal *et al.*, 2018). Stromal cells secrete a range of ECM proteins, chemokines, cytokines, and growth factors that can aberrantly activate autocrine and paracrine loops, which influence the behaviors of the tumor cells in a paracrine or juxtacrine fashion. These interactions between stroma and tumor cells, along with underlying genetic defects of the tumor cells, affect the growth characteristics, morphology, and invasiveness of the tumor (Mittal *et al.*, 2018). Therefore, there is increasing interest in developing new therapeutics that target TME since it can promote tumor invasiveness and metastatic progression.

Malignant transformation requires a fertile microenvironment in which tumor cells proliferate, and under certain circumstances, form a highly invasive and metastatic tumor. The diffusion of oxygen and nutrients from the surrounding tissue is a necessity for the growth of the tumors at the early stage of tumor development. However, this is only enough for the tumor to grow to a size of 1–2 mm^3. Further tumor growth requires the development of new blood vessels (Janic and Arbab, 2010). Neovascularization is a vital process needed for tumor growth and metastases, which requires a contribution from bone marrow-derived endothelial progenitor cells (BM-EPC) (Zhao, X. *et al.*, 2016). EPCs are increased in patients with stage III and IV breast cancers (Naik *et al.*, 2008). Moreover, the expression of EPC markers was found in breast tumors but not in the respective adjacent healthy tissue indicating that breast tumors recruit EPCs in a very targeted and focal fashion (Botelho and Alves, 2016). EPCs can differentiate to functional ECs; therefore, they contribute to the

tumor vasculogenesis by providing structural support. Moreover, they can secrete numerous pro-angiogenic factors that promote blood vessel formation, tumor growth, and metastasis. Mobilization and homing of EPCs from the bone marrow are influenced by different regulatory molecules such as vascular endothelial growth factor, SDF-1, CCL-2, CCL-5, and adiponectin (Zhao, X. *et al.*, 2016). Estrogen is also implicated in mediating the mobilization of bone marrow-derived endothelial progenitor cells to the endothelium after arterial injury by stimulating the expression of EPC nitric oxide synthetase (Iwakura *et al.*, 2003).

Estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. Approximately, 84% of breast cancers are E2-dependent. Most of the estrogen effects on the normal and abnormal mammary cells are mediated via estrogen receptors, ER-α and ER-β, including control of cell proliferation. However, there are also alternative pathways of estrogen actions that do not involve ERs. Estrogen is documented to enhance proliferation of the cells by controlling several protooncogenes and growth factors that induce the cells to enter the cell cycle. It also acts on cyclins that control the cell cycle (Ciocca and Fanelli, 1997). In breast cancer, estrogen binding to either membrane-bound and/or cytosolic estrogen receptors causes activation of several intracellular signaling pathways and promotes the development of the metastatic phenotype (Hua *et al.*, 2008). The link between estrogen, EPC mobilization, and breast cancer was investigated for the possible involvement of E_2 in EPC mobilization to tumors. It was found that E_2 induces homing of EPCs to tumor tissues, and this induction is associated with enhanced expression of angiogenic and matrix metalloproteinase RNA transcripts indicating the important role of this hormone regarding the functional activity

of EPCs. The connection between estrogen and EPCs opens the path for the use of anti-estrogens to inhibit EPC-mediated tumor neovascularization for breast cancer treatment.

Based on the fact that EPCs circulate in the peripheral circulation and home to the tumor bed to participate in new blood vessel formation by providing structural support to nascent vessels and by paracrine secretion of several pro-angiogenic growth factors and cytokines, we hypothesize that EPCs interact with breast cancer cells and determine their phenotype. We propose that interactions between EPCs and breast cancer cells result in more invasive cancer cells, metastasis, and poor patient prognosis. The mechanisms underlying breast cancer cell-EPCs interactions require further investigation, which might identify a novel mediator of tumor progression that can be exploited clinically. To start with, this work focused on EPCs which were defined as a subpopulation of CD34+ cells that are CD133 positive (CD34+/CD133+).

