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Mechanisms Of Hormonal Regulation Of Invasiveness And Metastasis Of Luminal Breast Cancer

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MECHANISMS OF HORMONAL REGULATION OF INVASIVENESS AND METASTASIS OF LUMINAL BREAST CANCER

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DEDICACTION

This dissertation is dedicated to my family and friends, who have been my role models

throughout my life.

To my mother, who has fought cancer and understands the true trials and tribulations of this

disease. She was my first teacher and was instrumental in my decision to study biological

sciences.

To my father who has taught by example to be resourceful, patient, loving, strong, and

independent.

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AF	Activation Function Domain
AI	Aromatase Inhibitor
CK	Cytokeratin
DCIS	Ductal Carcinoma In Situ
E_1	Estrone
E_2	Estradiol
E_3	Estriol
EMT	Epithelial to Mesenchymal transition
ER	Estrogen Receptor
ERE	Estrogen Response Element
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HER2	Human Epidermal Growth Factor
HRT	Hormone Replacement Therapy
Hsp	Heat Shock Protein
IDC	Invasive Ductal Carcinoma
IHC	Immunohistochemistry
LBD	Ligand Binding Domain
LN	Lymph Node

miR	micro RNA
miRNA	micro RNA
MPA	Medroxy Progesterone Acetate
PR	Progesterone Receptor
PR-A	Progesterone Receptor Isoform A
PR-B	Progesterone Receptor Isoform B
PRE	Progesterone Response Element
RISC	RNA Induced Silencing Complex
SERD	Selective Estrogen Receptor Down Regulator
SERM	Selective Estrogen Receptor Modulator
SFM	Serum Free Media
SHBG	Serum Hormone Binding Globulin
TNBC	Triple Negative Breast Cancer

CHAPTER 1: BACKGROUND

1.1 Clinical pathology of breast cancer Breast cancer is the most common type of cancer diagnosed in women and the second leading cause of cancer related deaths in the United States (1). Due to the dependence on female sex hormones for growth and progression, this disease is diagnosed predominantly in women; however, it is still observed in men, accounting for less than one percent of all diagnosed breast cancers.   Herein, we will discuss the biological properties of breast cancer from origin within the breast tissue to developed lesions and what factors drive this progression.

1.1.1 *Histological subtypes:* In order to understand breast cancer as a disease, it is important to know the biological composition of the breast and that not all breast cancer is the same. Although being classified under the umbrella of breast cancer, the origin of the tumor within the breast is critical for diagnosis and treatment of the disease. Breast tissue comprises lobules, ducts, adipose tissue, and connective tissue. The lobules produce milk, and the ducts carry milk to the nipple, whereas the connective tissue and adipose tissue provide structural support to hold other tissues in proper orientation to deliver milk (2). Breast cancer usually develops in the lining of the ducts, which comprises differentiated epithelial cells (3). The predominant histological subtypes of breast cancer are ductal carcinoma *in situ* (DCIS)*,* invasive ductal carcinoma (IDC), and invasive lobular carcinoma (ILC) (4). Ductal carcinomas (DCIS, and IDC) are the most commonly diagnosed breast cancers. DCIS is intra-ductal, confined to the duct structure and has not yet invaded through the basement membrane (5), whereas IDC is invasive and has left the confines of the duct (6). DCIS is commonly treated by surgical resection involving lumpectomy (minimal tissue removal), or mastectomy (partial or complete), followed by radiation (7). IDC is the most common type of invasive breast cancer (80%) and its prognosis is variable, depending on

histopathological grade/subtype, tumor size, lymph node positivity, hormone receptor status and HER2 status (4). Treatment for IDC includes surgery (lumpectomy or mastectomy) followed by radiation, chemotherapy and hormone therapy. ILC is less common (<15% of invasive breast cancers) and predominantly occurs in post-menopausal women and usually maintains hormone receptor status throughout progression (7). Treatment of ILC is identical to IDC but the metastatic tissue profile is quite distinct. IDC preferentially metastasizes to the lungs, bone and central nervous system, whereas ILC metastasizes to gastrointestinal sites and organs located in the peritoneal cavity (8–10).

1.1.2. *Breast cancer staging*: After determining the histological subtype of the disease, it is important to diagnose the stage, an indication of how advanced the disease is for both diagnostic tools and for therapeutic intervention. The most common classification of staging for breast cancer is the TNM system (T= tumor size, N= lymphnode status, and M= metastasis). Tumor size can be denoted as "tis" (which represents ductal/lobular carcinoma in situ) or be given a numeric value. A value of 1 represents a tumor size less than 2cm. A value of 2 represents a tumor larger than 2cm but less than 5cm. A value of 3 is a tumor larger than 5cm, and a value of 4 is any tumor that has spread beyond the breast. Lymph node (LN) status is divided in to 4 subcategories; N0 (no LN metastasis), N1 (1-3 axillary LN are positive for cancer cells), N2 (4-9 axillary LNs are positive), and N3 (10 or more axillary LN are positive, or a supraclavicular LN is positive). Metastasis is subcategorized into 3 groups: MX (cannot be assessed), M0 (no metastasis), and M1 (metastasis). Based upon this classification system, breast cancers can be subdivided into stage I-IV. Stage I is classified as early detected breast cancer whereas stage II and III are defined as locally advanced breast cancer. Stage IV is any breast cancer that has metastasized.

1.1.3. *Molecular classification***:** Estrogen has long been thought to be the primary culprit in breast oncogenesis, beginning with the discovery in 1902 that removal of ovaries in women with breast cancer resulted in regression of tumor size (11). Furthermore, it was discovered that estrogen was one of the primary hormones produced by the ovaries (12) and this led to strategies in the early 20th century to focus on surgery and oophorectomy which resulted in positive outcomes (30% survival). In 1968 the estrogen (ER) receptor was discovered (13), paving the way to classification of breast cancer on the basis of hormone receptor status. ER was used as a predictive marker for outcome of estrogen ablative therapy (14). This early classification was the foundation for the modern molecular classification of breast cancer, which also takes into account, expression of the progesterone receptor (PR) and the cell surface tyrosine kinase protein HER2/neu (human epidermal growth factor 2). A majority of breast cancers are hormone receptor positive (60% pre-menopausal, and 80% post-menopausal) (15). Hormone receptor positive breast tumors rely upon estrogen for growth and survival; hence anti-estrogens and estrogen depletion by non-invasive methods have become a mainstay in treating ER+ breast cancer. Breast cancers can express HER2 either in conjunction with or independently of ER and PR. HER2 is amplified in 30% of all breast cancers (16–18) and is associated with greater morbidity (19,20). Due to the high incidence of HER2 amplification, HER2 targeting antibodies have been produced and are used in the clinic. Humanized monoclonal antibodies targeting HER2 include pertuzamab which targets subdomain 2 of HER2 and trastuzumab which targets subdomain 4 of HER2. These monoclonal antibodies inhibit dimerization with HER3 and HER2 and ultimately cause the internalization of HER2 (21). Combining these antibodies with chemotherapy improves median survival from 20 months to 25 months (22). When ER, PR and HER2 are all absent in the tumor, the breast cancer is classified as triple negative breast cancer (TNBC). TNBC has a poor prognosis and can only be treated with

conventional chemotherapy, surgery and radiation due to the absence of drug target receptors (1). Taking into account histological grade and receptor status, breast cancer is classified as Luminal A, Luminal B and Basal like, which is included in a broader classification as triple negative breast cancer.

Luminal A breast cancers are highly differentiated and strongly resemble the cells of the inner lumen of the breast duct. Luminal A tumors tend to be ER positive, PR positive, and HER2 negative. This type of tumor is often classified as a low tumor grade. Roughly 65% percent of breast cancers are luminal A (23,24). Luminal A tumors have the best prognosis, with 97% 10-year relapse free survival (24). These tumors frequently have low histological grade, low degree of nuclear pleomorphism, low mitotic activity and are associated with good prognosis. Luminal-A is characterized by very high levels of ER and lower levels of high mitotic genes. Genetic profiling has shown that Luminal A breast cancers have high expression of cytokeratins (CK) 8 and 18 and other luminal associated markers including ER regulated genes. These genes include LIV1, ZIP6, FOXA1, XBP1, GATA GATA3, BCL2, erbB3 and erbB4 (25). Luminal A subtype is defined as ER-positive and PR-positive tumors with negative HER2 and low Ki67 index by immunohistochemistry (26). Recurrence is common in bone, whereas liver, lung and central nervous system metastases occur in less than 10% of patients and treatment is mainly based on hormonal therapy (27). Luminal A breast cancers are often treated with surgery and anti-estrogen therapy. Chemotherapy is not commonly used, as it has shown no additional benefit (28).

Luminal B tumors comprise of 15-20% of all cases of breast cancer and are considered more aggressive than Luminal A breast cancers. Luminal B breast cancers are also highly differentiated and originate from the inner lumen of the breast duct. Luminal B tumors tend to be estrogen receptor positive, progesterone receptor positive, and can be HER2 negative or positive.

They have a higher grade, faster progression, and worse prognosis than Luminal A cancers (29). Furthermore, these tumors have a higher rate of relapse (30). The largest difference between the two luminal subtypes is that Luminal B has increased expression of the genes v-MYB, GGH, LAPTMB4, and CCNE1. Furthermore this subtype also expresses HER2 in a third of the cases (31,32). Histologically Luminal B is defined as ER-positive, HER2-negative and Ki67 high. Also, Luminal B breast cancers can be classified as ER and HER-2 positive with low Ki67 staining intensity. It is important to note there is currently no standard way to define Luminal A and Luminal B by Ki67 cutoffs alone (33). The overall survival of untreated Luminal B cancers is similar to TNBC and HER2+/ ER- cancers (34). Luminal B tumors tend to not be as sensitive to hormone therapy as Luminal A tumors. Furthermore, this subtype is more resistant to microtubule targeting taxanes when compared to HER2+/ER- and basal like breast cancers. This resistance has been attributed to the fact these cancers rely upon alternate growth pathways through PI3K, FGFR1, HER1, and Src signaling pathways (35–40). Fibroblast growth factor 1/ and 2 (FGFR1 and FGFR2) are amplified in Luminal B breast cancers and is associated with a higher resistance to endocrine therapy. Diagnosis occurs at a younger age with advanced stages of disease (40). Patients tend to have larger tumors and more lymph node metastasis. Furthermore Luminal B subtypes are more likely to relapse (40). Treatment options for Luminal B tumors include; surgery, chemotherapy, immunotherapy (trastuzumab) and anti-estrogen therapy (41). Once metastasis has occurred, patients are usually aggressively treated with anti-estrogen therapy, followed by single agent chemotherapy (anthracycline or taxane) (42). There are few proven standards of care in in metastatic breast cancer management, resulting in variable treatment regimens depending on physicians and patients (42).

Basal like breast cancers account for roughly 35% of breast cancer cases (45,46). This subtype is associated with a high histological grade, poor tubule formations, and has necrotic cores. Furthermore, basal like tumors have high mitotic rates with solid growth patterns. They are marked by their high aggressiveness and high rates of metastasis to the brain and lungs (43,44). Basal like tumors also have high expression of myoepithelial markers, such as laminin, CK5, CK14, and CK17. Furthermore, these cells do not express high levels of ER, PR, or HER2 (70% of cases) and are also grouped as TNBC (44) . Though not required for basal-like identification, this subtype has frequent P53 mutations, inactivation of Rb, and dysregulated integrin expression (44).

Oftentimes it is thought that basal-like breast cancers are triple negative, but the two classifications are not synonymous. Roughly 20-30% of basal-like breast cancers are not triple negative. TNBC is a classification used for immunohistochemical (IHC) identification of disease, and only relies upon the three markers ER, PR, and HER2. Basal-like breast cancer are identified by their gene expression, via microarray or realtime-PCR. It is important to note the basal-like classification is currently not in use in the clinical setting. This is in part due to the fact that all therapies currently used rely on the three markers identified by IHC for indication of various treatments. The classification of "triple negative" is considered to be a reliable surrogate for basal-like for all treatment purposes (45). There is a large amount of research to explore putative biomarker candidates for basal-like breast cancers, however, there have not been any reliable complementary biomarkers used in the clinic thus far (46). Researchers have also added the subtype of basal-like cancer as claudin-low. Claudins are needed for tight junctions between cells, and are commonly found in epithelial-like cells. Claudin-low basal-like breast cancers tend to be more mesenchymal-like and have much poorer outcomes when compared to other basal-like breast cancers (47).

Next generation sequencing, microarrays, and IHC analyses have shown that basal-like breast cancers make up roughly a third of *breast cancer 1 (BRCA*1) related breast cancers. BRCA1 is a gene located on chromosome 17. When BRCA1 is expressed, it plays a pivotal role in the DNA damage sensing process, and facilitates DNA damage repair. BRCA1 deficient tumors tend to be triple negative with P53, EGFR, and X-chromosome abnormalities. Basal-like tumors and BRCA1 related breast cancers both behave similarly and have early relapse and similar pattern of metastatic disease (48). BRCA1 is specifically involved in DNA double strand break repair. BRCA1 deficient cells are highly sensitive to agents that create double strand breaks, when used with a poly-ADP ribose polymerase (PARP) inhibitor (induce synthetic lethality) (49). PARP is an enzyme that repairs DNA single strand breaks, and inhibiting PARP increases the chances of creating multiple nicks in the DNA to create double strand breaks. Thus, inhibiting PARP in BRCA1 deficient cells can increase the number of double strand breaks, creating a lethal phenotype. PARP inhibitors are currently indicated for BRCA1 deficient advanced ovarian cancer, with several clinical trials on breast cancer ongoing. TNBC also includes secretory, adenoid, medullary (BRCA1 related), and high grade invasive ductal carcinomas. Triple negative breast tumors are treated with surgery, adjuvant chemotherapy, and radiotherapy (50). Triple negative non-basal like tumors have 93% 10-year relapse free survival, whereas basal-like has an 86% 10-year relapse free survival (24). More importantly if relapse occurs, it happens within three years. Women with BRCA1/2 deficiency have shown to benefit from prophylactic use of tamoxifen, and showed a significant reduction (10%) of contralateral breast cancer incidence after initial diagnosis (51). The benefits of tamoxifen have been attributed to the possible role of ERβ in TNBC (52), but further investigation is needed.

1.1.4. *Breast Cancer Etiology*: Despite the fact that the etiology of breast cancer is not well understood, reports have described it as a culmination of factors such as hereditary-predisposition, somatic mutations, reproductive physiology, and environmental pressures. Germ line mutations account for a small proportion of predisposition to breast cancer, and are primarily attributed to BRCA1 (1% of cancers) and BRCA2 (4% of cancers), and carriers with susceptibility mutations have an 80% life time risk of developing breast cancer, and women with a primary family member with BRCA1 and/or BRCA2 mutations are twice as likely to develop breast cancer than the female population (53–55). More importantly BRAC1/2 mutations are more strongly associated with developing TNBC, resulting in prophylactic measures such as mastectomy, tamoxifen, and oophorectomy (56).

Increased exposure to estrogen (earlier onset of menarche and later onset of menopause) has shown to increase risk of developing breast cancer (57). Furthermore, exposure to estrogenic like compounds, aromatic amines, polyaromantic hydrocarbons, tobacco smoke and radiation are risk factors associated with breast cancer (58). More recently it was shown that hormone replacement therapy (HRT) which is used to mitigate menopausal symptoms plays a role in breast cancer risk. The women's health initiative study of 2007, showed women receiving estradiol (E_2) and medroxyprogesterone acetate (MPA) had increased incidence of advanced stage invasive breast cancer when compared to non-users. Furthermore, women who received hysterectomies and received estrogen alone had decreased late stage IDC breast cancer when compared to non-users (59,60). These studies were essential to the decision to decrease HRT in the general public to reduce the incidence of IDC.

1.2 Estrogen Biology. Estrogens are the primary female sex steroid hormones and are responsible for the development and regulation of the female reproductive system, secondary sexual characteristics and development of the central nervous system. There are three naturally occurring estrogens in women; Estrone (E_1), Estradiol (E_2), and Estriol (E_3) (61). E_3 is the most abundant estrogen, yet the weakest in terms of estrogenic activity. E_2 is 80 times more potent and plays the predominant role in estrogen signaling during sexual maturation and development (62). The lower circulating plasma levels of E_2 when compared to E_1 and E_3 is because of E_2's high affinity for steroid hormone-binding globulin (SHBG) (63). In pre-menopausal women, E_2 is primarily produced by the ovaries and has dynamic concentration ranges depending on the stage of the menstrual cycle. Plasma levels range between 1.4-1.6 nM during the follicular phase and 3.6-4.2 nM during the luteal phase (64). During pregnancy, estrogen levels increase about 40 times in the form of E_3 due to heightened production in the placenta (65). Synthesis of estrogens is stimulated by the secretion of gonadotropin releasing hormone (GnRH) which is synthesized in the hypothalamus. GnRH stimulates the release of follicle stimulating hormone (FSH) from the anterior portion of the pituitary gland. FSH then acts as a signaling molecule to increase estrogen synthesis in the granulosa cells of the ovary (66). High levels of circulating estrogen acts as a negative regulator on GnRH production, creating a negative feedback loop (66). Biochemical synthesis of estrogens starts in the theca-interna of the ovary, whereas androstenedione is synthesized from cholesterol. Androstenedione then passively diffuses to the granulosa cells where it is converted into either E_1 or testosterone. In an additional step, testosterone is converted to E_2 by the enzyme aromatase (67). Both E_1 and E_2 can be readily converted to E_3 by redox reactions in the liver or placenta (68–70). Adipose tissue, smooth muscle and liver express high levels of aromatase which can convert circulating androgens to estrogens, and this is the primary source of

estrogens post-menopause (69,71). Though circulating E_2 plasma levels are profoundly low post-menopause (~0.1nM), breast tissue estrogen levels are significantly higher (1.4+0.7 nM) (72–74). Higher breast tissue concentrations are maintained by local *de novo* synthesis and what is believed to be a retention capture mechanism that is not well understood (73).

Estrogen plays a diverse role in vertebrate development which includes sexual maturation and cognitive health. The human female relies upon estrogen and progesterone for the development of the breast in both pre-pubertal and post-pubertal stages (75). During the development of the breast, the ductal system is primarily under the control of E_2 and progesterone. Duct elongation is stimulated by both estrogen and progesterone whereas ductal branching is stimulated by growth hormone and progesterone (75). The proliferative effects of E_2 also occurs in the endometrium, where E_2 acts as the initiator of the menstrual cycle. E_2 is required for endometrial wall thickening during the follicular and early luteal stages of the menstrual cycle (76). The endometrium then becomes much more vascularized to support the implantation of the blastocyst and for placental development (76).

Estrogen functions are not limited to reproductive health and have critical roles in maintaining bone mineralization and density. Estrogen signaling alters population pools of osteoclasts (responsible for bone resorption) and osteoblasts (responsible for bone formation)(77) (77). Estrogen limits osteoclasts formation by decreasing their population through apoptosis, and increases osteoblasts by supporting their proliferation. Post-menopausal osteoporosis is primarily the result of low circulating estrogen, causing a shift in balance resulting in osteoclasts out numbering osteoblasts (77). Prolonging estrogen exposure with exogenous hormone has shown to be beneficial in fighting osteoporosis (78). Estrogen also acts as an important regulator of cardiovascular health by maintaining a lipid profile that is high in HDL-cholesterol and low in

LDL-cholesterol (79). The shift in lipid profile has had a significant effect in lowering risk of coronary heart disease (79). Furthermore, estrogen is known to be important in cognitive memory, mood, libido, and obsessive compulsive disorder (80–82). Estrogens have diverse roles in human development and the need for their stringent regulation is essential in normal human physiology throughout a woman's life.