EPCs can be found in bone marrow (BM), peripheral blood (PB), and umbilical cord blood (UCB). UCB contains a significantly higher number of EPCs than PB or BM (Hristov *et al.*, 2003). UCB was selected in this study because it is a significant reservoir of stem and progenitor cells. Progenitors derived from cord blood have several superior characteristics compared to the progenitors derived from adult bone marrow. They are more likely to form colonies, proliferate longer and faster, and have longer telomeres. Cord blood collection does not involve any invasive techniques, either. Transplanted cord blood-derived EPCs efficiently promote postnatal neovascularization *in vivo* (Murohara *et al.*, 2000). In these experiments, EPCs were expanded in suspension cultures since the alternative method of *in vitro* expansion utilizing attached EPCs was associated with increased senescence and inferior proliferation capacity (Janic and Arbab, 2012).

In Aim 1 of this study, it was demonstrated that progenitor cells positive for $CD133^{+}$, isolated from human cord blood mononuclear cells by immunomagnetic positive selection and phenotypically characterized as $CD133^{+/}CD34^{+}$, are highly proliferative and can differentiate into functional endothelial cells. The acquisition of the endothelial phenotype was indicated by changes in cellular morphology, expression of specific mature endothelial markers, uptake of acetylated-LDL, and tubule formation. In addition, experiments in Aim 1 demonstrated that EPCs express ER-α and are estrogen-responsive. Seven nM and 10 nM concentrations of 17 β-E_2 resulted in significant increases in EPC proliferative and invasive capacity suggesting that these cells might be responsive to the growth regulatory effects mediated by the E_2–ER interaction. To determine the biochemical rationale for the estrogen responsiveness of these cells, western blot analysis on whole cell lysates demonstrated that EPCs expressed only ER-α. Therefore, we concluded that EPCs are estrogen-responsive owing to their expression of estrogen receptor-alpha. Following stimulation by estrogen, EPCs secrete proinflammatory cytokines such as ANG, EGF, GRO, IGF-1, IL-8, and TIMP2 that can directly support tumor growth. Therefore, E_2 participates not only in recruiting EPCs but also by inducing them to release cytokines that may promote a microenvironment conducive to tumor survival and growth. The cytokines released by estrogen stimulated EPC's and their potential role in tumor progression are discussed below.

Angiogenin (ANG), a member of ribonuclease A superfamily, is a multifunctional pro-angiogenic protein. It is a strong inducer of blood vessel formation and plays roles in several physiological and pathological processes, including tumorigenesis, neuroprotection, inflammation, host defense, reproduction, wound healing, and

hematopoietic regeneration. Released ANG binds to the actin on the surface of vascular endothelial cells to enable the activation of the matrix protease cascades. The activation of matrix proteases results in the degradation of the basement membrane and extracellular matrix, enabling the migration and invasion of the ECs. Then, the migrating endothelial cells upregulate the expression of ANG receptors resulting in ANG nuclear translocation. ANG nuclear translocation increases the biogenesis of the ribosomes resulting in endothelial cell proliferation. Finally, the proliferating endothelial cells form new blood tubules (Yu *et al.*, 2018).

The expression of EGF and EGFR has been shown to associate with aggressive tumor progression, metastasis formation, and chemotherapy resistance. When the receptor is bound by the EGF ligand, it activates various signaling pathways, including Ras/mitogen-activated protein kinase, 3-kinase/Akt phosphatidylinositol, and nuclear factor-κB. Tumor aberrant expression of EGFR usually gives a more aggressive phenotype and hence is indicative of a bad prognosis (Sasaki *et al.*, 2013).

Malignant cells express and secrete various CXC chemokines, including CXCL1, CXCL2, CXCL3 (GRO family chemokines), CXCL5, CXCL7, and CXCL8. These molecules act on CXCR1 and CXCR2 receptors. They set up a pro-tumorigenic tissue microenvironment and facilitate progression and metastatic dissemination of cancer via autocrine and paracrine loops. The CXC chemokine GRO plays a critical role in wound healing, inflammation, angiogenesis, tumorigenesis, and metastasis. The upregulation of GRO is attributed to the constitutive activation of NF-B in various types of tumors. Other CXC chemokines play a significant role in the regulation of angiogenesis during many pathologic processes, such as tumor growth, ischemia, and wound healing. Chemokines,

such as CXCL8 and CXCL1, have pro-angiogenic functions mediated by binding to the CXCR2 receptor. CXCL8 and CXCL1 are also functionally involved in Bcl2-mediated angiogenesis via an autocrine signaling pathway (Fimmel *et al.*, 2007). CXCL8 can modulate the tumor microenvironment towards an immunosuppressive state by the trafficking of neutrophils, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages, which can locally reduce the anti-tumor immune response (Alfaro *et al.*, 2017).

IGF-IR mediates the biological activities of the IGF ligands. IGF-1R activation leads to phosphorylation of the insulin receptor substrate (IRS) protein family and stimulation of phosphatidylinositol-3 (PI-3) kinase and mitogen-activated protein kinase (MAPK) pathways. This activation results in cyclin D1 upregulation contributing to the phosphorylation of the retinoblastoma protein and the expression of downstream target genes such as cyclin E. Additionally, IGF-1R activation decreases the cell cycle suppressors such as PTEN, suggesting that various pathways are engaged in creating its mitogenic activity. Activated IRSs cause two intracellular signaling networks to be activated: Ras / Raf / Mek / Erk, and PI3K. Ras / Raf / Mek / Erk primarily mediates the mitogenic effects of insulin and IGFs, whereas the PI3 K pathway regulates metabolic and cell growth responses via Akt (Brahmkhatri *et al.*, 2015).

TIMP-2 is a small secretory glycoprotein whose main action is inhibition of MMP activity. These proteases have the ability to degrade extracellular matrix proteins, including collagens, laminin, fibronectin, vitronectin, aggrecan, enactin, tenascin, elastin, and proteoglycans. Most data from model systems indicate that high levels of this inhibitor prevent metastasis. However, in human breast cancers, high levels of TIMP-2 correlate

with both shortened disease-free interval and overall survival (Remacle *et al.*, 2000). In melanoma and lung cancer cells, TIMP-2 expression directly upregulates the transcriptional activity of NF-κB. The effects include decreasing the basal level of IκBα, increasing phosphorylation of IκBα and NF-κB, increasing NF-κB transcriptional activity, and elevated CXCL8 levels (Sun, J. and Stetler-Stevenson, 2009). These data suggest that the balance of downstream activities affected by TIMP2 expression may dictate whether tumor cells acquire a more aggressive phenotype. Based on the fore-mentioned information, the capacity of estrogen to stimulate EPCs to secrete pro-angiogenic proinflammatory cytokines and chemokines can enhance EPC capability in supporting tumor growth and can also result in the recruitment of inflammatory cells to the tumor site to promote a microenvironment conducive to tumor survival and growth.

In Aim 2 of this study, the modulation of EPC functions was confirmed to be an E2-dependent effect, according to the opposite event found in these cells when they were treated with DIM. 3,3-diindolylmethane (DIM) is a natural compound that is found in cruciferous vegetables. *In vitro* studies have found that DIM has anticarcinogenic effects against various cancer cells, including prostate, breast, endometrial, colorectal and pancreatic cancers, and leukemic cells (Zhang, W. W. *et al.*, 2014). The anti-proliferative effect from DIM is mediated through its abilities to induce G2/M arrest of the cell cycle, induce apoptosis, inhibit angiogenesis, regulate sex hormones and their receptors. DIM exerts anti-inflammatory activity, and also shows anti-migration effects by downregulating uPA and its receptor uPAR in prostate cells (Zhang *et al.*, 2014). DIM acts as an anti-estrogen by probably targeting the genomic and non-genomic actions of estrogen: estrogen receptor complex. A variety of synthetic anti-estrogen drugs are available, including

tamoxifen and fulvestrant. However, these anti-estrogens have undesirable toxic side effects. Tamoxifen side effects include mood swings, hot flashes, vaginal dryness, or discharge. Less common side effects of tamoxifen are uterine cancer, blood clots, deep vein thrombosis, pulmonary embolism, and stroke (Waks and Winer, 2019). Prolonged use of fulvestrant causes osteoporosis (PDQ Adult Treatment Editorial Board, 2019). Fortunately, DIM is a non-toxic alternative that exerts antiproliferative and anti-estrogenic effects. Therefore, it was chosen to confirm the estrogen dependency of EPC functions. **In Aim 2**, the efficacy of DIM in abrogating the pro-angiogenic effects of estrogen was demonstrated using a well-established model of *in vitro* angiogenesis.