1.3 Estrogen receptor Structure and function. Estrogens have great potential in changing physiology and cellular phenotypes in relatively short spans of time. Estrogen signals through the estrogen receptors. Estrogen actions can be mediated by non-genotropic estrogen receptor proteins which are primarily membrane bound G-coupled protein receptors. Many have been identified, but the most significant one is considered to be GPR30, which is expressed in most cell types. In general, when using the nomenclature "ER" henceforth, reference is made to the nuclear receptor subtype coded by the ESR genes. ER is a ligand-activated transcription factor which preferentially binds the more potent estrogen E_2. ER is a nuclear receptor that belongs to the steroid/thyroid/retinoid/orphan receptor superfamily. There are two main isoforms; ERα and ERβ. ERα is encoded by the gene *ESR1* located on 6q24 and spans nearly 300kb with eight coding exons (about 140kb) and seven introns (83). ERβ is encoded by *ESR2* on chromosome 14q21 and comprises eight exons (84). Both receptors comprise an activation function domain 1 (AF1-A/B), a DNA binding domain (C Domain), a hinge region (D Domain) and activation function domain 2 (AF2-E Domain). They are homologues with 17% similarity in the A/B domain, 97% in the C-domain, 30% in the D domain, 55% in the E domain and 18% in the F-domain (85). The AF-2 domain of both receptors is responsible for ligand activated functions. E_2 binding creates a unique conformation change resulting in repositioning of helix 12 in the AF-2 region. The change in position of helix 12 is what promotes dimerization and co-regulator binding. A ligand's ability to

alter the position of helix 12 is what distinguishes an agonist from an antagonist (86). The ESR1 gene has at least seven known promoters, and none of them have a CCAAT, GC, or TATA box. The diversity in promoters is thought to be responsible for tissue and cell specific regulation by distinct signaling pathways. The ESR2 promoters have not been well studied, but two (GO and ON) are known to strongly regulate its expression (87). The two receptors are found in the breast tissue, cardiovascular system, bone, and ovaries. However, distinct tissue specific regions of expression have been reported. ERα is primarily expressed in the liver and thecal cells of the ovary, whereas ERβ is the predominant isoform in the smooth muscle and granulosa cells (88). In normal breast tissue, ER+ cells are largely non-dividing but utilize E_2 signaling to upregulate paracrine factors to support ER-negative cell growth (89).

The functional canonical signaling of ER is primarily based upon location, ligand binding, and cofactor recruitment. Initially ER is bound by chaperone heat shock proteins (Hsps) Hsp70 and Hsp90 (90). Ligand binds to ER in the ligand binding domain creating a three-dimensional structural shift releasing ER from Hsp grasp. A heterodimer or homodimer composed of ERα and ERβ can bind to estrogen response elements (EREs) in DNA directly. EREs are 13 nucleotide palindromes consisting of two half sites separated by 3 nucleotides: GGTCAnnnTGACC (91). Once ER binds to the ERE it recruits cofactors for expression or repression of target genes. The majority of co-activators belong to the p160 family which consists of SRC1/2/3, ACTR, A1B1 and GRIP1. These proteins then bind to the AF-2 domain of ER by utilizing their NR-box (92). This complex in-turn recruits and binds to the core transcription machinery to express ER target genes downstream. In contrast, ER can also bind to the co-repressors SMRT and NCoR which recruit histone deacetylases (HDACs) to keep downstream genes inaccessible (93). Most of ER target genes are targeted through ERE specific binding and regulation, yet a large minority (30%)

of genes do not contain EREs. ERE-independent regulation is due to ER's ability to bind to other transcription factors and regulate a distinct subsets of genes. ER binding can be mediated by SP1, Fos, Jun and NF-κB to target a distinct sets of genes (94). All of these tethering mechanisms still rely on P160 proteins for transactivation.

ER can act as a transcription factor in a ligand-independent manner. Growth factors signaling through insulin-like growth factor-1 (IGF-1), epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR) activate MAPK. Active MAPK signaling results in phosphorylation of ER at ser118. Phospho-ser118 causes ER to act as a transcription factor in the absence of ligand and stabilizes ER by decreasing proteolytic turnover (95). Rapid non-genomic signaling does play a role in ER signaling, but most long-term ER functions rely upon classical ligand-dependent canonical signaling.

1.4 Adjuvant therapy in Hormone receptor positive breast cancer. When initially diagnosed, breast cancer is generally localized. However, after metastasis has occurred patients are virtually incurable and succumb to metastatic disease. First line therapy includes surgery and chemotherapy and is usually followed by adjuvant therapies to minimize growth of cells that have already spread. In hormone receptor positive breast cancer, anti-estrogen therapy is a critical tool and is divided into three categories: selective estrogen receptor modulators (SERMs), Aromatase inhibitors, and Selective estrogen receptor down regulators (SERDs).

1.4.1 *SERMs* are a group of compounds that bind ER and act as partial agonists/antagonists. SERMs are primarily used because they repress the canonical signaling by recruiting co-repressors at ERE sites in breast tissue. The most widely used SERMs are Tamoxifen and Raloxifene. Tamoxifen is indicated for use in both pre-and post- menopausal women and was the first SERM used in ER+ breast cancers in the U.S. (96). Tamoxifen has antagonist effects on

breast tissue but acts as a partial agonist on bone, cardiovascular and the uterine tissues (97). The agonist or antagonist effects of Tamoxifen are based upon the promoter context and cell type (98). Tamoxifen at a dose of 20mg/day reduces mortality in breast cancer patients by 26% and decreases contralateral breast cancer incidence by 50% (99). Benefits are reported to persist for an additional 10 years after treatment. Due to the estrogenic effects of Tamoxifen on the endometrium and cardiovascular system, women are twice as likely to develop endometrial cancer when treated with Tamoxifen for longer than 5 years (100) and are at increased risk for vascular thrombotic events (101).

Raloxifene is a second generation SERM initially used to treat osteoporosis. However, a large comparative clinical trial of Tamoxifen and Raloxifene showed the efficacy of Raloxifene was no different from Tamoxifen in preventing hormone receptor positive breast cancer (102). When comparing the two SERMs, Raloxifene had a lower toxicity profile and less side-effects when compared with Tamoxifen. Patients using Raloxifene have lower incidence of thrombotic events, and lower risk of endometrial cancer when compared to Tamoxifen (102). There are third generation SERMs available and studied, however, there is no additional benefit when compared to Tamoxifen and Raloxifene.

1.4.2 *Aromatase Inhibitors* block the activity of the protein aromatase, a cytochrome P450 enzyme responsible for converting testosterone to E_2 and androstenedione to E_3 (103). Aromatase is expressed in a variety of tissues and plays a necessary role in estrogen production. Following menopause, the synthesis of estrogens by the ovaries stops but peripheral tissues can still maintain low systematic levels of estrogen through the expression of aromatase (103). There are two types of aromatase inhibitors; Type 1 and Type 2 inhibitors (104). Type 1 inhibitors include exemestase and formestane, which act as competitive inhibitors of androstenedione. Type 2 inhibitors include

anastrozole and letrozole, which reversibly bind to the heme-group of the aromatase enzyme (105). Aromatase inhibitors are able to decrease intratumoral estrogen levels 30-fold (106), and virtually ablate circulating hormone levels in post-menopausal women (107). Pre-menopausal women have an intact hypothalamic-gonadal feedback loop which can compensate for the reduction of estrogen by aromatase inhibitors, thus aromatase inhibitor use is restricted to post-menopausal women (108). Aromatase inhibitors are indicated for postmenopausal women with local ER+ breast cancers and have been shown to decrease relapse by 40% (109).

1.4.3 *SERDs* are indicated for ER+/Her2- tumors that rely on ER for proliferation independent of E_2. Most tamoxifen and aromatase inhibitor-resistant breast cancers develop resistance by non-classical signaling as described above. SERDs such as Fulvestrant are steroidal anti-estrogens used in second-line therapy of ER positive breast cancers. Fulvestrant binds to ER, and is a pure competitive inhibitor with no agonist activity. It functions by binding to ER in the ligand binding domain and inhibits ER dimerization and signals proteolytic degradation with its long hydro-carbon side chain (110). Fulvestrant decreases cancer progression by 34% in patients resistant to first line therapies (111). Use of Fulvestrant is limited to second line treatment, primarily because of the global loss of ER, which is needed in normal healthy tissue.

1.4.4 Luminal Breast Cancer: Resistance to Endocrine Therapies. Resistance to endocrine therapy has been addressed by several theories of how hormone receptor positive breast cancer may behave over the course of disease progression. ER loss over time occurs in 20% in patients originally diagnosed with Luminal subtypes of breast cancer (112–114). These tumors are no longer reliant on estrogen and utilize pathways that are used in lieu of estrogen. In some cases, upregulation of HER2 occurs and, although it is rare (113,115), can actually drive down the expression of ER and PR, rendering cells less responsive to anti-estrogen therapy (116,117). Pre-

clinical work has suggested the possibility that tumors switch back and forth between ER and HER2 positivity by selective pressure from neo-adjuvant therapies (29,113,115,116,118,118). This may require continuous monitoring with use of multiple biopsies.

ER splice variants (ERα36) and estrogen related receptors ERR, though not commonly seen, have been implicated in resistance to tamoxifen therapy. ER coregulators are thought to play a more important role in tamoxifen resistance. Overexpression of A1B1 has shown to be an important for tamoxifen resistance both in vitro and in the clinical setting (119,120). Furthermore, the silencing of NCoR is associated with lack of transrepression by Tamoxifen, resulting in Tamoxifen resistance (93). ER can localize outside of the nucleus where it can act as an activator of cytoplasmic and membrane signaling complexes (120–123). These pathways have been shown to be a part of a feed forward loop which increases ER cytoplasmic localization, enhancing ER's non-genomic signaling. Interestingly both tamoxifen and estrogen can activate this cascade, creating an "off target" effect that supports tamoxifen resistance (120,121,123). Lastly ER can remain active in the presence of estrogen antagonists via tethering by NF-kB and AP-1 to maintain its genotropic effects (121,124,125).

Loss of PR is common during hormone therapy and is associated with worse outcome. ER upregulates PR, and if a tumor is ER+/PR-, it indicates ER is functioning independent of ligand (117).This is in part to be due to ER splice variants, and gain of function mutations. PR loss is also associated with increased PI3K signaling, which is known to downregulate PR expression (126). In some instances when patients progress on antiestrogen therapies (Tamoxifen and aromatase inhibitors), high dose estrogen is given to stimulate a switch to estrogen dependence and upregulation of ER. Following estrogen treatment, patients are withdrawn from estrogen. This induced estrogen dependence and deprivation causes cells to become less proliferative. Though

this is not common practice, a single patient is reported to have metastatic bone disease controlled for 8 years by continuous sequences of estrogen therapy followed by withdrawal (119). Other resistant theories include the small population of tumor like stem cells, which may play a crucial role in shifting the balance of tumor cell populations between endocrine dependent and independent pathways.

Though there is no clinical evidence, pre-clinical studies have shown that upregulation of mitotic signaling pathways may play a role in endocrine therapy resistance. For example, upregulation of Myc, cyclin D and cyclin E cause resistance to anti-estrogens *in vitro*. Furthermore, loss of function mutations in P27 and P21, followed by inactivation of pRb is also indicative of resistance (127,128). In parallel, it is expected that growth factor receptor signaling from the tumor microenvironment plays an essential role in resistance. Tyrosine kinase families such as IGF1, FGF, and VEGF, as well as cellular Src, AKT, and stress-related kinases have been implicated (117,129–131). Despite knowledge of all of these possible pathways and mechanisms of resistance, the most reliable and clinically relevant mechanism established to date is the EGFR/HER2 pathways as therapies that target this signaling axis are effective.

1.5 ER+ breast cancer dormancy and the role of anti-estrogen therapy

Breast Cancer Dormancy and metastasis is the primary reason for mortality in ER+ breast cancer cases. Dormancy is defined as a period when tumors are undetectable and can remain in a quiescent state below the threshold for identification in the clinic. Roughly 20-40% of ER+ breast cancer metastasizes to distant sites, with half occurring after 5 years of dormancy (upwards to 20 years) before reappearing (132). ER- tumors recur much more quickly and reappear in less than 3 years (133). ER+ breast cancers rely upon E_2 for growth and proliferation and anti-estrogen therapies have powerful effects in decreasing recurrence (134). However, recurrence is simply

postponed with increased time of exposure to anti-estrogens. This suggests that limiting E_2 signaling through ER does not kill the cancer cells but keeps cells in a state of dormancy (132). E_2 is not required for basal growth and survival of ER+ cells. The ER apoprotein can maintain low state of basal growth through the upregulation of the RARα apoprotein, directing cell survival and growth pathways (135). Though anti-estrogen therapy increases duration of survival in ER+ breast cancer cases, the issues of metastasis and dormancy remain as major problems throughout the remaining years of a patient's life.

1.6 Progesterone Biology

Progesterone is a steroid sex hormone that is often thought of as estrogen's opposing counterpart. It belongs to a group of hormones called progestogens and is the most functionally relevant and abundant progestogen in the human body (136). Progesterone acts as a signaling molecule by binding to its receptors, the progesterone receptors (PR). There are two major nuclear PR isoforms and several membrane PRs that act as strong intracellular signal transducers (as discussed below).

Progesterone is synthesized from its precursor pregnalone by the enzyme 3-beta-hydroxysteroid dehydrogenase (137). During pre-menopausal years the corpus luteum secreted progesterone disseminates through the entire body. Most of the systemic progesterone is bound to albumin and transcortin with only 10% being unbound (138). Progesterone is often called the hormone of pregnancy because of its important roles prior to blastocyst implantation and post-implantation (76). Progesterone is responsible for converting the endometrium into the secretory phase, a time when the endometrial epithelium is prepared for implantation. It however plays an anti-mitogenic role in opposing the trophic effects of E_2 on the endometrium. However, progesterone promotes a state of stasis that supports vascular composition following implantation

for fetal development and placental growth (139). If implantation does not occur, progesterone levels decrease due to loss circulating human chorionic gonadotropin (developed by the blastocyst) signaling to the corpus luteum. Loss of progesterone signaling results in atrophy and shedding of endometrial lining and marks the beginning of menses (140). Progesterone is also responsible for reducing the maternal adaptive immune system in order to protect the fetus from rejection (141). The presence of progesterone is required for pregnancy and inhibition of fetal partition throughout the gestational term. During this protective period, progesterone reduces smooth muscle contractility, inhibits lactation, and opposes onset of labor. Progesterone is essential for secondary sexual characteristics such as proper breast development. Progesterone is responsible for lobuloalveolar development in the mammary gland (described further below) by the upregulation of paracrine factors such as RANKL (142).

1.7 Nuclear Progesterone Receptor Isoforms and their functions

The progesterone receptor (PR) is encoded by the *PGR* gene located on chromosome 11q22. It has three isoforms which are all encoded by the same gene. The expression of each isoform is under the control of alternate promoter usage (143). The expression of the progesterone receptors is primarily regulated by the distinct estrogen response elements located upstream of the *PGR* locus. The three isoforms include the progesterone receptor A (PR-A), the progesterone receptor B (PR-B) and the progesterone receptor C (PR-C). PR-A and PR-B are the major isoforms expressed in human tissues and have a ligand dependent role. The long-term genotropic actions of progesterone are mediated through PR-A and PR-B and act in a similar fashion as other steroid receptors. In the absence of ligand, the PRs are sequestered by heat shock proteins Hsp-90,-70 and P59 (144–146). Upon progesterone binding, the receptor undergoes a conformational change resulting in a monomeric receptor absent of Hsp's (147). The receptors spontaneously dimerize

(both in hetero- and homo-dimers) and bind to specific DNA sequences called progesterone response elements (PREs) (147,148). PR/PRE occupied sites recruit co-activators and co-repressors to regulate gene expression. PR-B is the longer isoform with a length of 933 amino acids and includes an additional AF3 domain (149). PR-A is identical to PR-B except that it lacks the N-terminal AF-3 domain and is 769 amino acids in length (149) (Figure 1.1). Both isoforms have identical transcriptional activation domains (AFs) with AF1 located in the N-terminus and AF-2 in the ligand binding domain. AF1 and AF2 can function independently or they can synergize through an intra-molecular interaction between the N- and C-termini (143,150–153). Both homodimers and heterodimers can bind to an imperfect PRE palindrome (G•ACA• • •TGT•C) (154). Each one of the three possible dimer pairs regulates a distinct set of genes (155,156). Both receptors have similar affinity for progesterone, but the transcriptional profile of each isoform has little overlap (157). In most cellular contexts, PR-A acts as a transcriptional repressor and PR-B acts as a transcriptional activator (158). PR-B has a higher affinity for transcriptional co-activators GRIP and SRC1, whereas these cofactors do not bind to PR-A.   The first 140 amino acids of PR-A contain the inhibitory domain (ID). The ID is completely exposed due to the lack of the AF-3 domain (158) . When deleterious point mutations are made in the ID, the transcriptional repressive function of PR-A is lost (158). The intact exposed ID is responsible for the high affinity between PR-A and the co-repressor SMRT (159). PR-A is considered a strong trans-dominant repressor of PR-B, glucocorticoid receptor (GR), androgen receptor (AR), mineralocorticoid receptor (MR), and estrogen receptor (ER) (160–162).
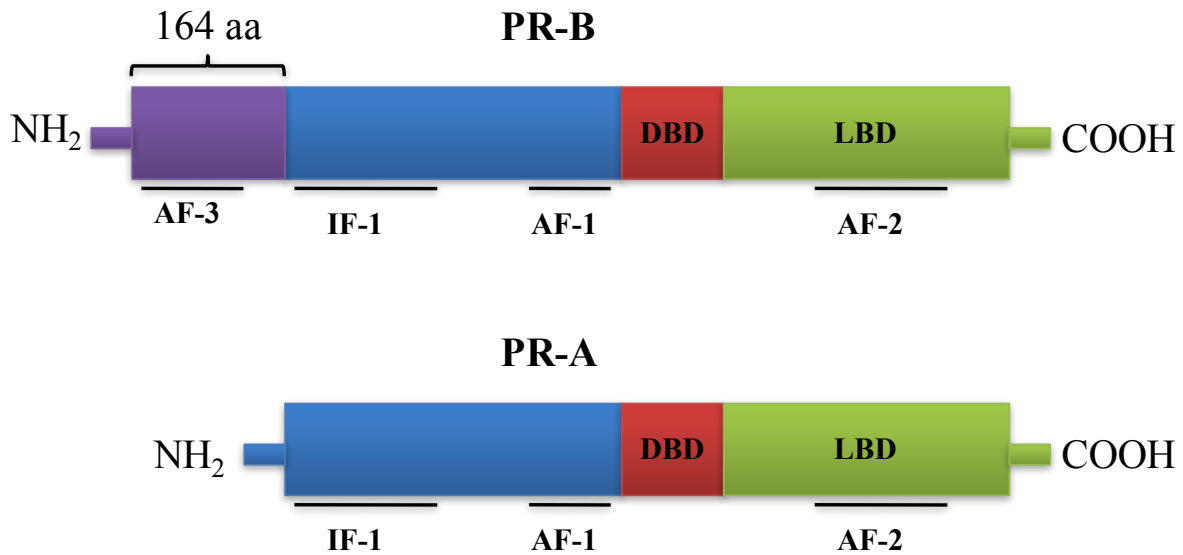
Figure 1.1. Progesterone receptor isoforms and functional domains

Unlike ER, PR is not inherently proliferative or anti-proliferative. Progesterone/PR activate the progression through one mitotic cycle followed by G1 arrest. The halt in cell cycle is caused by the induction of P27, P21 and reduction of cyclins D and E. During G1 arrest, progesterone upregulates growth factor receptors to promote paracrine stimulation of growth. Progesterone can be paradoxical and can act as an inhibitor of growth, or a necessary prerequisite for growth depending on the environmental context (163,164). The inhibitory effects of progestins on tumor growth were being pursued as a therapeutic in the early 1980s, and was observed to be as useful as anti-estrogens (165). However, it is important to note these early studies were using tumor growth as an endpoint and not tumor progression (invasion and metastasis) which will be discussed below.

Both PR-A and PR-B are normally expressed in vivo, however the ratio of expression is variable based upon tissue/cell type, physiological states, and disease. PR-A and PR-B are known to have unique tissue specific roles, and can have altered expression ratios depending on the anatomical and physiological context. Isoform levels change drastically in the endometrium depending on the stage of the menstrual cycle. In the CNS PR-A is the predominant form in the pituitary gland and PR-B is the predominant isoform in the hypothalamus (167). In the breast, overexpression of PR-A causes severe hyperplasia and disorganized basement membrane of the duct (168). In contrast, overexpression of PR-B causes growth arrest of the duct and inhibition of lobulo-alveolar differentiation (169). Furthermore, PR-A knockout in mice has no effect on normal breast development. Clinical evidence has shown different roles for each isoform expression in cancer as well. Overexpression of PR-A in human breast tumors is associated with more aggressive breast cancers and lower disease-free survival (170). In contrast, over-expression of PR-B is associated with more aggressive endometrial, cervical and ovarian cancers (171). Though the PR isoforms are similar in structure, the distinct role of each is quite different depending on the cell type and tissue.