The essential events in angiogenesis involve enzymatic degradation of the basement membrane, endothelial cells (ECs) activation by pro-angiogenic growth factors, ECs proliferation, directed ECs migration, and tubule formation (Cook and Figg, 2010). In this study, we observed that E_2 was able to significantly enhance *in vitro* tubule formation invasion, pro-angiogenic factors secretion, proliferation, and migration of HUVECs. On the other hand, DIM was able to interfere with all essential events involved in blood vessel formation. Most importantly, **Aim 2** results demonstrated that estradiol enhancement of blood vessel formation is dramatically nullified When DIM and estrogen were applied together to cell cultures.

The physiological functions of estrogen are modulated largely by the estrogen receptors subtypes alpha (ERα) and beta (ERβ). Both receptors are nuclear transcription factors that are involved in the regulation of different physiological processes in humans. ERα is primarily expressed in the mammary gland, uterus, thecal cells of the ovaries, bones, testes, epididymis, stroma of the prostate, liver, and adipose tissue. On the other

hand, ERβ is expressed predominantly in the prostate epithelia, bladder, granulosa cells of the ovaries, colon, fat tissue, and immune system. ERα activation by estrogens is generally considered responsible for enhanced proliferation, whereas this is counteracted by the presence of ERβ, which exerts an antiproliferative effect. In this study, we observed that HUVECs express ERβ, and treatment with DIM resulted in increased expression of ERβ, which exerts antiproliferative effects. Moreover, the effect of DIM was accompanied by the induction of G2/M cell cycle arrest in HUVECs. To validate this observation, HUVECs were treated with PHTPP, which is a selective estrogen receptor β (ERβ) antagonist with 36-fold selectivity for ERβ over ERα. Blocking ERβ in HUVECs abolished the potent DIM mediated cell cycle inhibition. Collectively, observations from **Aim 2** indicate that *in vitro* angiogenesis by HUVECs is estrogen-dependent, and the use of anti-estrogen DIM successfully inhibits the estrogen-mediated increase in *in vitro* tubule formation, invasion, pro-angiogenic factors secretion, proliferation, and migration of HUVECs. These results support the induction of ERβ expression as the mechanism behind DIM's effect on HUVECs.

After confirming the effectiveness of DIM in inhibiting the pro-angiogenic effects of estrogen, this anti-estrogen was used to treat EPCs to validate the estrogen dependency of EPC functions. This study demonstrated that E_2 significantly enhanced pro-angiogenic factor secretion, proliferation, and invasion of EPCs. Most significantly, estradiol mediated enhancement of EPCs function was drastically annulled by DIM when DIM and estrogen were applied together to cell cultures. This finding confirms that EPCs' function is estrogen-dependent.

These results highlight the role of estrogen in breast cancer, even in the subtype that is estrogen receptor-negative (TNBC). These results open the venue for novel antitumor strategies using anti-estrogens for the treatment of estrogen negative breast cancer to target the estrogen-responsive cells in the tumor microenvironment. These results also indicate that the natural anti-estrogen DIM may be a safer alternative for the clinically available anti-estrogens, which are associated with unwanted side effects. In HUVECs, we found that DIM is possibly acting through the ERβ pathway. However, EPCs do not express ERβ, but DIM exerted potent effects suggesting that DIM might work via different functional pathways, which makes it a powerful anti-estrogen treatment in neutralizing tumor angiogenesis and neovasculogenesis.