1.8 Role of estrogen in invasion and metastasis

ER positive breast cancer accounts for the majority of breast cancers (75-80%) and strongly relies upon E_2 for growth. Growth is supported by activation of genes that support mitosis, cell cycle progression, and survival (172). Tamoxifen and aromatase inhibitors as described above have had significant impact in increasing survival. However, ER+ breast cancers can become growth adapted through multiple pathways (173) to grow independent of E_2 (135). In over 90% of cases with lymph node positive metastasis, cancer cells still retain ER even if growth is not reliant on E_2 (174).

The majority of studies of E_2 mediated effects on breast cancer have been devoted to the genotropic effects on growth (175). However, it is important to note that E_2/ER play multiple roles unrelated to proliferation. In the ductal epithelium, E_2 signaling through ER is required for terminal differentiation and morphogenesis of the duct. In ER expressing breast cancer cells, E_2 represses invasion both *in vitro* and in *vivo* (176–178)*.* Furthermore, clinical evidence from *The Women's Health Initiative* has shown that women with prior hysterectomy receiving estrogen hormone replacement monotherapy have decreased risk for invasive ductal carcinoma when compared to non-users (60).

Transcriptional regulation by E_2 through ER can activate genes but a majority of E_2 regulated genes are repressed. Most studies of E_2-repressed genes have focused on a subset of genes thought to be negative regulators of growth and survival(179–181). However, E_2 also represses critical genes that are necessary for invasion *in vitro* (182). This repressive effect on invasion was clearly defined by the ability of ER to bind co-repressors and down regulate genes needed for invasiveness (182). Furthermore, the use of tamoxifen blocked the repressive role of ER increasing invasion (182). Ectopic overexpression of ER in the presence of estrogen reduced invasive capacity in hormone receptor-negative cells (183). Furthemore, ER's ability to repress invasion occurs even if cells do not rely on E_2 for growth (182). The hormone regulated repressive effects of ER rely upon the AF-2 domain, as mutations in this region (LBD) result in loss of regulation of invasiveness (184) (Figure 1.1). Clinical studies have shown that tamoxifen did reduce mitotic capabilities of ER+ breast cancer, but also increased the risk of contralateral ER-breast tumors 5 fold (134). E_2 and ER play an important dual role in supporting growth/survival but repressing invasiveness/metastasis in breast cancer cells.

1.9 Role of progesterone in breast cancer invasiveness and metastasis

Progesterone and other progestins increase invasiveness and metastasis. During normal post-pubertal development breast cells are required to proliferate, migrate, and invade during the luteal phase and pregnancy (185). Both of these events are marked by high levels of plasma progesterone. This lead to the hypothesis that progesterone could take advantage of these pathways and utilize them for the progression of breast cancer. Women receiving hormone replacement therapy with both E_2 and progestin have a higher incidence of invasive breast cancer when compared to non-users and women receiving E_2 monotherapy (59). Progestins at high doses cause rapid cellular changes through non-genomic signaling. These rapid signaling cascades increase migratory capacity by increasing focal adhesion (FA) complexes needed for lamellopodia and fillopodia (186). FAs are mediated by Focal Adhesion Kinases (FAKs), which activate the recruitment of the stress fiber mediators vinculin, talin and paxillin (187,188). High dose progesterone (>10nM) through PR increases FAK phosphorylation resulting in rapid increased cellular protrusions into the extracellular space. Furthermore, increased migratory ability is mediated by liganded nuclear PRs utilizing Src signaling (189). Interfering with each PR, Src, RHOA, ROCK2, MAPK and MEK1/2 independently resulted in the blocking of progestins' ability to promote FA complexes. In addition to FAK signaling, high doses of progestin (>20 nM) has been reported to reduce E-cadherin protein in MCF7 cells, resulting in a more mesenchymal phenotype. Non-genomic signaling clearly results in an increased metastatic phenotype, however, all studies were performed at doses well above post-menopausal plasma and correspond to HRT scenarios.

Malignant ER+ breast cancer tissues often have a dysregulated expression of the PR isoforms. Normal PR+ breast tissue cells express equal amounts of each PR isoform. However,

70% of metastatic tumors have an excess of PR-A or PR-B (170,190). Moreover, the over-expression of PR-A is associated with lower disease-free survival (170). Interestingly, tumors overexpressing PR-A often manifest in post-menopausal years (170), a time when circulating progesterone levels are in the sub-nanomolar ranges (191). Most studies of progesterone receptor-dependent invasiveness have relied upon the ectopic expression of PR-B with progestin doses well above physiological levels (192–196). Knock out models in mice show that PR-B expression alone allows for normal breast ductal morphogenesis (197). In contrast, the expression of PR-A alone leads to malignant transformation (198). Furthermore, overexpression of PR-A leads to ductal hyperplasia and dysplasia at normal endogenous progesterone levels (168,199). It was long thought that following menopause, progesterone was virtually absent (~1 nM) when compared to their normal physiological ranges during reproductive years (4 nM in the follicular phase and increases up to 50 nM in the luteal phase) (200). This lead to the hypothesis that the mere expression of either progesterone receptor isoform in the absence of hormone could regulate a gene profile that supported invasiveness. It has been reported that apo-PR-A has a distinct regulatory profile that is sufficient to alter cell morphology into a more migratory phenotype (201). Historically all of the *in vitro* evidence supporting the role of progesterone on breast cancer cell invasiveness has relied upon the forced expression of the progesterone receptors in triple negative breast cancer, or with progesterone doses well above normal tissue concentrations throughout a woman's life. Furthermore, progesterone signaling has been primarily studied in the absence of E_2, and little is known about the cross talk between ER and PR and their combined role in breast cancer progression.

1.10 The Role of EMT in ER+ Breast Cancer Metastasis

The current understanding of the role of reproductive hormones on ER+ breast cancer progression and invasiveness has primarily focused upon the role of E_2 signaling through its receptor ER. ER has become a quintessential biomarker in predicting the use of antiestrogens and aromatase inhibitors in the clinic (202). E_2 is known to function through ER to activate the expression of growth and survival genes (203), and conversely recruit co-repressors to trans-repress genes required for invasiveness (182). Hormonal regulation of invasiveness in ER+/PR+ breast cancers has been well studied but little ground has been gained on the role of these receptors and ligands on the epithelial-to-mesenchymal transition (EMT) thought to occur during metastasis.

EMT is often regarded as an essential step in the progression of breast cancer to a more invasive phenotype (204). This transition is marked by loss of polarity, loss of cell-cell adhesion with gain in migratory and invasive capacities (204). This process is occurs in the development of the mesoderm (205), neural tube formation (206), wound healing (207) and cancer metastasis (208). The mesenchymal like phenotype is marked by loss of E-cadherin, beta catenin, and claudin-1, along with a gain of expression of N-cadherin, vimentin, slug, and snail (209). EMT has become an important topic in the progression of breast cancer oncogenesis. However, the relationship between EMT and hormonal regulation in ER+ breast cancers has become less clear with many confounding interpretations (175,210–214).

The loss of E-cadherin is considered to be necessary for the EMT to begin (215) . Some reports have shown that low dose E_2 decreases the expression of E-cadherin, resulting in increased migratory capacity. However, there was no investigation into its regulation on invasiveness (183,216–218). Initiation of metastasis requires cells to become more invasive, and EMT confers a more invasive phenotype. However, EMT has been reported to not be required for metastasis

(204,219,220). Moreover, pathological analysis of IDC tissues have shown that cells within a lesion retain tertiary structures such as acinars and luminal spaces (221). These structures maintain tight junctions, desmosomes, and cellular polarity (221,222). Most important, the epithelial characteristic in these patients (N=149) showed no correlation with metastatic disease (222). These finding have been partially reconciled by the concept of epithelial reversion from the mesenchymal phenotype (223,224). Until recently this has been the justification for the lack of mesenchymal-like cells seeded in meta-sites. Mechanistic fate mapping in both breast and pancreatic cancer have shown that EMT is unnecessary for invasion and metastasis to occur (219,220). It is important to note that EMT can lead to cellular dissemination but is not required (219,220). EMT is reported to actually play a crucial a role in chemo-resistance during metastasis (219,220). EMT in breast cancers has been widely attributed to loss of ER. The ER apoprotein is considered to be the master regulator of maintaining a epithelial-like phenotype and epithelial markers (211–213,225,226). This is at odds with the few reports stating E_2 signaling through ER is a primary component of advanced disease progression (227,228). Hormonal regulation of invasiveness has been clearly defined, but whether EMT plays a part has not been clearly elucidated.

1.11. Current status of treatments for advanced breast cancer and their limitations

1.11.1 Survival of patients with metastatic breast cancer

Patients with estrogen receptor (ER)-positive metastatic breast cancer often respond to endocrine therapy (ET), which can reduce tumor burden and symptoms with generally fewer side effects and toxicities when compared to chemotherapy. However, few if any patients with metastatic breast cancer will be cured, and the goal of therapy is principally for palliative purposes. Efforts are made to reduce the number of side effects and still stabilize or reduce the burden of

disease. Once a patient develops progressive breast cancer that is not responding to endocrine therapy, chemotherapy regimens are implemented in the same manner as hormone receptor negative breast cancer as discussed below.

Following surgery of breast cancer, disease can recur at both regional and distant sites. Local recurrence is defined as cancer that reappears on the ipsilateral chest wall. Furthermore, recurrence can also include a tumor that involves regional lymph nodes such as ipsilateral axillary, supraclavicular and less commonly the infraclavicular and/or internal mammary nodes. Roughly 5-10% of patients who have received surgery will have locoregional relapse within a 10 year time (229–235). Half of these patients will have recurrence isolated in the chest wall (236,237). Of these patients, 30-40% will have regional metastases in the supraclavicular, axillary, or internal mammary nodes. It is estimated that patients with locoregional metastasis have synchronous distant metastasis as well (234). The median time to locoregional recurrence after surgery is 3 years, and over 90% arise within five years (238–241). However, relapse can be delayed with the use of Tamoxifen in hormone receptor-positive disease (242). For women with locoregional metastasis, the most consistently accurate prognostic factor is the interval between initial diagnosis and recurrence. Disease-free survival of at least two years is associated with a significantly better outcome when compared to earlier recurrence regardless of breast cancer subtype (229,233,238). Furthermore, long-term control of disease is dependent upon the disease volume. Disease control rates are highest with either limited chest wall or axillary node involvement, and when supraclavicular nodes are not involved (238,241).

There are few prospective randomized trials, and most available data come from single institute studies (238). A post-mastectomy study in 145 women showed that survival depended on the location of metastasis, and was comprised of patients with diverse subtypes of breast cancer.

Though hormone positive breast cancers are less likely to relapse at meta-sites, if locoregional disease persists aster initial treatment, survival rates are similar to other breast cancers (hormone receptor negative). Overall survival rates for breast cancer identified in the chest wall alone, or axilla alone is 50%. Survival rates for supraclavicular nodes alone and chest wall plus axilla is 28%. Lastly, survival rates for supraclavicular nodes plus chest wall/axilla are 7% (238).

1.11.2 Treatment of metastatic breast cancer

Surgical intervention: Whenever possible, guidelines suggest wide excision of all nodules that are amenable to resection (230,238,240,243). Resections have shown to reduce the required dose of radiotherapy (RT) and increase long-term disease control (as discussed below). Though conservative surgery is often considered, complete excision results in less frequent recurrence (21% vs. 60%) (241). However, extensive surgery for locally recurrent disease is only performed if other options are limited (244–246), and if surgery cannot be performed, systemic treatments are preferable (discussed below). Following wide local excisions, a second local recurrence occurs in 60-70% of cases (247,248). The likelihood of success is increased when a recurrence pattern in a patient has fewer lesions with a relapse period of greater than 2 years for all breast cancers (244,249–252).

Radiotherapy (RT) is an essential part of treatment for women with breast cancer recurrence. Optimal RT management generally involves treatment to the entire chest wall and draining lymph node areas. Conservative treatment of the chest wall increases the risk for future recurrences in the supraclavicular and axillary regions(238,241,243). For patients who have a non-resectable chest wall recurrence, radiation alone can be attempted. However, 60 to 70 percent will experience a second local recurrence (239,241,248,253). Because of the high risk of a second recurrence in these patients, systemic therapy (discussed below) is preferred.

*Systemic therapy***:**

It is commonly agreed that higher response rates are seen with chemotherapy when compared to endocrine therapy in advanced hormone receptor positive cancer with poor prognosis (especially with visceral metastasis). A meta-analysis that included eight randomized trials, compared chemotherapy alone to endocrine therapy alone (254). Patient response was far superior for chemotherapy over endocrine therapy (relative risk 1.25, 95% CI 1.01-1.54). In general, endocrine therapy is beneficial but has to be timed correctly. Endocrine therapy has fewer side effects, but its benefits only occur after many months of treatment. Therefore, chemotherapy with anthracyclines, taxanes, platinums, and etoposide are first line in advanced metastatic disease. After (if) chemotherapy stabilizes disease progression, a switch to maintenance endocrine therapy can be used. Endocrine therapy is a preferable method when the option is available because of reduced treatment side effects without compromising overall survival (255–257). It is important to note no survival benefit was seen when chemotherapy and endocrine therapy were combined.

Treatment approach and success

As discussed above, breast cancer treatment options can vary among individuals, but systematic treatments of early stage breast cancers are quite distinct systematic. Most of the treatment regimens primarily depend upon hormone receptor and HER2 status. However once distal metastasis has occurred (Stage 4), almost all cancers are aggressively treated with chemotherapy with follow up advanced hormone therapy(ER+), or with experimental drugs (Table 1). Furthermore, success rates for 5-year survival are dependent upon stage and disease progression, and less dependent upon disease subtype, although this may change for more recently developed treatments when prospective studies with endpoints of up to 20-year survival are completed.

Breast Cancer Stage	Step 1	Step 2	Step 3	Step 4	Step 5	Overall 5 year Survial
Stage 1 (ER+)	Breast Conserving Surgery	Radiation (<70 YO)	Chemotherpay (tumor > 1cm)	hormone therpay (5 years)	NA	
Stage 1 (HER2+)	Trastuzumab /Pertuzumab	Breast Conserving Surgery	Local Radiation (<70 YO)	Trastuzumab /Pertuzumab	NA	100%
Stage 1 TNBC	Breast Conserving Surgery	Chemotherapy	NA	NA	NA	
Stage 2 (ER+)	neo-adjuvant therapy	Breast non-conserving	Local Radiation (<70 YO)	Chemotherapy	hormone therpay (5 years)	
Stage 2 (HER2+)	Trastuzumab /Pertuzumab	Breast non-conserving	Chemotherpay (tumor > 1cm)	Trastuzumab /Pertuzumab	Hormone therpay (if ER+)	93%
Stage 2 TNBC	Chemotherapy	Surgery	Chemotherapy	Tamoxifen in some instances	NA	
Stage 3 ER+	Neo-adjuvant chemo	Advanced Surgery	Regional Radiation	Chemotherapy	hormone therpay (5 years)	
Stage 3 HER2+	Neo-adjuvant chemo	Advanced Surgery	Regional Radiation	Chemotherpay (tumor > 1cm)	Trastuzumab /Pertuzumab (1 year)	72%
Stage 3 TNBC	Aggressive chemotherapy	Regional Radiation	Aggressive chemotherpay	NA	NA	
Stage 4 ER+	Aggressive chemotherapy	Regional Radiation	bisphosphonates (inidcated for bone Met)	Fulvestrant (if no stabelization)	Clinical trial	
Stage 4 HER2+	Aggressive chemotherapy	Regional Radiation	bisphosphonates (inidcated for bone Met)	capecitabine	Clinical trial	22%
Stage 4 TNBC	Aggressive chemotherpay	Regional Radiation	bisphosphonates (inidcated for bone Met)	Aggressive chemotherpay	Clinical trial	

Table 1.1. Treatments steps for molecular subtypes of breast cancer and 5-year overall survival

1.11.3 Molecular subtype-site specific distal metastases and overall survival.

Though advances in breast cancer have been made in the past 20 years, roughly 30% of patients with early diagnosed breast cancer will experience relapse with distant metastatic disease. Triple negative cancers are often thought of as the more aggressive disease due to limited treatment options. However, even with lower relapse rates, ER+ positive tumors make up the majority of metastatic breast cancers. A study with a sample size of 3,726 patients found that of all breast cancers, 22% of breast cancers metastasized to distant organs and were ER+, whereas the other subtypes accounted for less than 5% of breast cancer patients (27). Furthermore, although women with ER+ breast cancers do have a higher 10-year overall survival rate (Table 1), 30% and 46% of the patients with luminal A and luminal B cancers respectively will not survive 10 years. This clearly demonstrates that ER+ breast cancers are not truly indolent, even though more treatment options are available for them.

Risk of recurrence is generally defined by tumor size, nodal involvement, and lymphovascular invasion (27). However, there has not been a large-scale analysis of spread and specific recurrence. Current EMSO and UpToDate guidelines focus on treatment strategies for specific subtypes of disease based upon the molecular subtype at initial diagnosis (i.e. hormone therapy vs chemotherapy). Characteristics of the primary tumor are generally retained at the meta-site. Yet as disease progresses with continuous relapse, a defined treatment scheme for each subtype fades. Though the total number of patients that succumb to breast cancer are similar between subtypes (Table 1), the disease progression in the final stages is quite different, with different specific sites of metastasis between subgroups (Table 2). Bone is the common site amongst all subtypes (except basal). A report using multivariate analysis (with luminal A as the control) showed luminal B tumors preferentially metastasizing to the brain and lungs when

compared to other tumor subtypes. However, all the other subtypes have more metastasis to the liver and bone when compared to Luminal B (27).

Total N = 3726	N	Percent of Patients	15 year Relapse Rate	10 Year Overall survival	Distant metastasis
Luminal A	1639	44%	28%	70%	12%
Luminal B	894	22%	43%	54%	10%
Luminal HER2+	242	7%	48%	46%	3%
HER2+ enriched	265	7%	51%	48%	4%
Basal like	365	10%	43%	53%	4%
TNBC Non Basal	317	9%	35%	63%	2%

Table 1.2 Patient relapse rate, overall survival and frequency of distant metastasis.

	No. Patients	Brain		Liver		Lung		Bone		Distant Nodal		Pleureal		Other	
Subtype	N	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Luminal A	458	35	7.6	131	28.6	109	23.8	305	66.6	73	15.9	129	28.2	62	13.5
Luminal B	378	41	10.8	121	32	115	30.4	270	71.4	88	23.3	133	35.2	73	19.3
Luminal HER2+	117	18	15.4	52	44.4	43	36.8	76	65	26	22.2	40	34.2	16	13.7
HER2+ (ER-/PR-)	136	39	28.7	62	45.6	64	47.1	81	59.6	34	25	43	31.6	23	16.9
Basal	159	40	25.2	34	21.4	68	42.8	62	39	63	39.6	47	29.6	38	23.9
TN nonbasal	109	24	22	35	32.1	39	35.8	47	43.1	39	35.8	31	28.4	28	25.7

Table 1.3 Metastatic site based upon breast cancer molecular subtype

It is important to note that current treatment regimens for luminal breast cancer focus primarily on reducing tumor burden, rather than inhibiting metastasis. Currently there are no treatments that specifically target the process of metastasis, which is the cause of 95% of breast cancer deaths. It is therefore the goal of this study to first seek a better understanding of fundamental mechanisms that determine the metastatic potential of luminal breast cancer. A little explored area in this regard is micro RNA-mediated regulation of metastasis in luminal breast cancer despite the known importance of micro-RNAs in many aspects of hormone signaling in breast cancer. It is the premise of this thesis that discovery of micro RNA mediators of hormonal control of invasiveness and metastasis of luminal breast cancer will enable a better mechanistic understanding of metastasis of this major cancer sub-type.