A previous study has shown that EPCs are vital determinants which contribute to breast cancer growth (Suriano *et al.*, 2008). EPCs form a structural component of the breast cancer neovasculature and maintain a supportive tumor microenvironment, thus playing a crucial role in tumor development. In this study, we demonstrate that a significant component of the pro-angiogenic paracrine activity associated with EPCs is mediated by exosomes. Using the XTT assay, we found that treatment of breast cancer cell lines MDA-MB-231 (Triple-negative breast cancer) and MCF-7 (Luminal B breast cancer) with conditioned media (CM) from endothelial progenitor cells (EPCs) resulted in a significant increase in cell viability and proliferation. This finding indicates that the presence of EPCs in the tumor microenvironment is not only to structurally support the growing vasculature, but the effect of these EPCs can go beyond that to the support of tumor growth, proliferation, and invasiveness through secretion of paracrine factors.

Using angiogenesis arrays to measure angiogenic factors in the CM of EPCs, we found that EPCs secrete growth factors, including basic fibroblast growth factor, EGF, platelet-derived growth factor, and TGF-β, which play essential roles in maintaining cancer stem cells and promoting tumor growth. EPCs also secrete IL-6, which enhances the metastasis to the bone by upregulating CXCR4 through STAT3 and c-Jun. Moreover, EPCs secrete VEGF. VEGF expression in usually correlates closely with microvessel density. Furthermore, microvessel density and VEGF expression are related to tumor grade and lymph node metastasis in patients with invasive ductal carcinoma. Currently, exosomes are known as crucial vehicles for intercellular communication. Therefore, we analyzed the conditioned media of endothelial progenitor cells for secretory exosomes. Exosomes are crucial components of extracellular vehicles (EVs), ranging in size from 30-150 nm (Kalluri, 2016). They have attracted considerable attention in the past decade due to their distinctive characteristics. Exosomes are characterized by immune compatibility, low toxicity, and nano-scale size, and are also relatively homogenous and stable. Exosomes are generated by almost all cell types and can be extracted from various biological fluids. Several proteins such as tetraspanins (CD63, CD9, CD81), heat shock proteins (Hsc70), lysosomal proteins (Lamp2b), and fusion proteins (flotillin, annexin) are identified on the surface of exosomes (Vlassov *et al.*, 2012) (van Dommelen *et al.*, 2012).

Exosomes are released from cells upon fusion of the multivesicular body (MVB), with the plasma membrane. The accumulated information indicates that exosomes are biologically important for cancer development and metastasis (Kikuchi *et al.*, 2019). Exosomes are crucial vehicles for intercellular communication by transferring proteins, RNA, or microRNA directly into the cytoplasm of target cells (Wang *et al.*, 2019). In order

to induce a response from recipient cells, exosomes might either fuse with the plasma (Wang *et al.*, 2019), be taken up entirely via endocytosis, or attach to the surface of recipient cells to stimulate a signaling response (Edgar, 2016).

In this study, we show that EPCs secrete exosomes. These exosomes are positive for exosomal protein markers (CD81 and CD63) and are morphologically similar in size and shape to exosomes described in the literature. Electron micrographs indicate the presence of several MVBs in the cytoplasm of EPCs. These MVBs contain exosome-like vesicles (intraluminal vesicles) similar to the standard size of exosomes (30-100 nm). The MVB membrane invaginates inward to start the biogenesis of exosomes. The MVBs carry these intraluminal vesicles to the cytoplasmic side of the plasma membrane, where the MVB fuses and releases the exosomes to the extracellular space near recipient cells.

The fact that EPCs secrete intact exosomes indicates that these cells can induce a response in the recipient cells. Therefore, we sought to determine the effect of exosomes derived from EPCs on breast cancer cells proliferation, invasion, and migration. Exosomes derived from endothelial progenitor cell significantly enhanced the proliferation of MDA-MB-231 and MCF-7 breast cancer cells. The proliferation enhancement effect of EPCs exosomes was comparable to the proliferation enhancement effect of the EPC CM. EPCs CM contains exosomes and secreted soluble proteins, and the fact that EPC exosomes had similar effects to the EPC CM indicates that the EPC exosomes are an important paracrine component of EPC induced breast cancer cell proliferation.