1.12 microRNA biogenesis and Regulation of mRNAs

Micro RNA (miRNA) is a small non-coding RNA molecule that is roughly 22 nucleotides in length. miRNAs are conserved in plants, animals, and viruses (258). These short RNAs primarily act as endogenous silencers of mRNA transcripts (258). miRNAs function by base pairing with complementary sequences found within mRNA sequences resulting in silencing by three different modes of action: (I) recruitment of mediators that cleave the mRNA into two pieces; (II) destabilization of the mRNA by shortening the poly(A) tail; and (III) significantly decreasing efficiency of ribosomal translation machinery (259,260). The human genome codes for over 1000 miRNAs which are found in both introns and exons (261). miRNAs in animals are very promiscuous with a single miRNA targeting up to 500 unique mRNA transcripts. It is estimated that 60 percent of the human genome is regulated by miRNAs (262,263). miRNA genes are transcribed by RNA polymerase II and require a promoter sequence upstream (264,265). The initial RNA transcript is called the pri-miRNA. This precursor is capped at the 5' end and has a poly (A) tail a the 3' end. Pri-miRNA is 80 nucleotides longs and folds back on itself to create a secondary stem and loop structure. The pri-miRNA is then processed by the enzymes Drosha, and Pasha, cleaving the 5'-Cap and Poly (A) tail. This shorter double stranded RNA structure is called pre-miRNA (264,266). It is important to note that each pri-miRNA can code for up to 7 miRNAs. However, expression patterns of miRNAs from the same pre-cursor can vary based on the flanking processing coding sequences (267). Pre-miRNAs are then exported from the nucleus by Exportin-5, which recognizes the enzyme Drosha and 3' overhang of the miRNA (268). Once in the cytoplasm, the loop joining the 3' and 5' segment is cleaved by the protein Dicer. This process creates an imperfect RNA duplex, and also unwinds the duplex (269,270). Only one strand is incorporated into the RNA induced silencing complex (RISC). This is in part decided by the

thermodynamic stability of base-pairing on the 5'end compared to the 3' end, and the location of the loop cleavage (271–274). The passenger strand is often rapidly degraded prior to RISC complex joining. Once the RISC has joined, the Argonaute protein (Ago) in the complex can cleave the mRNA target or sequester the mRNA to inhibit translation (275). There are 8 Ago proteins in humans, and all have unique co-factor binding abilities that result in various levels of mRNA translational repression (276). The functional guide miRNA is thought to be retained in the cleft of the Ago protein and can target multiple mRNAs. Thus, a single miRNA can be much more potent than the previously expected 1:1 miRNA/mRNA degradation (275,276). Degradation of mature miRNAs and miRNA duplexes outside of the scope of the RISC complex have been discovered in C-elegans and plants. However, human homologues are yet to be elucidated (277).

1.13 miRNA Mediators in Breast Cancer

miRNAs can be deemed oncogenes or tumor suppressors depending on the context of mRNA inhibition and pathway regulation. After the discovery of miRNAs, it was observed that various breast cancer subtypes exhibited unique miRNA profile signatures (278–281). Most studies on miRNAs are focused on associations to identify signatures for tumor subtype. However, functional studies have shown that most miRNAs act as tumor suppressors and play a strong role in opposing progression in various tumor types (282). For instance, miR-125b down regulates HER2 but is absent in most HER2+ tumors (283). miR-205 regulates HMGB3 and its ectopic expression significantly promotes apoptosis in breast cancer (284,285). Expression of the miR-17-92 cluster in breast cancer is associated with significantly reduced metastasis (286) and the miR-200 family is essential for maintaining epithelial phenotype (287,288). Furthermore reintroduction of miR-200 family members have decreased aggressiveness of various tumor subtypes (289–291).

It is very clear that tumor suppressor miRNAs in one cancer type can act as an oncogene

in another cancer cell type. The actions of many miRNAs are dependent upon cell, tissue, and environmental contexts. These miRNAs can have various different roles system-wide (281). This has been considered to be the result of inconsistent studies in the miRNA field, as high-throughput studies are highly flawed based on limitations in technology (281). Furthermore, low throughput studies using specific primer/probes for mature miRNAs have shown context specific regulation of miRNAs. For instance, miR-146 acts a tumor suppressor by targeting NF-κB in gastric cancers, but also strongly down regulates BRCA1 in breast cancers (292–294). These studies truly underscore the importance of miRNA investigation in tissue specific contexts when attributing functional roles to each miRNA.

Most studies on miRNAs have relied on the basic observation of virtual complete loss, or gain of miRNA expression in disease tissues when compared to normal tissues. Very little is known about the modulation of expression of miRNAs by hormones and their respective receptors. A compilation of six studies has shown that E_2 was able to regulate 43 miRNAs (295), but little was done to explore the functionality of any of the miRNAs. It was identified that miRNAs that are present in triple negative breast cancers and absent in ER+ breast cancers were important for the identification of ER regulation. Inhibition of miRs-221/222 in MDA-MB-468 restored ER expression and increased tamoxifen sensitivity (296). Furthermore, estrogen signaling can sel-regulate oneself by negative feedback loops utilizing miRNAs. E_2/ER signaling negatively regulates itself by upregulating miR-206 which targets ER mRNA(297). E_2 regulation of miRNAs has been well noted and is hypothesized to play a critical role in breast cancer development. In contrast, progesterone regulated miRNAs are understudied in the breast tissue with a majority of studies focusing on miRNA expression in endometrial tissue (298).

1.14 Current challenges of directly using miRNAs in cancer treatment

A described above many miRNAs are tumor suppressors and oncogenes (onco-miRs), and reintroducing miRNAs/inhibitors in the *in vitro* setting has been demonstrated to cause modulation of tumor growth and invasion to overcome chemoresistance. Re-introducing miRNAs is achieved by using synthetic RNA duplexes (mimics) that can be chemically modified to be more stable and amenable to different delivery methods. To inhibit a miRNA, antimiRs, and antigomirs are used. AntimiRs are single stranded RNAs that are complementary in sequence to the mature form of miRNA and often reduce the overall targeted miRNA. Furthermore, antigomirs are miRNA inhibitors with the 2′-O-methoxyethyl modification. This modification creates a much stronger bond to the miRNA, but also stabilizes the entire duplex.

The most common mode of delivery is the use of lipid nanoparticles, and is currently in use in a phase I clinical trial (NCT01829971). The tumor suppressor miR-34 has been successfully delivered to lung(299), prostate(300), and liver (300) in mouse models. Studies were performed with local injection and systemic delivery, which both resulted in decreased tumor growth. In other reports researchers have delivered miRNAs using 1,2 dioleoyl-*sn* glycero-3 phosphatidylcholine (DOPC) liposomal carriers. In mouse tumor model studies, DOPC delivery of miR-200 family members increased radiosensitivity and decreased total tumor burden when compared to control mice (301).

In addition to the classical nanoparticle delivery systems, miRNAs can be linked to a peptide backbone that is sensitive to pH (pHLIP). When the miRNA-pHLIP encounters a low pH environment, a conformation change occurs. The peptide carboxyl terminal end is then inserted into the cell membrane and the miRNA is released. This creates a unique delivery system with the miRNA remaining stable during transport to the tumor microenvironment (302).

miRNAs are viewed as desirable therapeutics due to their low toxicity profile, and chemical stability. Given the current rapid advances in understanding each miRNA and its role in cancer, numerous drug targets within their regulatory pathways will eventually likely be identified. The current limitations of direct clinical applications of miRNAs in treating disease primarily relate to efficiency of delivery of their mimics and inhibitors, but it may only be a matter of time before successful delivery methods are developed. **As it pertains to this thesis, studies of miRNAs can provide fundamental insights into molecular mechanisms governing cancer progression. Novel regulatory pathways involving miRNA mediators of metastasis of luminal breast cancer could reveal protein targets for selective interventions to suppress metastasis of luminal breast cancer.**

CHAPTER 2- ROLE OF THE SHORT ISOFORM OF THE PROGESTERONE RECEPTOR IN BREAST CANCER CELL INVASIVENESS AT ESTROGEN AND PROGESTERONE LEVELS IN THE PRE- AND POST-MENOPAUSAL RANGES

2.1 Introduction

The process of breast oncogenesis is believed to span up to several decades. Most (> 78 percent) of newly diagnosed breast cancer cases occur in women whoare older than 50 years (303) and the median age at diagnosis is 61 years (304). In most cases the tumors express the estrogen receptor (ER). ER+ tumors are exquisitely sensitive to anti-estrogen therapy. However, ER+ breast cancer is often metastatic at the time of diagnosis and metastatic ER+ tumors also frequently appear after many years of dormancy (132,305). In either case, the metastatic disease is generally incurable and even targeted therapies are generally only palliative. Therefore, it is necessary to understand more about deregulated molecular mechanisms that confer invasive properties on ER+ breast cancer cells. Clearly, both pre-menopausal and post-menopausal events that influence breast tumor invasiveness are clinically highly significant in breast tumor progression. Profound decreases in the levels of circulating estrogen and progesterone are a hallmark of post-menopausal physiology although, in post-menopausal women, breast, endometrial and adipose tissues contain much higher levels of estrogen and progesterone, compared to plasma levels of the hormones (73,74,306–308).

As the progesterone receptor (PR) gene is a target of estrogen, the PR expression status of ER+ breast tumors is believed to reflect the robustness of ER signaling and hence predict patient response to anti-estrogen therapy. Nevertheless, PR agonists do directly support invasiveness and metastatic potential in ER+/PR+ breast cancer cells, as demonstrated using *in vivo* experimental models (309,310). The physiological relevance of these model systems is supported by the observation that in postmenopausal women, hormone replacement therapy with the combination

of estrogen and progestin was associated with increased incidence of invasive breast cancer and breast cancer mortality compared with non-users (59) whereas, estrogen monotherapy in women with prior hysterectomy was associated with a persistent decrease in the onset of invasive breast cancer (60). However, in post-menopausal women who are not undergoing hormone replacement, the role of the endogenous hormones in the progression of ER+/PR+ breast tumors is unclear.

PR has two isoforms, A and B, that are expressed by alternative promoter usage from a single gene; PR-B is identical to PR-A except for the presence of an additional 164 amino acid amino-terminal segment that contains within it, an additional activation function, AF3 (311). PR-B and PR-A exhibit both distinctive and overlapping patterns of agonist-induced gene activation or gene repression, depending on the variable contexts of the target promoters and the nature of the associated chromatin sites of PR binding (156,311,312). In cells expressing equal amounts of PR-A and PR-B, a substantial proportion of the two proteins are sequestered by forming a heterodimer; the heterodimer regulates a smaller and unique set of genes compared to the homodimers (155,156). Clinical studies have shown that although in normal breast PR-A and PR-B are expressed at comparable levels, this balance is commonly altered during breast oncogenesis with a predominance of a high PR-A:PR-B ratio in early, as well as progressed lesions (190). An elevated PR-A:PR-B ratio, which is frequently due to overexpression of PR-A, is associated with a lower rate of disease-free survival (170).

In vitro molecular studies have shown that when hormone-depleted breast cancer cells are treated with PR agonists, they induce invasiveness through several non-genomic and genomic signaling pathways of progestin (192,313–318). Some of those studies have further reported that it is PR-B that mediates progestin-induced invasiveness *in vitro* (192,193). The progesterone doses that were used to demonstrate substantial PR-B dependent effects on invasiveness *in vitro* were

relatively high, corresponding to the plasma range of the hormone levels associated with only the luteal phase of the menstrual cycle or with pregnancy. Horwitz and co-workers have also elegantly demonstrated *in vitro* that the mere overexpression of PR-A confers an inherently more aggressive phenotype in breast cancer cells, including adhesion to extracellular matrix, migratory capacity and survival, due to hormone-independent gene regulation by PR-A (201).

Most breast tumors are ER+ (319) and continue to retain ER expression even as they progress to hormone-independence (320,321). Estrogen supports the growth of ER+ breast tumors but it suppresses invasiveness of the tumor cells whether or not their growth is hormone-sensitive and also suppresses breast tumor progression (321–327). However, *in vitro* studies of the role of PR in breast cancer cell invasiveness have generally been investigated mechanistically in the absence of estrogen signaling. The studies have either used ER+ cell line models in the absence of estrogen or they have relied on forced expression of PRs in ER-negative cells (192–196). The relative contributions of PR ligands to invasiveness through opposing the suppressive effect of estrogen and the underlying mechanisms are still unclear in the literature.

Further, although the gene regulatory profile of ER has been shown to be estrogen dose-dependent (328), it is less clear whether PR has distinct mechanisms of action that depend on progesterone dose. The plasma levels of estrogen in pre-menopausal women is 1.4 nM - 1.6 nM in the follicular phase and 3.6 nM - 4.2 nM in the luteal phase (64). Plasma levels of estrogen in post-menopausal women is 0.027 ± 0.01 nM whereas breast tissue levels of estrogen in post-menopausal women is $1.4+0.7$ nM (72–74). The plasma level of progesterone ranges from 0.6 nM to 4 nM in the follicular phase and increases up to > 50 nM in the luteal phase(200) whereas post-menopausal women have a wide range of 0.047nM to 0.318nM (median 0.127nM) (191). The breast tissue level of progesterone in post-menopausal women is above an order of magnitude greater than its

plasma levels (306). Therefore, further investigation of the role of the individual PR isoforms on ER+ breast cancer cell invasiveness in the context of estrogen signaling and in the physiological range of breast tissue hormone levels was needed to more fully understand early events in hormonal regulation of breast cancer progression.

The ER+ model cell lines used in this study included T47D (ER+/PR+), ZR-75-1 (ER+/PR+) and BT474 (ER+/PR+/HER2+) cells. All three cell lines express both PR-A and PR-B. To dissect the actions of the individual PR isoforms, we also used recombinant T47D cells generated by Dr. Kathryn Horwitz and co-workers that virtually exclusively expressed PR-A or PR-B in addition to ER (329).

2.2 Materials and methods

2.2.1 Chemicals and reagents.

Dulbecco's modified Eagles medium (DMEM) and phenol red-free DMEM, glutamine, penicillin, streptomycin, Fetal Bovine Serum (FBS), charcoal stripped FBS and TaqMan probes were purchased (Life Technologies, Carlsbad, CA). 17β-estradiol (E$_2$), R5020, RU486, progesterone and medroxyprogesterone acetate (MPA) were purchased from Sigma Aldrich (Saint Louis, MO). Growth factor reduced matrigel (Cat# 356231) and calcein AM fluorescent dye (Cat# 354216) were purchased from BD Biosciences (San Jose, CA). PR-B directed siRNAs (339,340) and control non-silencing siRNA (Cat# SIC001) were ordered from Sigma Aldrich (St. Louis, MO). siRNAs targeting TM4SF1 (Cat# S8367), HES1 (Cat# S6920), PRKCH (CAT#S1107), and ELF5 (CAT# S4629) were purchase from Life Technologies (Carlsbad, CA).

2.4.2 Cell culture and treatment.

BT474, T47D and ZR-75-1 breast cancer cells (American Type Culture Collection) were cultured in DMEM supplemented with FBS (10%) penicillin (100 unit/ml) streptomycin

(100ug/ml) and L-glutamine (2mM). T47D-A and T47D-B cells were a generous gift from Dr. Katherine Horowitz (University of Colorado, Denver, CO) and were cultured as previously described (312). The cell lines were all cultured at 37°C with 5% CO_2. Before hormone treatment, cells were plated in 6-well plates at 30% confluence in phenol red-free media supplemented with charcoal-stripped FBS and incubated at 37°C with 5% CO_2 for 48 h. Cells were then treated with vehicle, progesterone, MPA, R5020, RU486 and E_2 alone or in various combinations at concentrations as indicated for each individual experiment for a duration of 48 h. The cells were then harvested for mRNA analysis, western blot analysis or cell invasion assays.

2.2.3 Western blot analysis.

Cells were lysed using RIPA Buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, and 50mM Tris pH 8.0) containing protease inhibitor cocktail (Pierce Biotechnology, Rockford IL). The lysates were chilled on ice and agitated by vortex every ten minutes for one hour. Total protein concentration was measured by Bradford assay (Bio-Rad laboratories, Hercules, CA). A total amount of 10-40 µg protein per sample was resolved by electrophoresis on a 8% SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore Corporation, Bedford MA). Membranes were probed with primary polyclonal rabbit anti-PR antibody (sc-539, Santa Cruz biotechnologies, CA), polyclonal rabbit anti-ERα antibody (sc-543, Santa Cruz Biotechnologies, CA), mouse monoclonal anti-GAPDH antibody (sc-4472, Santa Cruz Biotechnologies, CA), rabbit polyclonal anti-HES1 (sc-25392, Santa Cruz Biotechnologies, CA) or mouse monoclonal anti-ELF-5(sc-376737, Santa Cruz Biotechnologies, CA). The blots were then probed with appropriate horseradish peroxidase conjugated secondary antibody (Vector Laboratories, MD). The protein bands were visualized using enhanced chemiluminescence reagent Hyglo Quick spray (Denville Scientific, South Plainfield, NJ) per the manufacturer's suggested

protocol. Relative protein expression was determined by ImageJ (National Institutes of Health, USA).

2.2.4 RNA isolation, reverse transcription PCR and real time PCR.

Total RNA was isolated using the RNeasy mini kit (Qiagen, MD). Reverse transcription PCR reactions were performed using high capacity complementary DNA archive kit (Life Technologies Corporation, Carlsbad, CA) according to manufacturer's protocol. cDNA was measured by quantitative real time PCR using the StepOne Plus Real time PCR system (Life technologies Corporation, Carlsbad, CA). All mRNA measurements were performed in biological triplicates, and all C_T values were normalized to intra-sample GAPDH. mRNA values were represented as fold difference, which is calculated using the formula = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ sample -ΔC_T calibrator ($\Delta C_T = C_T$ of gene of interest- C_T of GAPDH).

2.2.5 Boyden chamber transwell invasion assay.

Cells ($1x10^5$) were re-suspended in the appropriate culture media devoid of serum and phenol red and added to the top chamber of the flouroblok inserts (Cat# 351152, 8 μM pore membrane: BD biosciences, Bedford, MA) coated with growth factor reduced matrigel (0.2 mg/ml). The chemoattractant comprised phenol red-free media supplemented with FBS (20%). The appropriate hormone treatment was included in both the top and bottom chambers. Each treatment was replicated in three wells and the entire experiment was replicated at least three times. Cells were allowed to invade for 24 h at 37°C with 5% CO_2. Cells that invaded to the bottom surface were stained with calcein AM (4ug/ml) in serum free media in the dark for 1 h at 37°C with 5% CO_2. Images were captured in an identical manner from each well in 5 non-overlapping fields (the middle of the well and surrounding fields) using a 4x objective. Images were analyzed

using ImageJ software (National Institutes of Health, USA) and the number of cells invaded was quantified by brightness and pixel size.

2.2.6 Migration Assay.

Pre-treatment and preparation of cells and the experimental protocol were identical to those described above for the Boyden chamber transwell invasion assay with the exception that the transwells were devoid of matrigel.

2.2.7 Lentiviral transduction.

293FT cells were used to generate lentiviral particles by transfection using lipofectamine 2000 (Life Technologies Corporation, Carlsbad, CA). Packaging plasmids pMD2G, PMDLg/RRE, and pRSV/Rev were cotransfected with pCDH PR-A expression plasmid, or pCDH empty vector plasmid. Lentivirus containing supernatant was harvested at 48 h and 72 h after transfection. T47D cells were plated in phenol red-free DMEM supplemented with heat-inactivated charcoal-stripped FBS (10%) and 2mM L-Glutamine two days before infection. For infection, T47D cells were transduced with either pCDH empty vector lentivirus or pCDH PR-A lentivirus with polybrene (8 μg/ml) for 5 h. A second transduction was performed similarly for another 5 h. The cells were then incubated in phenol red-free DMEM supplemented with charcoal-stripped serum (10%) and L-Glutamine (2mM) for 48 h. Following infection, cells were harvested for western blots and cell invasion assays as described previously.

2.2.8 siRNA Transfection.

Cells were plated to 30% confluence without antibiotic in phenol-red free DMEM medium supplemented with 10%charcoal-stripped FBS. 24 hours later cells were transfected with siRNA directed against specific gene targets or non-silencing siRNA using lipofectamine (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

2.2.9 mRNA expression profiling.