The effects of exosomes on cancer cell aggressiveness remain uncharacterized. **Experiments in Aim 3** showed that CD81, CD63 positive exosomes derived from EPCs promoted the migration of breast cancer cells, and significantly enhanced breast cancer cell

invasion. Both migration and invasion are essential events involved in cancer cell metastasis. EPCs exosomes might contribute to the aggressiveness of breast cancer by promoting EMT, as evidenced by enhancing the migration and invasion of MDA-MB-231 and MCF-7.

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells lose their cell-cell contacts and apicobasal polarity, and acquire mesenchymal properties, coupled to the ability to migrate and to invade the surrounding tissues. EMT permits differentiated cells to acquire the ability to migrate from the primary tumor, invade the basement membrane, and enter the vasculature. Transitional tumor cells can then exit from circulation and migrate into the tissue parenchyma forming metastatic secondary tumor sites. It is known now that metastasis is the major cause of death in cancer patients. Therefore, identifying the factors that induce EMT and target them may represent a strategy to prevent metastasis.

Exosomes have the capacity to stimulate both receptor-mediated and genetic mechanisms by transferring proteins, RNA, or microRNA into the cytoplasm of target cells. Growing data suggest that miRNAs, small non-coding RNAs, are packed into the exosomes and can be efficiently transmitted to the recipient cells to cause post-transcriptional gene expression regulation by attaching to the complementary sequence in the 3′ UTR of mRNAs. miRNAs are synthesized through different pathways. All pathways eventually form a functional miRISC complex (miRNA induced silencing complex). miRISC is a multi-protein complex that incorporates a functional miRNA strand, known as the guide strand, which targets the complementary mRNA transcripts. The miRISC

complex can regulate gene expression either by mRNA degradation, translational repression, heterochromatin formation, or DNA elimination.

A number of studies have independently identified miRNAs whose expression levels correlated with tumor aggressiveness and poor prognosis (Schoof *et al.*, 2012). miRNAs whose expression is upregulated in tumors are termed oncogenic miRNAs (oncomirs) and are proposed to assist cancer progression in a number of ways such as inhibiting the expression of tumor suppressor genes involved in different biological processes. Other oncogenic miRNAs influence the cell cycle and contribute to cell proliferation by affecting CDK inhibitors or transcriptional repressors of the retinoblastoma family proteins (Frixa *et al.*, 2015). Some oncogenic miRNAs are implicated in the regulation of the p53 transcriptional activator. It regulates the expression of many target genes involved in different cellular processes including cell cycle arrest, apoptosis, DNA repair and senescence. It has been found that some miRNA function as negative regulators of p53 through direct binding to two sites of p53 promoter (Frixa *et al.*, 2015). Other miRNAs are induced by MYCN, a member of the MYC family, and positively correlate with MYCN-amplification in neuroblastoma. MYC, proto-oncogene, encodes transcription factor that regulates cell proliferation, growth and apoptosis (Frixa *et al.*, 2015).

qRT-PCR analysis performed in this study showed that exosomes derived from endothelial progenitor cells are enriched with oncogenic microRNAs or oncomirs including hsa-miR-21-5p, hsa-miR-142-3p, hsa-miR-16-5p, hsa-miR-106b-5p, hsa-miR-181b-5p, hsa-miR-17-5p, and hsa-miR-15b-5p. These oncogenic miRNAs were identified through a search of OncomiR, an online resource for exploring pan-cancer microRNA

dysregulation. These miRNAs are upregulated in invasive breast cancer compared to normal tissues suggesting a functional role in breast cancer progression. The characteristics and proposed mechanism for oncogenesis of each of these oncomirs is discussed below.

miR-21 is one of the most commonly reported oncogenic miRNAs in several solid tumors where upregulation is commonly associated with adverse outcomes. Functional studies have also demonstrated that miR-21 upregulation can induce cell proliferation, migration, and apoptosis inhibition. miR-21 target genes are tumor suppressor genes, including PTEN and PDCD4 (Anwar *et al.*, 2019).

hsa-miR-181b-5p is one of the miR-181 family members that contains four highly conserved mature miRNAs. miR-181s are aberrantly highly expressed in several tumor tissues, including pancreatic, head and neck, and bladder cancer. Transient expression of miR-181b activates the epigenetic switch and inhibits the cylindromatosis (CYLD) tumor suppressor gene from negatively modulating NF-κB activity (Liu *et al.*, 2014).