T47D-A and T47D-B cells were depleted of hormone for 48 h as described above. Cells were then either treated with vehicle, 1nM E_2, 1nM R5020, or 1nM E_2+ 1nM R5020 for 48 h. Total RNA was isolated using the RNeasy mini kit (Qiagen, MD). Sample identities were randomized for blinded analysis. The samples were analyzed at the Wayne State University School of Medicine Applied Genomics Center (AGTC) using the HumanHT-12 v4 Expression BeadChip with the Illumina HiScan System (Illumina, San Diego, CA). A total of 47,000 probes were used to analyze the transcriptome expression for each treatment group. Data was analyzed using Partek V6.6 software (St. Louis, MO), and processed using genome Studio (Illumina, San Diego, CA). Expression values were normalized using quantile-normalization, with background subtraction. Log transformation to the base of 2, followed by one way ANOVA was used to determine error. The differentially expressed genes were identified by comparing E_2 treatment with vehicle treatment, R5020 treatment with vehicle treatment and E_2 treatment with E_2+R5020 treatment (repressed or activated with a fold difference of 1.5 and a p value < 0.05). Genes that had activated expression in E_2+R5020 treatment but were repressed by E_2 treatment were identified. Genes that were activated in E_2 treatment but repressed by E_2+R5020 were also identified. Gene ontology analysis was performed by literature mining by searching the MEDLINE database (National institutes of Health, USA) with a query of "name of gene" followed by the term "AND Cancer" or " AND Breast Cancer". All articles under the specified query were examined to determine gene function in breast cancer. Validation of Microarray Data was performed by real-time RT-PCR as described above using TaqMan probes.

2.2.10 Statistical analysis.

Experimental values were presented as mean +/- standard deviation using triplicate treatment sets. The statistical difference between values was determined by using one way ANOVA followed by post hoc paired t-test. The significant *P* values are noted in the figures. Concordant results were obtained from at least three repetitions of the experiments conducted on different days.

2.2.11 Supplemental Tables 1-13. mRNA profiling of estrogen (1nM) and progesterone (1nM) regulated genes in cells expressing ER and either PR-A or PR-B .

Expression values from genome expression profiling in tables can be found:

http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=vie
w&path%5B%5D=508

2.3 Results

2.3.1 Estrogen dose dependence for inhibition of invasiveness.

Estrogen (E_2) is known to inhibit breast cancer cell invasiveness (326,330–333). To relate the effect of E_2 on invasiveness to physiological E_2 levels, the E_2 dose response for inhibition of invasiveness was determined in BT474, T47D and ZR-75-1 cells. E_2 was able to inhibit invasiveness of the cells in the sub-nanomolar range with most of the inhibition occurring below 0.01 nM and virtually complete inhibition occurring at 0.1 nM in all three cell lines (Figure 2.1-A, B, C). Thus the E_2 dose that was required for substantial or virtually complete suppression of invasiveness in the three ER+ cell lines is at the low end of the literature consensus for both plasma and breast tissue levels of E_2 in pre-menopausal (1.4nM- 4.2nM) or post-menopausal (0.027 ± 0.01 nM in plasma; $1.4nM \pm 0.7$ in breast tissue) women (64,72–74). Invasiveness remained completely suppressed at higher concentrations of E_2 (10nM and 20nM) (Figure 2.2).

2.3.2 Dose-dependent dual regulation of invasiveness by natural and synthetic progestins.

Plasma levels of progesterone are known to change throughout a woman's menstrual cycle ranging from 0.6 nM to 4 nM in the follicular phase and upwards to greater than 50 nM in the luteal phase (200). Furthermore, the median plasma concentration of progesterone in post-menopausal women is 0.127 nM (191) with breast tissue concentrations an order of magnitude greater than in the plasma (306). To examine the effects on progesterone in the context of estrogen signaling, BT474, T47D and ZR-75-1 cells were treated at varying concentrations (0 nM-100 nM) of progesterone either alone or in the presence of a fixed concentration (1 nM) of E_2 (Figure 2.1D-F). In all the three cell lines E_2 alone inhibited invasiveness. However, progesterone at 0.5 nM at least partially rescued invasiveness from the effects of E_2 and showed virtually complete rescue in all cases at a concentration of 1 nM. It may be noted that progesterone alone (in the absence of E_2) did not influence invasion below a concentration of 2.5 nM - 5 nM but only rescued invasiveness from E_2 regulation in the low concentration range (Figure 2.1-D, 2.1E, 2.1F). At higher concentrations, progesterone progressively increased invasiveness of the cells independent of estrogen (Figure 2.1D-F).
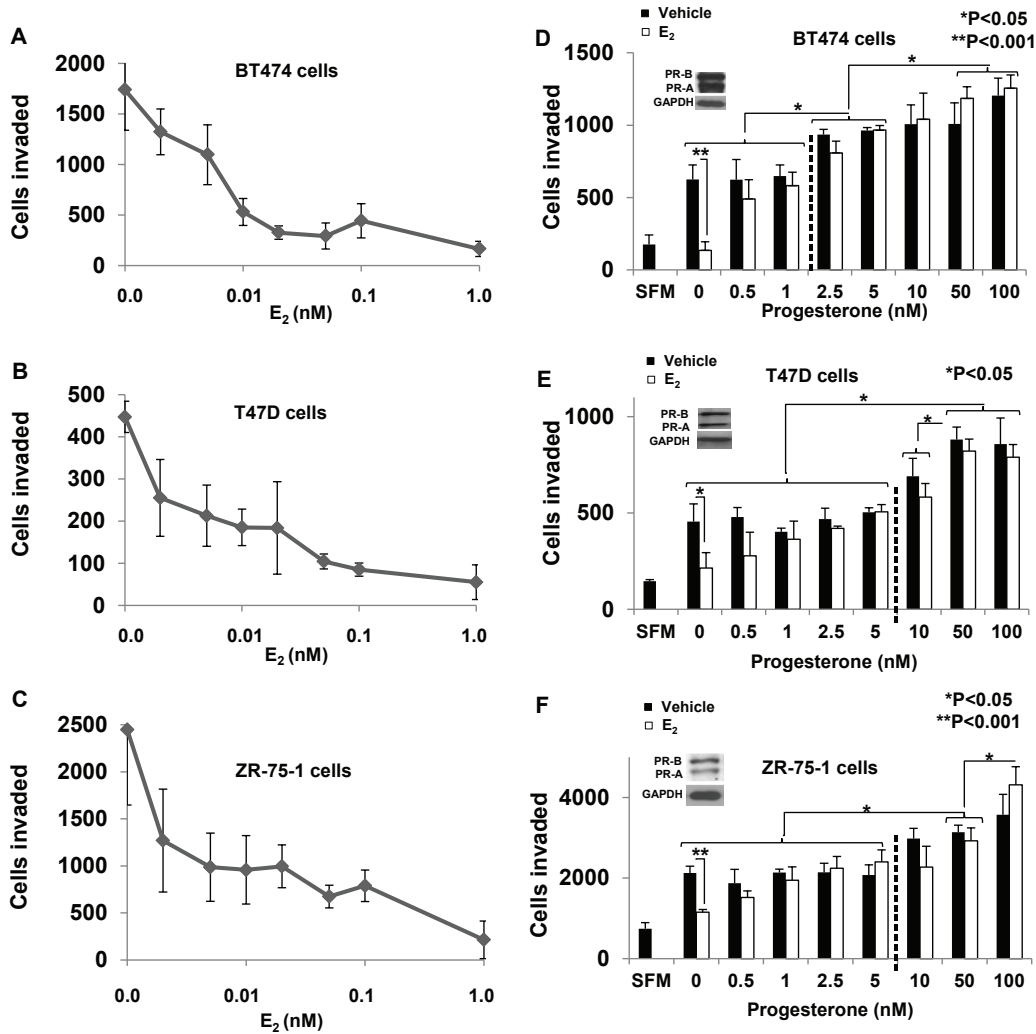
Figure 2.1 Dose response of regulation of breast cancer cell invasiveness by estrogen and progesterone. In panels A-C, hormone depleted BT474 cells (*Panel A*), T47D cells (*Panel B*) and ZR-75-1 cells (*Panel C*) at 30% confluence were treated with vehicle or the indicated concentrations of E_2 for 48 h. Cells were trypsinized and subjected to the matrigel transwell invasion assay with vehicle or the appropriate concentration of hormone present in the top and bottom chambers, as described under Materials and Methods. In the negative control, serum free media (SFM) was used instead of the FBS chemoattractant. Data points in the plots in *Panels A-C* represent values for invasiveness represented as average number of cells invaded with the background (SFM) values subtracted. In panels D-F, Hormone depleted BT474 cells (*Panel D*), T47D cells (*Panel E*) and ZR-75-1 cells (*Panel F*) at 30% confluence were treated with vehicle or E_2 (1 nM) and the indicated concentrations of progesterone for 48 h. Cells were trypsinized and subjected to the matrigel transwell invasion assay.
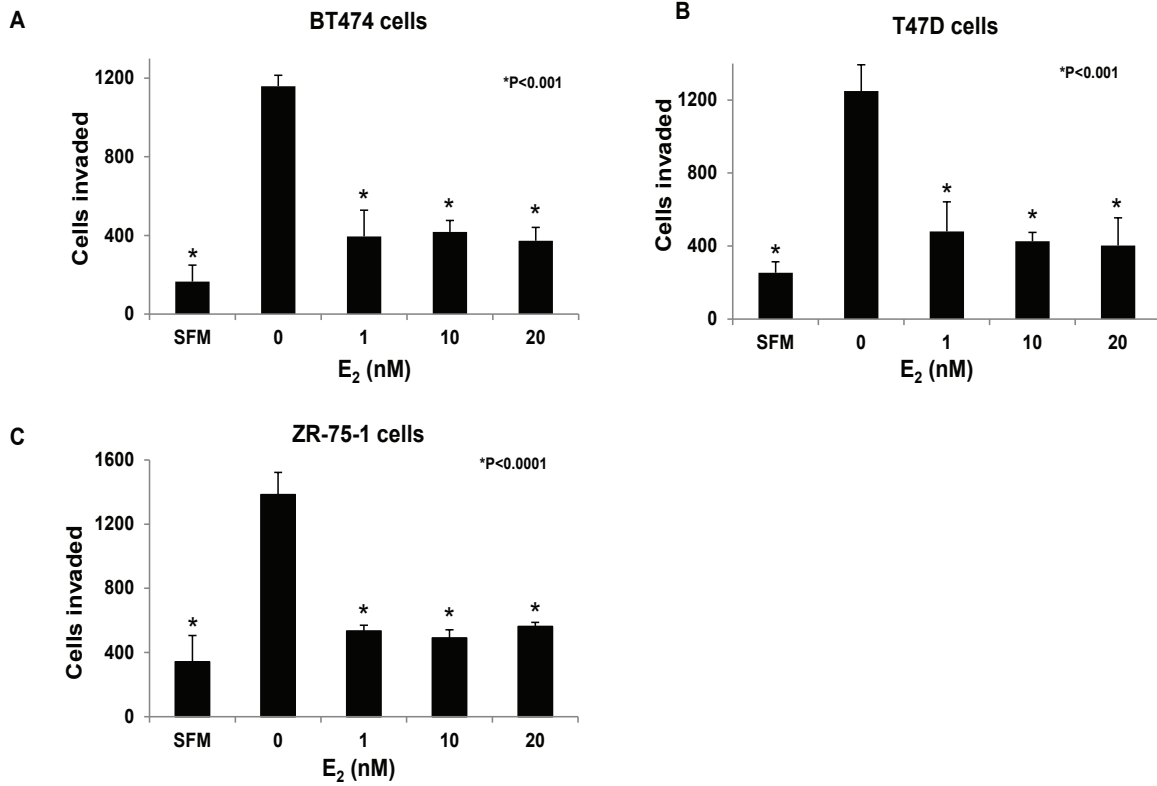
Figure 2.2 Inhibition of invasiveness by estrogen at different concentrations. Hormone depleted BT474 cells (*Panel A*), T47D cells (*Panel B*) and ZR-75-1 cells (*Panel C*) at 30% confluence were treated with vehicle or the indicated concentrations of E_2 for 48 h. Cells were trypsinized and subjected to the matrigel transwell invasion assay with vehicle or the appropriate concentration of hormone present in the top and bottom chambers, as described under Materials and Methods. In the negative control, serum free media (SFM) was used instead of the FBS chemoattractant. Values are represented as average number of cells invaded from experimental triplicates and the error bars represent standard deviation. P Values are indicated.

The hormones regulated invasiveness of the cells without affecting their migratory capacity (i.e., in the absence of matrigel in the transwells) (Figure 2.3). Thus, the data in Figure 2.1 (D-F) reveals two components of progesterone's effect on invasiveness *in vitro* in the three cell line models studied: (i) at low concentrations, progesterone rescues invasiveness from suppression by E_2; and (ii) at higher concentrations, progesterone also induces invasiveness independent of E_2.

As noted above, during the luteal phase of the menstrual cycle, the E_2 level is elevated to about 4 nM along with an increase in the progesterone levels from about 4 nM to about 50 nM. Therefore, we tested the effect of 4 nM and 50 nM R5020 (a more stable synthetic progestin), in the presence of 4 nM E_2 on invasiveness of T47D, ZR-75-1 and BT474 cells (Figure 2.4A-2.4C). In all the three cell lines, suppression of invasiveness by E_2 was completely prevented by both concentrations of R5020 and at 50 nM R5020, there was a further increase in invasiveness (Figure 2.4A-2.4C).

The dual effect of progesterone on invasiveness was recapitulated in all the three cell lines using the potent synthetic progestin medroxyprogesterone acetate (MPA). When MPA is used as a contraceptive (intramuscular route of administration) it has a mean plasma concentration of 2.58 nM (334) and has a 10-20 fold higher plasma concentration when administered orally during hormone replacement therapy (335). At a concentration of 1 nM, MPA only reversed suppression of invasion by E_2, but at higher concentrations (10 nM and 100 nM) MPA induced an increase in invasiveness well above the basal level whether or not E_2 was present (Figure 2.4D-2.4F).
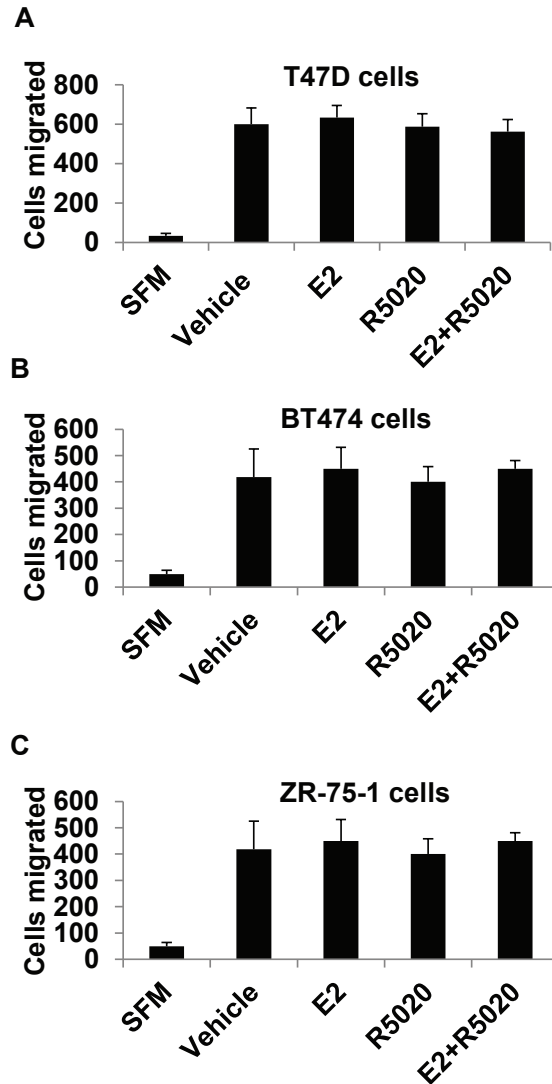
Figure 2.3 Absence of hormonal control of migration capacity of ER+/PR+ model cells. Hormone depleted T47D cells *(Panel A)*, BT474 cells *(Panel B)* and ZR-75-1 cells *(Panel C)* at 30% confluence were treated with vehicle, E_2 (1 nM), R5020 (1 nM), or E_2 (1 nM) plus R5020 (1 nM) for 48 h. Cells were trypsinized and subjected to transwell migration assay (i.e., in the absence of matrigel) with vehicle or the appropriate concentration of hormone present in the top and bottom chambers, as described under Materials and Methods. In the negative control, serum free media (SFM) was used instead of the FBS chemoattractant. Values are represented as average numbers of cells migrated from experimental triplicates and the error bars represent standard deviation. One-way ANOVA was performed and there was no significant difference between the treatment groups.
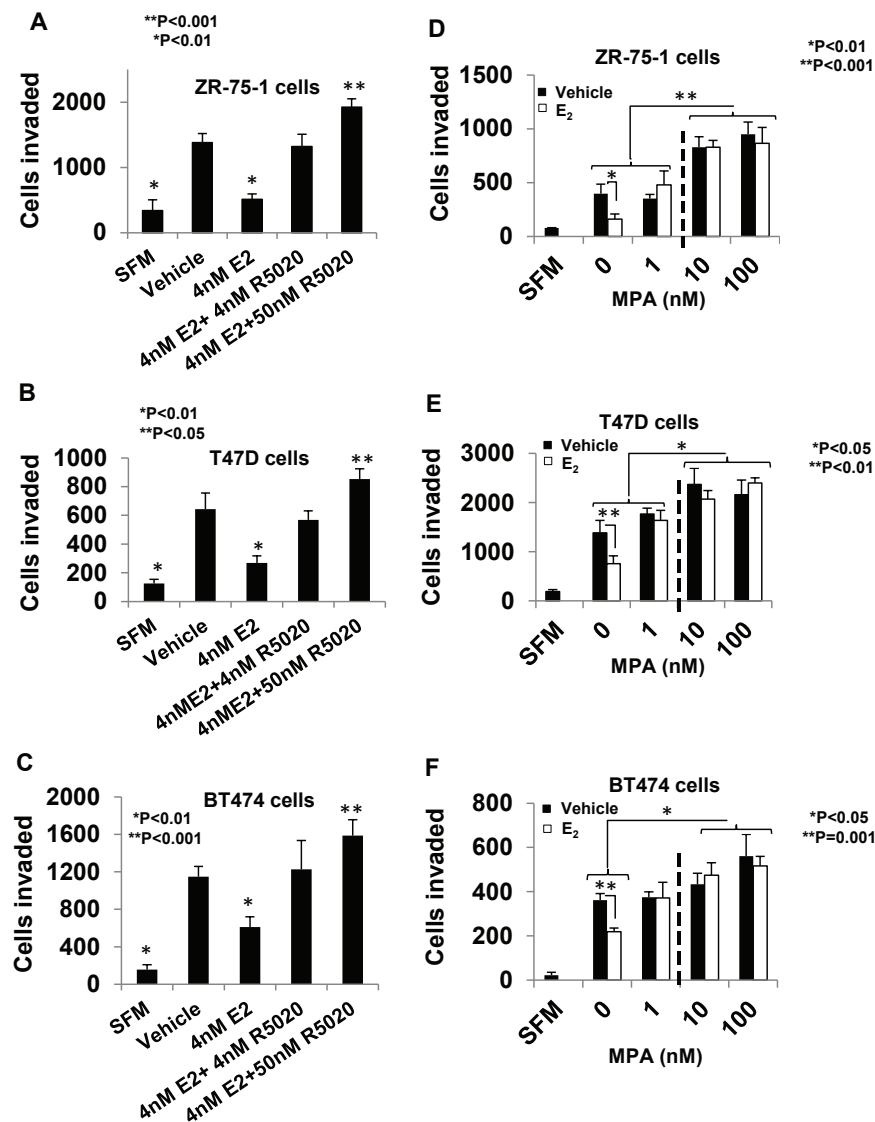
Figure 2.4 Regulation of breast cancer cell invasiveness by pre-menopausal concentrations of estrogen and progestin and dose-dependent effects of medroxyprogesterone acetate. In panels A-C, hormone depleted ZR-75-1 cells *(Panel A)*, T47D cells *(Panel B)*, and BT474 cells *(Panel C)* at 30% confluence were treated with vehicle or E_2 (4 nM), alone or in combination with R5020 (5 nM or 50 nM) for 48 h. Cells were trypsinized and subjected to the matrigel transwell invasion assay with vehicle or the appropriate concentration of E_2 and/or R5020 present in the top and bottom chambers, as described under Materials and Methods. In the negative control, serum free media (SFM) was used instead of the FBS chemoattractant. In panels D-F, hormone-depleted ZR-75-1 cells *(Panel D)*, T47D cells *(Panel E)*, and BT474 cells *(Panel F)* cells at 30% confluence were treated with vehicle or the indicated concentrations of MPA either with or without 1nM E_2 for 48 h. Cells were trypsinized and subjected to the matrigel invasion assay as described under Materials and Methods. One-way ANOVA was performed on triplicate treatment sets and P values are indicated.