This investigation focused on the oncomir miR-142-3p since it was observed that miR-142-3p was specific to the exosomes derived from the EPCs when compared to the exosomal miRNA content of HUVECs (as differentiated ECs). Moreover, it was highly enriched in exosomes derived from EPCs compared to the endogenous controls cel-miR-39-3p, cel-miR-39-3p, SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6-6P, and miRTC. miR-142 was first identified in hematopoietic stem cells and is critical for the formation and differentiation of hematopoietic stem/progenitor lineages, and the fact that it is significantly upregulated in invasive breast cancer suggests that exosomes secreted by endothelial progenitor cells may shuttle miR-142-3p into breast cancer cells. miR-142-3p has different functions in cancer, virus infection, inflammation, and immune

tolerance. miR-142-3p is highly expressed in undifferentiated cells, but it is downregulated in differentiated cells. Two mains consequences take place in the embryonic stem cells if miR-142 is deleted. Deleting miR-142 results in the differentiation of these cells into neuroectoderm, mesoderm, and endoderm layers. This effect is accompanied by complete inhibition of the expression of the pluripotency marker *Oct4*. Low levels of miR-142 induce KRas and Erk phosphorylation which initiates differentiation. This suggests that miR-142 can play an essential role in maintaining stem cell pluripotency (Shrestha *et al.*, 2017). In non-small cell lung carcinoma cell lines, miR-142-3p downregulated TGFß-induced growth inhibition by targeting the TGFß-R1 (Lei *et al.*, 2014). Moreover, miR-142-3p was found to induce the progression of tumors in human breast stem cells by targeting APC. Loss of APC expression induces the activation of the canonical WNT/CTNNB1 signaling pathway leading to excessive cell growth in breast tissue (Isobe *et al.*, 2014).

This study supports a profound impact of EPCs on breast cancer. Further studies are needed to confirm our hypothesis that miR-142-3p is shuttled to the breast cancer cells via exosomes of endothelial progenitor cells. The identification and verification of these cellular interactions may provide additional strategies to negate the tumor-promoting functions of EPCs by targeting those regulators and/or effectors, which could have significant potential in anticancer therapies.

The cellular interactions between EPCs and breast cancer cells are not a one-way interaction. The co-culture of EPC with breast cancer cells increased EPC proliferation and was associated with an increase in the percentage of the cells undergoing differentiation, as evidenced by morphological changes and flow cytometry results. The co-culture of

EPCs with breast cancer cells resulted in the production of functional ECs capable of taking up acetylated-LDL. These findings explain how cells in the tumor microenvironment interact with each other to assist tumor growth. Angiogenesis is an essential process for tumor growth and metastasis. Tumors have to establish a blood supply, or they cannot grow more than 1-2 mm^3. It was shown here that breast cancer cells induce the endothelial progenitor cells to differentiate into functional ECs and thus maintain a sufficient blood supply. Therefore, the secretome of breast cancer cells must be determined.

Analysis of breast cancer cells CM indicated that these cells have the capacity to secrete exosomes, which have proliferative effects on EPCs when cultured together. Moreover, further analysis of the conditioned media from breast cancer cells indicated that the more aggressive breast cancer type, MDA-MB-231, produced more growth factors. These growth factors include TIMP-2, GRO, IL-6, CXCL8, LIF, IGF-1, and MCP-1, which can all enhance tumor blood vessel formation. This study identified more angiogenic factors in the CM of breast cancer cells other than VEGF. These factors can be exploited in a multi-drugs approach to inhibit tumor-associated angiogenesis and neovasculogenesis since breast cancer was partially sensitive for antiangiogenic therapy (Lupo *et al.*, 2017).

The tumor microenvironment is a complex and dynamic environment. Different cells have been recognized for their role in supporting this milieu. In this study, we uncovered new roles for the endothelial progenitor cells in the tumor. Different targets have been discovered in this study that might have clinical translatability. Moreover, the findings in this study indicate that DIM is an effective antiangiogenic and anti-proliferative agent and may be useful for TNBC patients if used in combination with standard therapy to inhibit breast cancer progression.

Limitations of this study

Although this study used an *in vitro* model to evaluate the interaction between breast cancer and endothelial progenitor cells, there are a number of limitations present. There are several important advantages to cancer cell lines. Cell lines have played a significant role in elucidating signaling pathways, easy and inexpensive to use, provide rapid experimental results, and can be genetically manipulated through homologous recombination, short hairpin RNA (shRNA) gene knockdown, or CRISPR-Cas9 gene editing. However, cell lines lack the functional and genetic heterogeneity of human cancers, which is a significant factor in resistance to targeted therapies. With time, a heterogenous cancer cell population may be selected for a clonal expansion of cells specifically with repeated cell culture. Therefore, there would be loss of breast cancer heterogeneity. *In vivo* studies using patient-derived xenografts (PDX) and orthotopic transplant models would create a better model to use to study tumor microenvironment.

Another limitation of the study is related to the use of total exosome isolation reagent to isolate exosomes. The kit works through exosome precipitation. This technique alters the solubility or dispersibility of exosomes. It uses water-excluding polymers to tie up water molecules and force less soluble components out of solution. Basically, samples are first pre-cleaned from cells and cellular debris. Next, samples are mixed with the total exosome isolation reagent. After incubation at 4 °C overnight, the precipitate containing exosomes is isolated by low speed centrifugation. This method is easy to use and does not require any specialized equipment. However, it is increasingly clear that this method results in the co-precipitation of other non-exosomal contaminants like proteins and polymeric materials.

In this study, we employed the Invitrogen™ Total Exosome Isolation Reagent to isolate exosomes. The isolated exosome preparations were highly enriched for the classical exosome markers CD63, CD81, and CD9 and devoid of gross contamination. According to the data provided to us from the manufacturer, exosomes isolated with Invitrogen™ Total Exosome Isolation Reagent (from cell culture media) have a yield and size distribution similar to exosomes recovered with a sucrose gradient using a standard ultracentrifugal procedure. Profiles, as examined on NanoSight™ LM10, show that all particles are smaller than 300 nm; the majority of them are approximately 50–150 nm in size. However, additional procedures need to be employed to further separate exosomes from non-vesicular (NV) components.

In their study, using 6%–30% iodixanol density gradient fractionation on pellets prepared using conventional differential centrifugation, Jeppesen *et al* were able to separate small extracellular vesicles (sEVs) ($CD63^+$ and $CD81^+$) from non-vesicular material (Jeppesen *et al.*, 2019). Direct immunoaffinity capture (DIC) can also be used to specifically isolate exosomes from other types of sEVs. The use of these methods on pellets derived from total exosome isolation reagent will enhance the quality of the obtained results.

Yet this study still fits into the therapeutic landscape of breast cancer. This study identifies endothelial progenitor cells (EPCs) as one of the main stromal cells within the TME that have the ability to influence breast cancer development through crosstalk initiated by secretory factors such as cytokines, chemokines, growth factors, and exosomes. EPCs interaction with breast cancer cells results in more invasive cancer cells. Secretory component derived from EPCs promoted the migration of breast cancer cells, and

significantly enhanced breast cancer cell invasion. Both migration and invasion are essential events involved in cancer cell metastasis. With this information, we may better characterize the factors that influence breast cancer metastasis- the leading cause of breast cancer-related deaths in women.

Another important feature of this study is that we identified EPCs as estrogen-responsive, which highlights the role of estrogen in breast cancer, even in the subtype that is estrogen receptor-negative (TNBC). We investigated the property of a natural dietary compound found in cruciferous vegetables, 3,3-diindolylmethane (DIM), to target the estradiol mediated enhancement of EPCs function. These results open the venue for novel antitumor strategies using anti-estrogens for the treatment of estrogen negative breast cancer to target the estrogen-responsive cells in the tumor microenvironment.

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