2.3.3 The distinctive role of each PR isoform in the regulation of invasiveness by progestins.

The above observations led to the question of which PR-isoform(s) could mediate each of the two components of the regulation of invasiveness by progestins. To address this question, the effect of progestin dose on breast cancer cell invasiveness was tested in the absence or presence of 1nM E_2 using recombinant T47D cells that exclusively express PR-A (T47D-A cells) or PR-B (T47D-B cells) (Figure 2.5A). The recombinant cells were a kind gift from Dr. Kathryn Horwitz who generated the cells as previously described (329). Due to possible variance in absolute values of the number of cells invaded across experiments for a given cell line, we compared the invasive capacity of the T47D-A and T47D-B cells plated together under the same conditions at the same time. There was no difference in the invasive capacity of the two isogenic cell lines (Figure 2.6). In T47D-A cells both, R5020 and MPA only rescued invasiveness from E_2 regulation at all concentrations tested (1nM, 10nM or 100 nM) but had no effect on invasiveness in the absence of E_2 (Figure 2.5B and 2.5C). In contrast, in T47D-B cells, 1 nM of either R5020 or MPA was unable to rescue invasiveness from E_2 regulation but at the higher concentrations (10 nM or 100 nM) they induced invasiveness well above the basal level and this was uninfluenced by the presence of E_2 (Figure 2.5D and 2.5E).
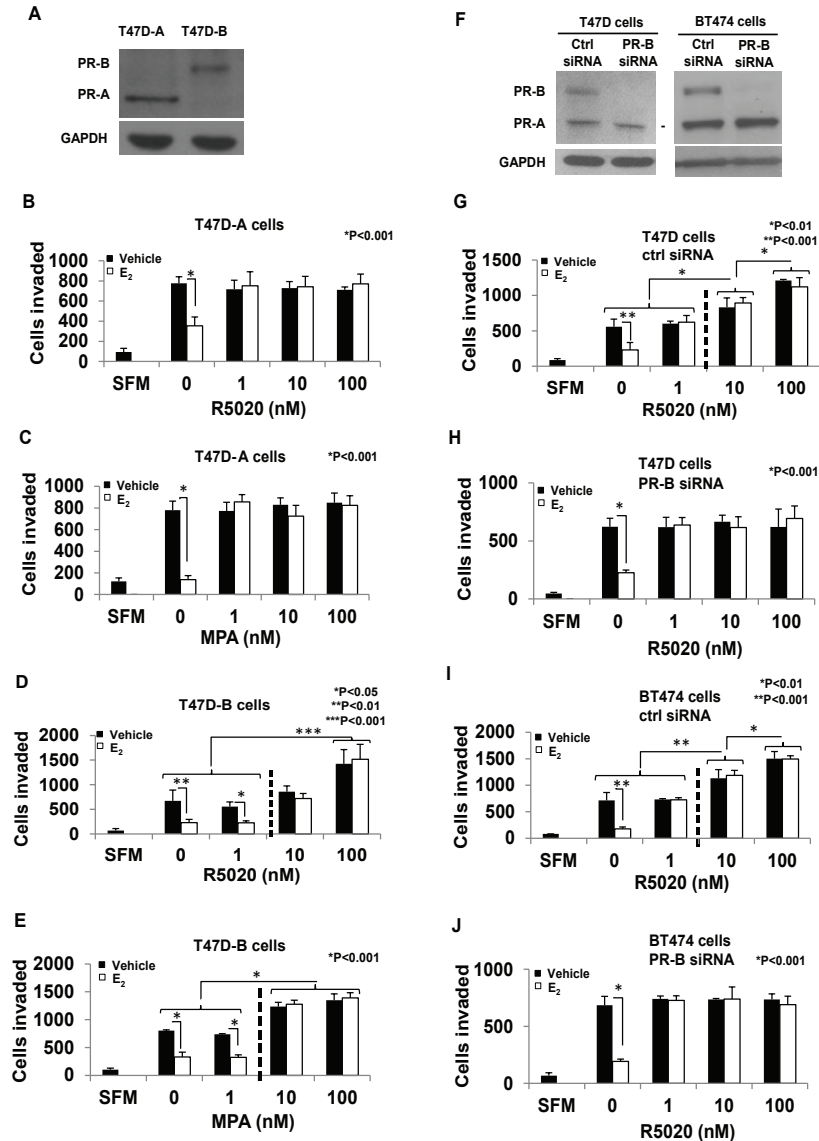
Figure 2.5 PR-A vs. PR-B mediated effects of progestins on invasiveness of breast cancer cells. Panel A shows a western blot of cell lysates from T47D-A and T47D-B. In panels A-E, hormone-depleted T47D-A cells *(Panels B and C)* and T47D-B cells *(Panels D and E)* at 30% confluence were treated with vehicle or 1 nM E2 in combination with the indicated concentrations of R5020 (*Panels B and D*) or MPA (*Panels C and E*) for 48 h. Cells were trypsinized and subjected to the matrigel transwell invasion assay, as described under Materials and Methods. In panels F-J, hormone depleted cells were transfected with either siRNA directed against PR-B or non-targeted control siRNA and incubated for 48h. In Panel F cell lysates from the transfected cells were analyzed by western blot for PR or GAPDH (loading control. T47D cells transfected with control siRNA (*Panel G*) or PR-B targeted siRNA (*Panel H*) and also BT474 cells transfected with control siRNA (*Panel I*) or PR-B targeted siRNA (*Panel J*) were treated with hormones and subjected to the matrigel transwell invasion assay. In panels B-E and G-J, One-way ANOVA was performed and P values are indicated.

As a complementary approach, we utilized siRNA knockdown of PR-B in parental T47D cells and in BT474 cells that express equal amounts of PR-A and PR-B. Both cell lines were transfected with siRNA directed against a target site in the unique 5' segment of the PR-B mRNA or with control non-silencing siRNA using Lipofectamine. Knockdown of PR-B (western blots in Figure 2.5F) resulted in a loss of E_2-independent induction of invasiveness at the higher concentrations (10 nM and 100 nM) of R5020 in both T47D cells and BT474 cells (Figure 2.5H and 2.5J) in contrast to the control siRNA transfected cells (Figure 2.5G and 2.5I). However, the selective depletion of PR-B did not alter R5020's ability to rescue invasiveness in the presence of E_2, at all concentrations (1nM, 10nM, and 100nM) of R5020 (Figure 2.5G-2.5J). This result is consistent with those observed above using T47D-A cells. The above results demonstrate that PR-A exclusively mediates the role of low dose progestins in opposing suppression of invasiveness by E_2, whereas PR-B exclusively mediates E_2-independent induction of invasiveness at high doses of progestins.
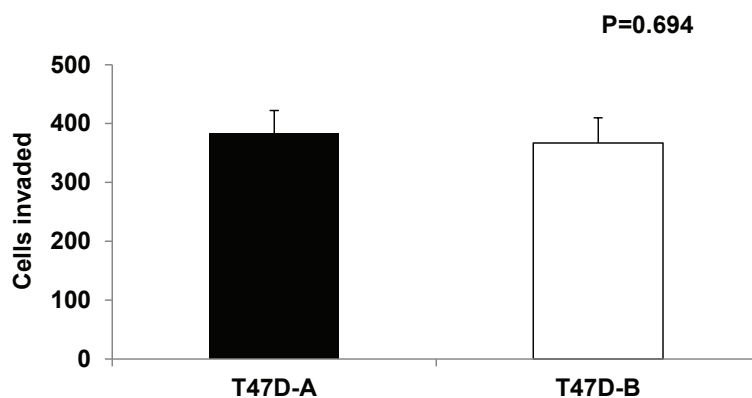
Figure 2.6 Invasive capacity of the isogenic T47D-A vs. T47D-B cell lines. T47D-A and T47D-B cells at 30% confluence were incubated in hormone-depleted media for 96 hours. Cells were trypsinized and subjected to the matrigel transwell invasion assay as described under Materials and Methods. Values are represented as average number of cells invaded from experimental triplicates and the error bars represent standard deviation.

2.3.4 Effect of RU486 on PR-A mediated induction of invasiveness by progestins.

RU486 is a synthetic antagonist of progesterone that is PR isoform selective in specific target gene contexts. Therefore it was of interest to test the effect of RU486 on the PR-A dependent actions of progesterone on breast cancer cell invasiveness. The cell lines T47D-A, T47D-B and BT474 were treated with either E_2, the progestin R5020, or the anti-progestin RU486, each at a concentration of 1 nM in the various combinations indicated in Figure 2.7. In T47D-A cells, RU486 disrupted the ability of R5020 to rescue invasiveness from E_2 suppression but did not have any effect by itself on invasiveness, in either the presence or absence of E_2 (Figure 2.7A). On the other hand, in T47D-B cells, RU486 had no effect on invasiveness under any of the conditions tested when each of the ligands was used at a concentration of 1 nM (Figure 2.7B). In BT474 cells, which express equal amounts of both PR isoforms, the effect of RU486 was similar to that observed in the T47D-A cells, demonstrating that agonist or antagonist actions that modulate the effect of PR-A on invasiveness are functionally independent of PR-B expression (Figure 2.7C).

2.3.5 Hypersensitization of PR-A to progestin through overexpression of the receptor.

As noted above, PR-A is frequently overexpressed in invasive clinical breast tumors. It was therefore of interest to examine the possibility that overexpression of PR-A in the tumor cells may sensitize PR-A mediated regulation of invasiveness to post-menopausal breast tissue levels of progesterone.
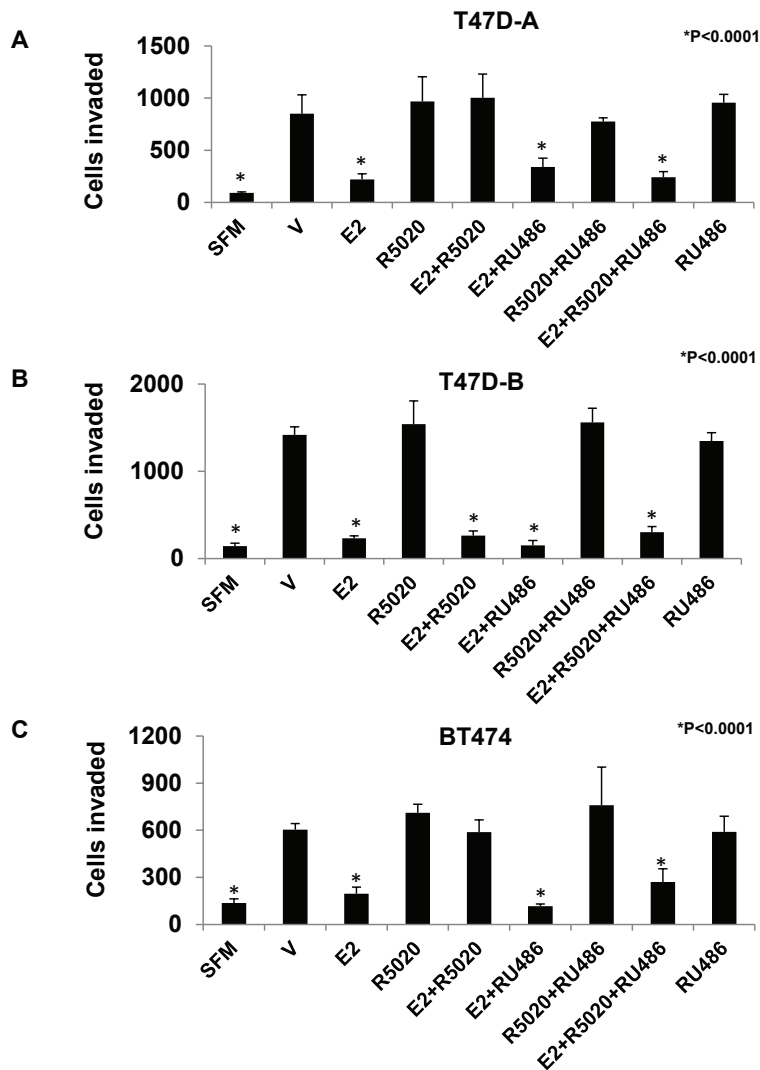
Figure 2.7 Effect of RU486 on regulation of breast cancer cell invasiveness by R5020 in relation to estrogen, PR-A and PR-B. Hormone depleted T47D-A *(Panel A)*, T47D-B *(Panel B)*, and BT474 *(Panel C)* cells at 30% confluence were treated with vehicle or the indicated combinations of E_2, R5020 and RU486, each at a concentration of 1nM for 48 h. Cells were trypsinized and subjected to the matrigel transwell invasion assay with vehicle or the appropriate concentration of E_2, R5020 or RU486 present in the top and bottom chambers, as described under Materials and Methods. In the negative control, serum free media (SFM) was used instead of the FBS chemoattractant. Values are represented as average number of cells invaded from triplicate treatment sets and the error bars represent standard deviation. One-way ANOVA was performed on triplicate treatment sets and P values are indicated.

A

B

C
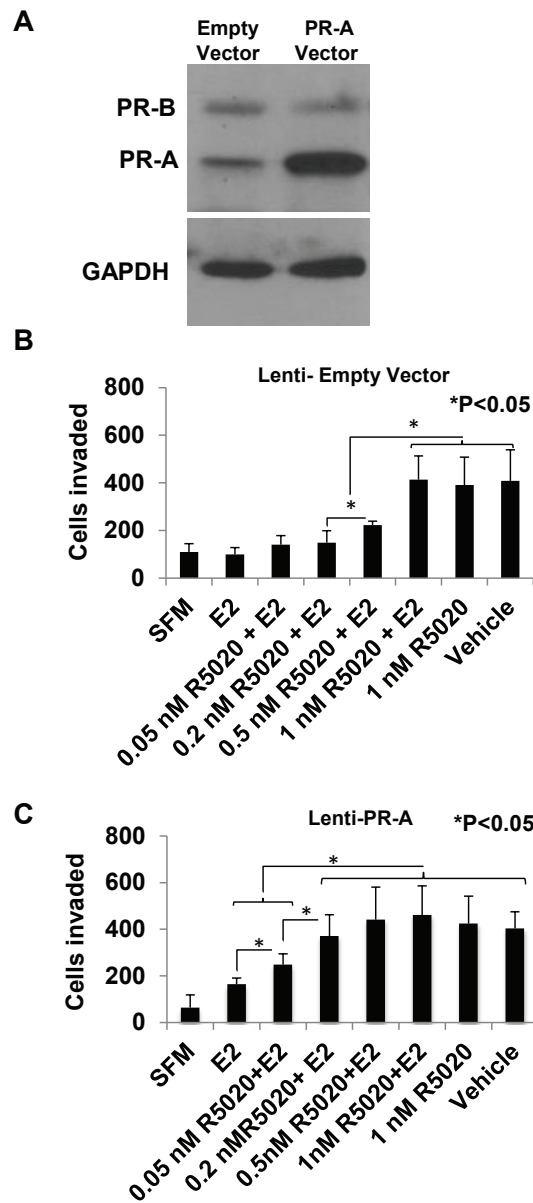
Figure 2.8 Effect of overexpressing of PR-A on the progestin dose response for rescue of invasiveness from estrogen regulation. PR-A was ectopically overexpressed in hormone-depleted T47D cells by lentiviral transduction, as described under Materials and Methods. Whole cell lysates from cells transduced with either the PR-A expression vector or the control empty vector were probed for PR and for GAPDH *(Panel A)*. Cells transduced with the control empty vector *(Panel B)* or PR-A expression vector *(Panel C)* at 30% confluence were treated with vehicle or the indicated concentrations of R5020 in the absence or in the presence of E_2 (1 nM) for 48 h. Cells were then trypsinized and subjected to the matrigel transwell invasion assay. Values are represented as average number of cells invaded from triplicate treatment sets and the error bars represent standard deviation. One-way ANOVA was performed on triplicate treatment sets and P values are indicated.

T47D cells express comparable amounts of PR-A and PR-B protein as observed on a western blot probed with an antibody against a common carboxyl-terminal peptide of the two receptor isoforms (Figure 2.8A). Lentiviral transduction of a PR-A expression plasmid increased the level of PR-A by approximately 3.7-fold, without altering the expression of PR-B (Figure 2.8A). The R5020 dose-dependence for rescue of invasiveness from E_2 regulation was compared between the PR-A overexpressing cells and the control cells transduced with the empty vector. Overexpression of PR-A clearly conferred hypersensitivity to R5020 as the progestin partially rescued invasiveness even at a concentration of 0.05 nM and fully rescued invasiveness at a concentration of 0.2 nM in the PR-A overexpressing cells (Figure 2.8C); in comparison, in the control cells a concentration of 0.5 nM - 1.0 nM R5020 was required to observe similar effects (Figure 2.8B).

2.3.6 PR isoform A-dependent regulation of E_2 target genes by progestin and their functional role.

E_2 acts through its receptor ER to repress expression of genes known to be involved in breast tumor invasion, EMT, and metastasis (182,332,333,336,337). The ability of progestins to oppose E_2 regulation of invasiveness did not involve a decrease in ER expression as evident from a western blot of T47D-A cells treated with R5020 (Figure 2.9A).

Next, we undertook to examine PR isoform-specific effects on transcriptional signaling by E_2 using T47D-A or T47D-B cells. A concentration of 1 nM R5020 was chosen because at this concentration the progestin completely rescued invasiveness from E_2 regulation (through PR-A) but did not exert E_2-independent effects on invasiveness (through PR-B). The cells were treated with vehicle, 1 nM E_2, 1 nM R5020 or 1 nM R5020 + 1 nM E_2 for an extended duration of 48 hours to examine expression of both direct and indirect target genes of the hormones. mRNA

expression profiles were examined by DNA microarray analysis using the Illumina platform and an arbitrary cut off value of 1.5-fold was applied to identify patterns of changes in mRNA expression. In T47D-A cells, among 631 genes that were repressed by E_2 (Supplemental Table 1) (Figure 2.9B), R5020 opposed the repression of 108 genes (Supplemental Table 2) (Figure 2.9B) including 48 genes that were activated by R5020, independent of E_2 (Supplemental Table 3) (Figure 2.9B). In T47D-B cells, among 311 genes that were repressed by E_2 (Supplemental Table 4) (Figure 2.9C), R5020 opposed the repression of 47 genes (Supplemental Table 5) (Figure 2.9C) including 21 genes that were activated by R5020, independent of E_2 (Supplemental Table 6) (Figure 2.9C). Inspection of these gene lists revealed that of the 108 E_2 repressed genes whose expression was rescued by R5020 in T47D-A cells, only 9 genes were also rescued by R5020 in T47D-B cells. The E_2 repressed genes that were activated by progesterone alone were also cell-type specific, with only 8 exceptions. Thus, repression of 99 genes by E_2 was opposed by R5020 in an exclusively PR isoform A-dependent manner. We next searched the literature to identify all the genes in this group that had suggested or established roles in breast tumor biology. A total of 19 genes were clearly known to be associated with breast tumor biology and they predominantly supported breast tumor progression, including invasiveness and metastasis (Supplemental Table 7). The DNA microarray data is validated for 4 representative genes (HES1, PRKCH, ELF5 and TM4SF1) by quantitative real time RT-PCR in Figure 2.10A and 2.10B using T47D-A and T47D-B cells. We also confirmed that these four genes were regulated in T47D (parental), BT474, and ZR-75-1 cells in the same pattern as that observed in T47D-A cells (Figure 2.10C-2.10E).

A T47D-A Cells

B T47D-A cells

C T47D-B cells

Figure 2.9 Effect of low dose progestin on the gene repression profile of estrogen in relation to PR-A and PR-B. Hormone depleted T47D-A or T47D-B cells at 30% confluence were treated with vehicle, 1 nM E_2, 1 nM R5020 (P) or 1nM E_2 plus1nM R5020 (E_2+P) for 48 h. In *Panel A*, whole cell lysates from the treated T47D-A cells were probed by western blot for ERα and for GAPDH. In parallel, total RNA was extracted from the treated T47D-A and T47D-B cells and subjected to mRNA expression profiling as described under Materials and Methods. The mRNA profiling data is represented in the Venn diagrams in *Panel B* (for T47DA cells) and in *Panel C* (for T47D-B cells). *Panels B* and *C* show comparisons among the gene set repressed by E_2 (E_2 repressed vs. Vehicle), the gene set activated by R5020 in the absence of E_2 (P activated vs. Vehicle) and the gene set activated by R5020 in the presence of E_2 (E_2+P activated vs. E_2). The data represents results from experimental triplicates.
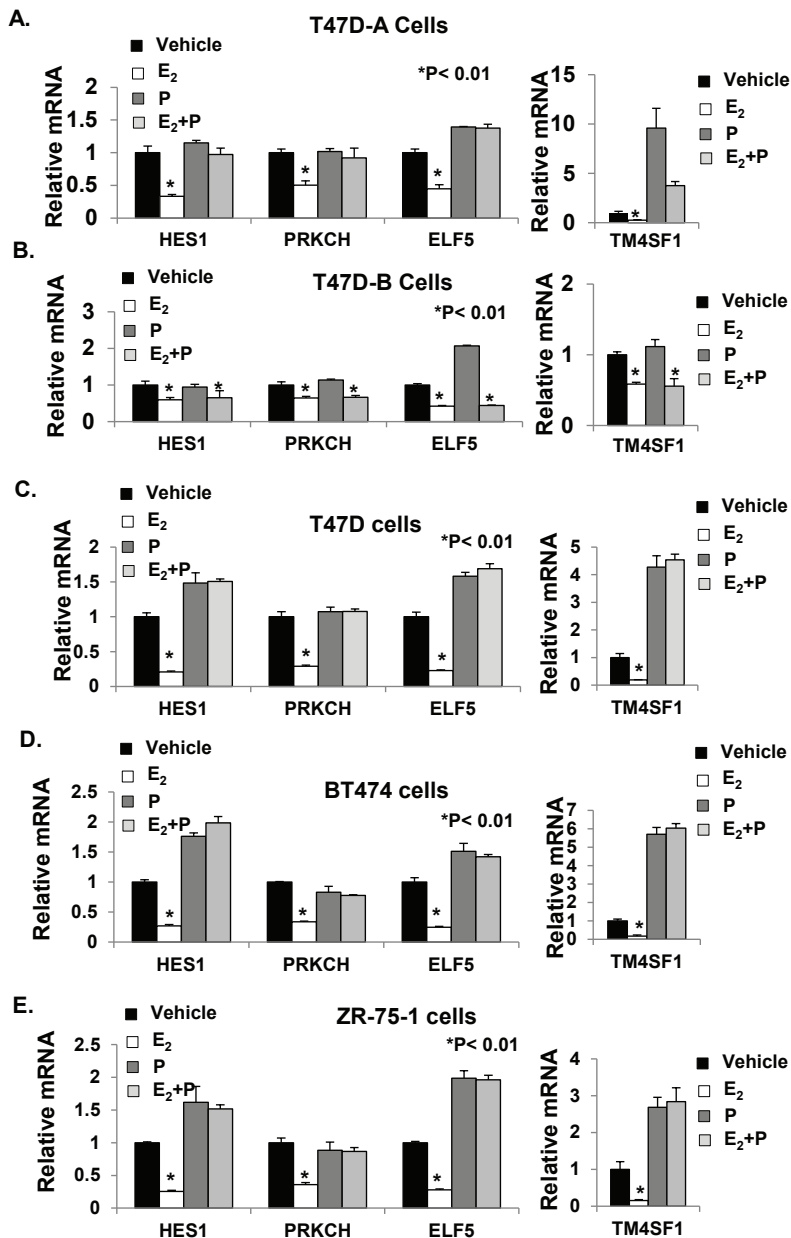
Figure 2.10 Validation of gene expression profiling. The total RNA samples used for mRNA expression profiling in Figure 6 were used for validation of the mRNA profiling data for selected genes in T47D-A cells *(Panel A)* and T47D-B cells *(Panel B)*. Validation of estrogen and progestin regulation of these genes in the PR-A+ cells was also extended to T47D (parental) cells *(Panel C)*, BT474 cells *(Panel D)* and ZR-75-1 cells *(Panel E)*. RNA purified from the treated cells was reverse transcribed and the cDNA was analyzed by real-time PCR using TaqMan Probes, as described under Materials and Methods. Relative mRNA levels were measured in the samples for HES1, PRKCH, ELF5, and TM4SF1 genes. All C_T Values were normalized to GAPDH and represented as fold change in comparison to vehicle treated controls.

The four genes (HES1, PRKCH, ELF5 and TM4SF1) validated above have all been associated with cancer progression. To directly test whether regulation of these genes by E_2 mediated the hormonal effects of E_2 on invasiveness in ER+ breast cancer cells, we used a loss-of-function approach. T47D and BT474 cells were transfected with siRNAs against the four genes either individually (Figure 2.11A and 2.11E) or together (Figure 2.11B and 2.11F); in all cases, the siRNAs effectively knocked down the genes, as observed by their mRNA levels compared to the cells transfected with control non-targeted siRNA (Figure 2.11A, 2.11B, 2.11E and 2.11F). Individually knocking down the genes decreased invasiveness of the cells to different degrees (Figure 2.11C and 2.11G) and the combined knockdown completely suppressed invasiveness (Figure 2.11D and 2.11H). The results indicate the functional relevance of genes whose regulation by E_2 was found in this study to be opposed by low dose progesterone acting through PR-A. Clearly the subset of E_2 repressed genes that are counter-regulated by progesterone/PR-A include genes that mediate hormonal regulation of invasiveness in breast cancer cells.

A similar analysis was then conducted for E_2 activated genes in T47D-A (Figure 2.12A and Supplemental Tables 8-10) and T47D-B (Figure 2.12B and Supplemental Tables 11-13) cells. We found that activation of 112 genes by E_2 was opposed by R5020 in an exclusively PR-A isoform dependent manner. Within this group, the small number of genes with better known functions in breast tumor biology tended to support growth and inhibit invasiveness.
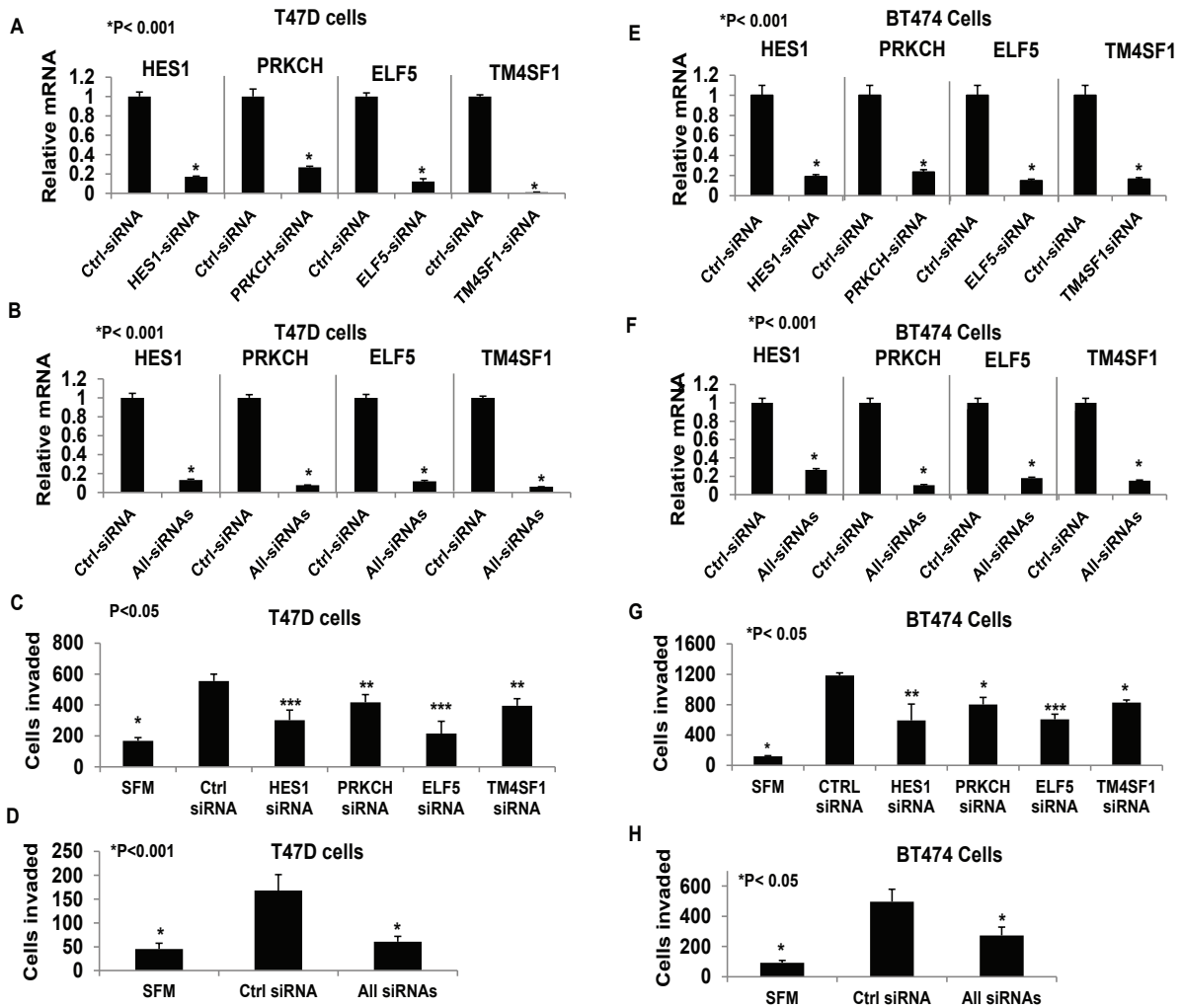
Figure 2.11 Functional testing of selected tumor progression genes. Hormone-depleted T47D cells (*Panels A-D*) and BT474 (*Panels E-H*) were transfected with control siRNA, TM4SF1 siRNA, HES1 siRNA, ELF5 siRNA and PRKCH siRNA independently (*Panel A, C, E, and G)* or all four targeted siRNAs in combination (*Panels B, D, F, H*). After 72 hours cell were subjected to the transwell matrigel invasion assay (*Panels C, D, G, and H*) as described under Materials and Methods. In the negative controls, serum free medium (SFM) was used instead of the FBS chemoattractant. Values are represented as average number of cells invaded from triplicate treatment sets and the error bars represent standard deviation. One way ANOVA was performed on triplicate treatment sets and P values are indicated.

A

T47D-A

E₂ activated vs. Vehicle   P repressed vs. Vehicle

683

527   28

84   687

273

E₂ + P repressed vs. E₂

B

T47D-B

E₂ activated vs. Vehicle   P repressed vs. Vehicle

3   402

254   12

333

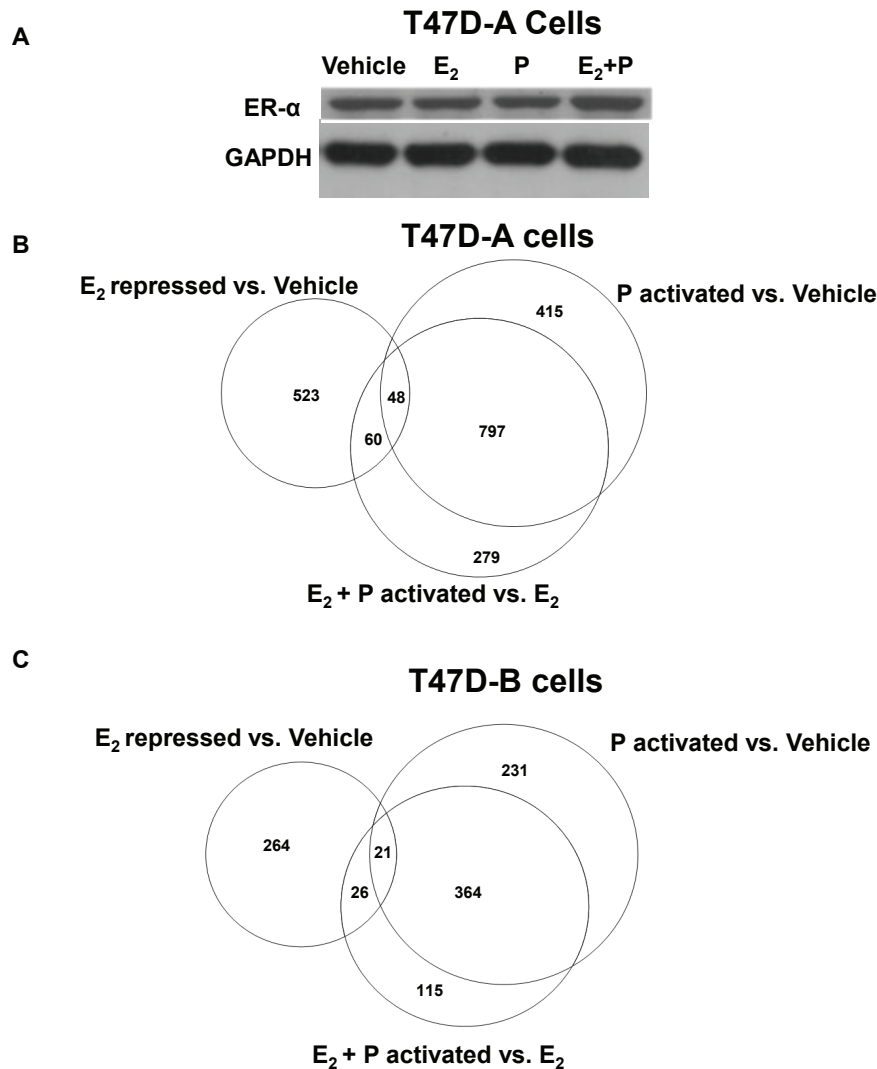24

100

E₂ + P repressed vs. E₂
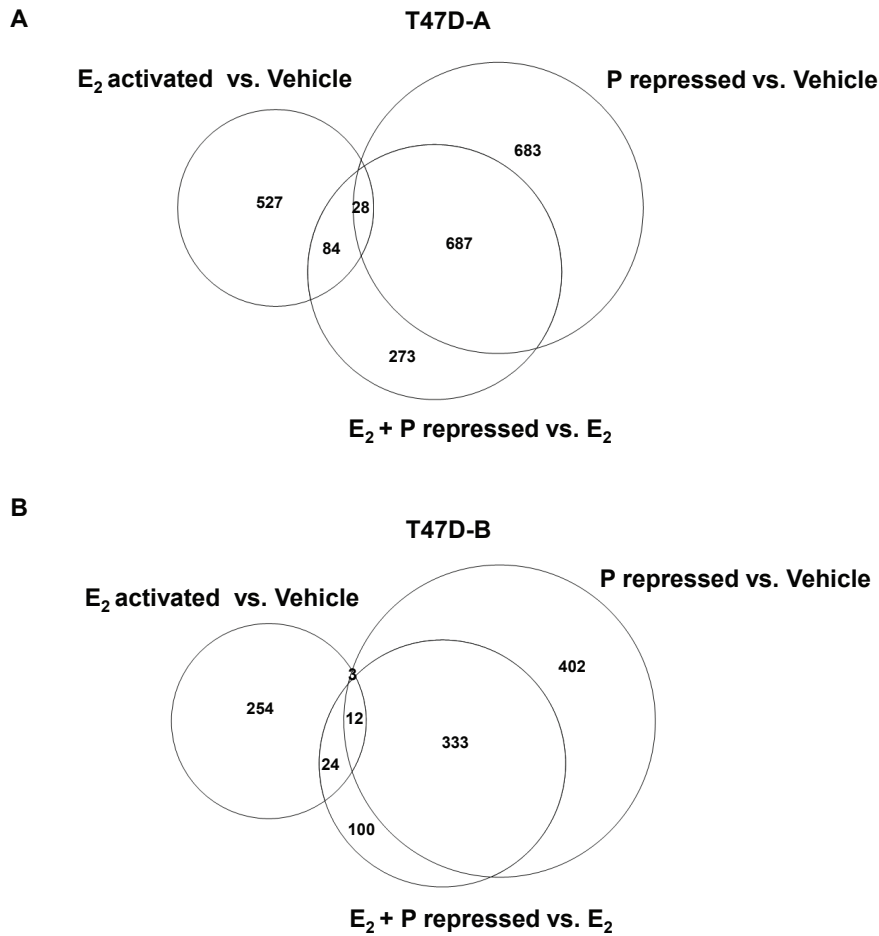
Figure 2.12 Effect of low dose progestin on the gene activation profile of estrogen in relation to PR-A and PR-B. Hormone depleted T47D-A or T47D-B cells at 30% confluence were treated with vehicle, 1 nM E₂, 1 nM R5020 (P) or 1nM E₂ plus1nM R5020 (E₂+P) for 48 h. Total RNA was extracted from the treated T47D-A and T47D-B cells and subjected to mRNA expression profiling as described under Materials and Methods. The mRNA profiling data is represented in the Venn diagrams in *Panel A* (for T47DA cells) and in *Panel B* (for T47D-B cells). *Panels A* and *B* show comparisons among the gene set activated by E₂ (E₂ activated vs. Vehicle), the gene set repressed by R5020 in the absence of E₂ (P repressed vs. Vehicle) and the gene set repressed by R5020 in the presence of E₂ (E₂+P repressed vs. E₂). The data represents results from experimental triplicates.

2.4 Discussion

The results of this study reveal that the positive effect of progestins on invasiveness of ER+ breast cancer cells has two components: 1. rescue of invasiveness from estrogen repression at relatively low progestin concentrations that is mediated exclusively by PR isoform A; and 2. estrogen-independent induction of invasiveness at high progestin concentrations that is mediated exclusively by PR isoform B. Moreover, PR-A was sensitized to even lower levels of progestin when this receptor isoform was overexpressed relative to PR-B. Similar to the observations here on PR isoforms, other steroid receptors are also known to induce distinct genotropic and phenotypic effects at different hormone doses as well as hypersensitization to hormone by a few fold overexpression of the receptor (328,338).

The relevance of the above findings to the physiological hormone status prior to and after menopause is apparent. The estrogen dose that was required for substantial or virtually complete suppression of invasiveness in ER+ cells is well under the plasma levels of estrogen in pre-menopausal women. It is also within the range of plasma and breast tissue levels of estrogen in post-menopausal women. The full effect of PR-A on the invasiveness of the various breast cancer cell lines occurred at < 1 nM progesterone and the dose requirement was reduced to < 0.2 nM when the expression level of PR-A was elevated. Thus, dysregulated PR-A has the potential to rescue invasiveness of breast cancer cells from estrogen regulation in response to post-menopausal plasma/breast tissue progesterone levels. This is in contrast to PR-B, which only induced invasiveness progressively with progesterone dose in the range of 5 nM to 50 nM. Thus, isoform A of PR plays the predominant hormone-dependent role in increasing invasiveness of ER+ breast cancer cells at progesterone concentrations that include the entire range of follicular phase, luteal phase and post-menopausal hormone levels, particularly when the cells overexpress PR-A. The

findings on the role of PR isoforms also extend to plasma progestin levels associated with the use of MPA, either in contraception or in hormone replacement therapy. Therefore in luminal breast cancer, prior to diagnosis or after cessation of treatment, PR-A may have a greater mechanistic role in promoting invasiveness than PR-B.

The unique ability of only isoform A of PR to oppose regulation of invasiveness by estrogen at low progesterone concentrations is clearly reflected in the differential abilities of PR-A and PR-B to mediate cross-talk between progesterone and estrogen with respect to patterns of gene regulation. Gene expression analysis using isogenic recombinant (T47D) cells that exclusively expressed either the A or the B isoform of PR revealed that the cross-talk between estrogen and low dose progesterone affected the expression of estrogen target genes with diverse functions. However, among these genes, the subsets that were regulated by progesterone through PR-A vs. PR-B were largely non-overlapping. The genes whose regulation by estrogen was opposed by progesterone in an exclusively PR-A dependent manner included both estrogen-activated and estrogen-repressed genes. The estrogen-repressed genes were more noteworthy in the context of this study as they included genes with established roles in progression of breast cancer. Moreover, we demonstrated that selected genes from this subset (HES1, PRKCH, ELF5 and TM4SF1) did support invasiveness in ER+ breast cancer cells.

In response to the binding of progesterone, several mechanisms could conceivably enable PR-A to oppose estrogen's action on a subset of estrogen target genes. The ligand-dependent activity of PR-A did not result in any change in ER expression. Rather, the exact mechanism of PR-A isoform dependent cross-talk between progesterone and estrogen signaling could depend on the target gene context. For example, (i) PR-A could compete with ER to bind to tethering proteins at repressive sites in the chromatin, either simply blocking repression by estrogen/ER or activating

the target gene; (ii) PR-A could bind at chromatin sites that are different from the repressive sites of ER binding and compete with ER for interaction with the pre-initiation complex of the target genes; (iii) PR-A could indirectly oppose gene regulation by estrogen by regulating transcription of other regulatory proteins or microRNAs. The amino-terminal truncation in PR-A could expose protein binding motifs that are unexposed in PR-B enabling unique or higher affinity interactions of agonist bound PR-A with other regulatory proteins in the chromatin. Similar chromatin interactions of PR-B may therefore require higher doses of progestins. More extensive studies including ChIP-seq analyses should help to establish specific mechanisms by which PR-A may de-regulate estrogen target genes.

In mice, selective ablation of PR-B revealed that PR-B was not required for the normal physiology of the uterus or the ovary but was necessary for pregnancy-associated mammary gland morphogenesis (198). That study demonstrated that the ability of progesterone to suppress estrogen-induced endometrial proliferation was due to PR-A. In contrast, when PR-A was selectively ablated, progesterone not only failed to inhibit estrogen-induced cell proliferation in the endometrium but actually further increased proliferation of the uterine epithelium, an effect mediated by PR-B (197). Therefore, given the necessary role of PR-A in endometrial physiology, selectively disrupting its actions in breast cancer cells vs. endometrial tissue will require a better understanding of tissue-specific molecular pathways by which PR-A opposes estrogen signaling in breast cancer. Identifying and narrowly targeting a critical cross-talk pathway between PR-A and ER may enable suppression of tumor progression without disrupting the protective role of PR-A in the endometrium or the adverse effects of a broader PR antagonist. Such an intervention may also be useful in combination hormone replacement therapy. A molecular signature of hyperactive PR-A may also more effectively predict tumor progression.

CHAPTER 3. THE SHORT PROGESTERONE RECEPTOR ISOFORM SUPPORTS INVASIVENESS AND METASTASIS OF LUMINAL BREAST CANCER BY SUPPRESSING REGULATION OF CRITICAL MICRO RNAS BY ESTROGEN

3.1 Introduction

Breast oncogenesis may span up to several decades. Most (> 78 percent) of newly diagnosed breast cancer cases occur in women older than 50 years (303) with a median age at diagnosis of 61 years (304). Most breast tumors express the nuclear receptors for estrogen (ER) and progesterone (PR) (341) even through progression (174). Primary ER+ tumors are highly responsive to anti-estrogen therapy. However, ER+ breast cancer is often metastatic at the time of diagnosis and metastatic ER+ tumors also frequently appear after many years of dormancy (132,305). Indeed, over a fifth of breast cancer patients harbor distally metastasized ER+ tumors(27). Unfortunately, the metastatic disease is generally incurable and even targeted therapies are generally only palliative. Therefore, it is necessary to understand more about deregulated molecular mechanisms that confer invasive/metastatic properties on ER+ breast cancer cells. However, in contrast to more aggressive cancers, studies of metastasis of luminal breast cancer are rather sparse, due in part to the inherently slow metastatic spread of the tumors in animal models(342). This has likely limited studies of metastasis of ER+ breast tumors to models in which the tumor cells are directly injected into the circulation, bypassing events in the initiation of metastasis at the primary tumor site (342). Therefore, it is also desirable to design an experimental strategy that can functionally link novel physiological mechanisms governing invasiveness of ER+ breast cancer cells to their ability to leave the primary tumor site.

Estrogen supports the growth of ER+ breast tumors but it suppresses invasiveness of the tumor cells whether or not their growth is hormone-sensitive and also suppresses breast tumor progression (183). High dose and potent synthetic forms of progestins directly support

invasiveness and metastasis in ER+/PR+ breast cancer cells, demonstrated using *in vivo* experimental models (309,310). These models may be physiologically relevant in postmenopausal women on high dose hormone replacement therapy, where the combination of estrogen and progestin was associated with increased incidence of invasive breast cancer and breast cancer mortality compared with non-users (59), in contrast estrogen monotherapy in women with prior hysterectomy was associated with a persistent decrease in the onset of invasive breast cancer (60). However, in post-menopausal women who are not undergoing hormone replacement, the role of the endogenous hormones in the progression of ER+/PR+ breast tumors have not been adequately studied. Compared to the knowledge on the influence of estrogen on breast tumor physiology, much less is known about the mechanisms of progesterone action, particularly in the presence of active estrogen signaling. Moreover, although the levels of estrogen and progesterone change throughout the menstrual cycle and decrease after menopause very little is known about the hormone actions on tumor invasiveness/progression in the context of this changing hormone status during a woman's lifetime. Our recent findings in chapter 2 have addressed these questions by identifying a fundamental role for cross-talk between ER and PR in governing invasiveness of a variety of model luminal breast cancer cell lines in the entire range (pre- and post-menopausal) of physiological levels of estrogen and progesterone (157).

PR has two isoforms, A and B, expressed by alternative promoter usage from a single gene; PR-B is identical to PR-A except for the presence of an additional 164 amino acid amino-terminal segment that contains within it, an additional activation function, AF3 (157). PR-B and PR-A induce both distinctive and overlapping patterns of agonist-induced gene activation or gene repression, depending on the variable contexts of the target promoters and the nature of the associated chromatin sites of PR binding (156,157,312). The heterodimer of PR-A and PR-B

regulates a smaller and unique set of genes compared to the homodimers (156). Clinical studies have shown that although in normal breast PR-A and PR-B are expressed at comparable levels, this balance is commonly altered during breast oncogenesis with an increase in PR-A in early as well as progressed lesions (190). Overexpression of PR-A is associated with increased invasiveness of clinical tumor lesions and a lower rate of disease free survival (170).

In vitro studies in the literature originally suggested that PR-B is the principal mediator of progesterone-induced invasiveness of breast cancer cells (192,193), at odds with the clinical observations noted above that implicate PR-A in tumor progression. However, the *in vitro* studies of PR-B were performed at high (luteal stage and pregnancy-associated) concentrations of progesterone and also were conducted in the absence of estrogen signaling (192–195,343). The plasma estrogen range in pre-menopausal women is 1.4 nM -1.6 nM during the follicular phase, and 3.6 nM - 4.2 nM during the luteal phase (64). Plasma progesterone ranges from below 4 nM during follicular phase, up to > 50 nM during the luteal phase (344). Post-menopause, there is a marked decrease in circulating hormone levels with median values of 0.14 nM for estrogen and 0.13 nM for progesterone, yet the breast tissue may retain up to about 1nM of each hormone (191,306). We have recently reported studies that were performed in the entire range of estrogen and progesterone concentrations corresponding to pre- and post-menopausal hormone status and in the presence of both estrogen and progesterone signaling (Chapter 2) (157). As the previous *in vitro* studies of high dose progesterone effects on metastasis were conducted in the absence of estrogen signaling, we considered the possibility that modulation of estrogen action may comprise a distinct aspect of the regulation of invasiveness by progestins in the range of its physiological levels (157) . Estrogen strongly suppressed invasiveness of ER+ breast cancer cells at concentrations below 0.01 nM. At low (< 1 nM) concentrations, progesterone/progestins

completely abrogated the inhibition of invasiveness by estrogen. It was only in a higher (5 nM -

50 nM) concentration range that progestins progressively induced invasiveness in the absence of

estrogen. The ability of progestins to rescue invasiveness from estrogen regulation was exclusively

mediated by PR-A and was uninfluenced by PR-B. On the other hand, PR-B mediated the estrogen-

independent component of progestin-induced invasiveness at either pharmacological (used in

hormone replacement) progestin levels or progesterone levels associated with pregnancy.

Overexpression of PR-A in PR-A+/PR-B+ cells lowered the progestin concentration needed to

completely rescue invasiveness (to < 0.2 nM). The studies demonstrate that progesterone

influences breast cancer cell invasiveness by rescuing it from estrogen regulation, exclusively via

PR-A, in the entire pre- and post-menopausal range of estrogen and progesterone concentrations

(157). These findings reconcile *in vitro* actions of PR isoforms with the clinically observed

association between PR-A and progression of luminal breast cancer.

Although progesterone, acting through PR-A, appears to be the major culprit in promoting

invasiveness of luminal breast cancer cells by counteracting estrogen, directly testing the effect of

this mechanism on metastasis *in vivo* by manipulating hormone levels is not possible because

estrogen depletion would prevent tumor formation. Instead, if a critical pathway of hormonal

cross-talk between PR-A and ER that regulates *in vitro* invasiveness could be identified, it should

be possible to test the effect of disrupting this pathway on metastasis using an appropriate *in vivo*

model. Additionally, such a cross-talk pathway(s) may reveal better therapeutic targets as clinical

interventions that broadly or systemically obstruct progesterone/PR signaling are precluded by the

need for progesterone for endometrial homeostasis (145,345) and off-target effects of progesterone

antagonists (346). In luminal breast cancer cells, ER strongly regulates tumor cell characteristics

by regulating micro-RNAs (miRNAs) (347–350) and is itself also regulated by miRNAs

(347,350,351).There is less information on the regulation of PR by miRNAs or on regulation of miRNAs by progesterone/PR. Indeed, miRNAs regulate up to a third of the human genome and have diverse roles in normal physiology as well as profound roles as tumor suppressors and oncogenes (352). This study was undertaken to explore the possibility that regulation of certain miRNAs could be critical for cross-talk between progesterone/PR-A and estrogen/ER in the specific context of hormonal control of invasion *in vitro* and to manipulate such a cross-talk pathway(s) as a means of establishing the role of hormonal cross-talk of PR-A with ER in metastasis of luminal breast cancer.

3.2 Materials and Methods

3.2.1 Cell Line Models and Breast Tumor Specimens

BT474, T47D and ZR-75-1 breast cancer cells were purchased from American Type Culture Collection (ATCC). T47D-A (ER+/PR-A+/PR-B-null) and T47D-B (ER+/PR-B+/PR-A-null) recombinant cells isogenic with parental T47D cells were a generous gift from Dr. Katherine Horowitz (University of Colorado, Denver, CO) and were cultured as previously described (329).T47D-PR-A++ cells were previously generated in our laboratory (157). Recombinant BT474 cell lines with stable expression of PR shRNA (PR-shRNA cells), miR-92a-3p (miR-92a-3p-On cells) or miR-26b-5p inhibitor (miR-26b-5p-Off cells) and control cells harboring Lenti-miR-Blank plasmid were generated using PR shRNA lentiviral plasmid (Cat# 0000436004, Sigma-Alrdrich, St. Louis MO) hsa-miR-92a-3p miRNA Lentivector (Cat# mh11076, ABM, Vancouver, BC) or LentimiR-Off-hsa-miR-26b-5p vector (Cat# mh30381, ABM, Vancouver, BC) or control LentimiR-blank vector (Cat# m007, ABM, Vancouver, BC) by lentiviral transduction methods described below. All human tumor samples, classified as ER+/PR+ ductal carcinoma, were obtained from Cooperative Human Tissue Network (CHTN).

3.2.2 Cell Culture and Hormone Depletion

Cell line models were cultured in DMEM supplemented with FBS (10%) penicillin (100 unit/ml) streptomycin (100ug/ml) and L-glutamine (2 mM) at 37°C in 5% CO2. To maintain selection pressure, the media for the recombinant BT474 cells contained $0.5\mu g$/ml puromycin and the media for T47D-A and T47D-B cells contained 200ug/ml geneticin. For hormone depletion, cells were plated at 30% confluence in phenol red-free media supplemented with charcoal-stripped FBS and incubated at 37°C in 5% CO_2 for 48 h.

3.2.3 Western Blot of Cells and Breast Tumor Tissues

Cell lysates were prepared as described (157). To prepare tumor tissue lysates, tissue (100mg) suspended in 500 ul of RIPA buffer was homogenized using the BioGen-PRO200 tissue homogenizer (Cat# 01-01200, Cambridge, MA) for 15 seconds on ice and centrifuged at 15,000x G and supernatant was used. Western blot was performed as previously described (157). The antibody probes include monoclonal rabbit anti-PR antibody (Cat# 8757, Cell Signaling, Danvers, MA), EMT sampler kit antibodies (Cat# 9782, Cell Signaling, Danvers, MA) or mouse monoclonal anti-GAPDH antibody (sc-4472, Santa Cruz Biotechnologies, CA) and appropriate horseradish peroxidase conjugated secondary antibodies (Vector Laboratories, MD). Relative protein expression was determined by semi-quantitative densitometry of auto-radiographic film using ImageJ software (National Institutes of Health, USA).

3.2.4 Boyden Chamber Transwell Invasion Assay

Cell invasion assays were performed as previously described (Chapter 2).

3.2.5 Isolation and Measurement of Micro RNA and mRNA

Total RNA was isolated from cells or tissues using the Exiqon miRCURY total RNA isolation kit (Vedebaek, Denmark). Breast tumor tissue lysates were prepared by suspending 50

mg tissue in 500ul of the lysis buffer and homogenized using the BioGen-PRO200 tissue homogenizer (Cat# 01-01200, Cambridge, MA) for 15 seconds on ice. Homogenized solution was centrifuged at high 15,000x G and supernatant was used for RNA extraction. Reverse transcription PCR reactions were performed using high capacity complementary DNA archive kit, or miRNA reverse transcription kit (Life Technologies Corporation, Carlsbad, CA). cDNA was measured by quantitative real time RT-PCR using TaqMan probes and the StepOne Plus Real time PCR system (Life technologies Corporation, Carlsbad, CA). All RNA measurements were performed in biological triplicates, and all CT values were normalized to intra-sample GAPDH (mRNA) or U6snRNA (miRNA). RNA values were represented as fold difference, which is calculated using the formula = $2-\Delta\Delta CT$, where $\Delta\Delta CT = \Delta CT$ sample $- \Delta CT$ calibrator.

3.2.6 Affymetrix Profiling of Micro RNAs Regulated by PR-A

Hormone depleted T47D-A and T47D-B cells were treated with vehicle, or R5020 (1nM). Total RNA samples, isolated using the Exiqon miRCURY isolation kit (Denmark), were analyzed at the University of Michigan Microarray Core using the Affymetrix miRNA microarray generation IV (Affymetrix, Santa Clara, CA). Expression values were normalized using quantile-normalization, with background subtraction. Log transformation to the base of 2, followed by one-way ANOVA was used to determine error. The differentially expressed genes were identified by comparing R5020 treatment with vehicle treatment (repressed or activated with a cutoff fold difference of 1.5 and a p value < 0.05). Real-time RT-PCR was performed as described above to validate miRNAs that were exclusively regulated by PR-A.

3.2.7 Lentiviral Transduction

Packaging of lentiviral particles with pCDH empty vector plasmid, pCDH-PR shRNA, hsa-miR-92a-3p miRNA Lentivector or LentimiRa-Off-hsa-miR-26b-5p vector and transduction of

cells ware performed as described (157). The pool of recombinant cells was selected by culturing in puromycin containing media for 6 weeks.

3.2.8 Transfection of siRNA or miRNA Inhibitor

Cells were plated to 30% confluence without antibiotic in phenol-red free DMEM medium supplemented with 10% charcoal-stripped FBS. 24 hours later cells were transfected with siRNA directed against specific gene targets, miRNA inhibitors (miRVana, Life Technologies, Carlsbad, CA) directed against specific miRNA targets or with appropriate non-silencing controls using lipofectamine (Life Technologies, Carlsbad, CA).

3.2. 9 Metastasis Pathway Gene Array Expression Analysis

Human Tumor Metastasis Fast TaqMan Real-Time PCR array (Life Technologies, Carlsbad, CA) was performed on StepOne Realtime (Applied Biosystems). cDNA samples from BT474 (control transduced cells), BT474 92a-3p-On cells and BT474 miR-26b-5p-Off cells were analyzed. The ΔΔCt method was used as described above to quantify gene expression.

3.2.10 Mouse Metastasis Model

Female athymic nude mice (Envigo, Indianapolis, IN) were implanted with 0.72 mg slow-release estradiol pellets (Innovative research of America, Sarasota, FL) on Day 0. On Day 3, 1×10^7- 2×10^7 cells were suspended in 300µl of equal parts DMEM and Matrigel and implanted subcutaneously in the right flank just below the right shoulder. Mice were sacrificed on day 17. Tumors, ipsilateral and contralateral axillary and inguinal Lymph-nodes and livers were harvested from the mice. All tissues were homogenized and total RNA extracted as described above for human tissues. The degree of tissue infiltration by the human tumor cells (metastasis) was measured in terms of the amount of mRNA present for human GAPDH by quantitative real time

RT-PCR using human and mouse species-specific high efficiency Taqman Probes. The mRNA values for human GAPDH was normalized to the mRNA for mouse GAPDH.

3.3 Results

3.3.1 Identification of micro RNAs that are uniquely regulated by low dose progesterone through PR-A.

Progesterone bound to PR-A could impinge on regulation of invasiveness by E_2/ER in a manner that is mediated by one or more miRNAs in the following ways (depicted as schematics in Figure 3.1A and 3.1B). First, Progesterone/PR-A could either activate or repress miRNA(s) (independent of estrogen) and this would then result in rescue of invasiveness from estrogen repression (Figure 3.1A). On the other hand, E_2/ER could either activate or repress miRNA(s) resulting in inhibition of invasiveness; in this case, progesterone/PR-A could suppress regulation of the miRNAs by E_2, resulting in rescue of invasiveness from estrogen repression (Figure 3.1B). To identify these putative miRNAs we used the following cell line models: T47D-A (ER+/PR-A+/PR-B-null), T47D-B (ER+/PR-A-null/PR-B+), parental T47D (ER+/PR-A+/PR-B+), BT474 (ER+/PR-A+/PR-B+) and ZR-75-1(ER+/PR-A+/PR-B+).

To identify candidate miRNAs in the putative pathways illustrated in Figure 3.1A, we first undertook Affymetrix miRNA profiling to screen for miRNAs activated or repressed by PR-A but not PR-B at a low dose (1 nM) of R5020. This was accomplished by using Affymetrix miRNA profiling of miRNA changes in hormone-depleted T47D-A vs. T47D-B cells treated with R5020 followed by data validation by quantitative RT-PCR. We found 5 miRNAs, that were activated by low dose progestin and PR-A (i.e., in T47D-A cells) but not by PR-B (i.e., in T47D-B cells). They are miR-6805-5p, miR-584-5p, miR-1228-5p, miR-501-5p and miR-668-5p. These 5 miRNAs were also strongly up-regulated in other PR-A-positive cell lines including

parental T47D cells, BT474 cells and ZR-75-1 cells (**Figure 3.1C**; values in *Table 3.1*). The same Affymetrix miRNA analysis did not reveal any miRNAs that were repressed via PR-A alone.

In contrast to progestin regulated miRNAs there is considerable literature data on miRNAs regulated by E_2 acting through ER (*not shown*). All of these miRNAs, that were either activated or repressed by E_2, were tested by real time RT-PCR analysis to identify miRNAs whose regulation by E_2 was prevented by R5020 in T47D-A cells (i.e., via PR-A) but not in T47D-B cells (i.e., via PR-B). In this manner, ten miRNAs were identified; five among them (miR-17-5p, miR-20a-5p, miR-92a-3p, miR-106a-5p and miR-106b-5p) were activated by E_2 and five (miR-26b-5p, miR-27a-5p, miR-27b-5p, miR-200c-5p and Let7a-5p) were repressed by E_2 in the absence of progestin/PR-A. The counter-regulation of these E_2-regulated miRNAs by progestin/PR-A was also confirmed in the other PR-A-positive cell lines including parental T47D cells, BT474 cells and ZR-75-1 cells (Figure 3.1D and 3.1E; values in *tables 3.2 and 3.3*). Finally, to ensure that higher doses of progestin did not enable PR-B to mimic regulation of the 15 miRNAs by PR-A, T47D-B cells were treated with vehicle, 1 nM $E_{2,}$ and 1nM E_2 plus R5020 (1nM-50nM). There was no progestin dose-dependent miRNA activation or opposition to E_2 regulation by PR-B (Figure 3.2).
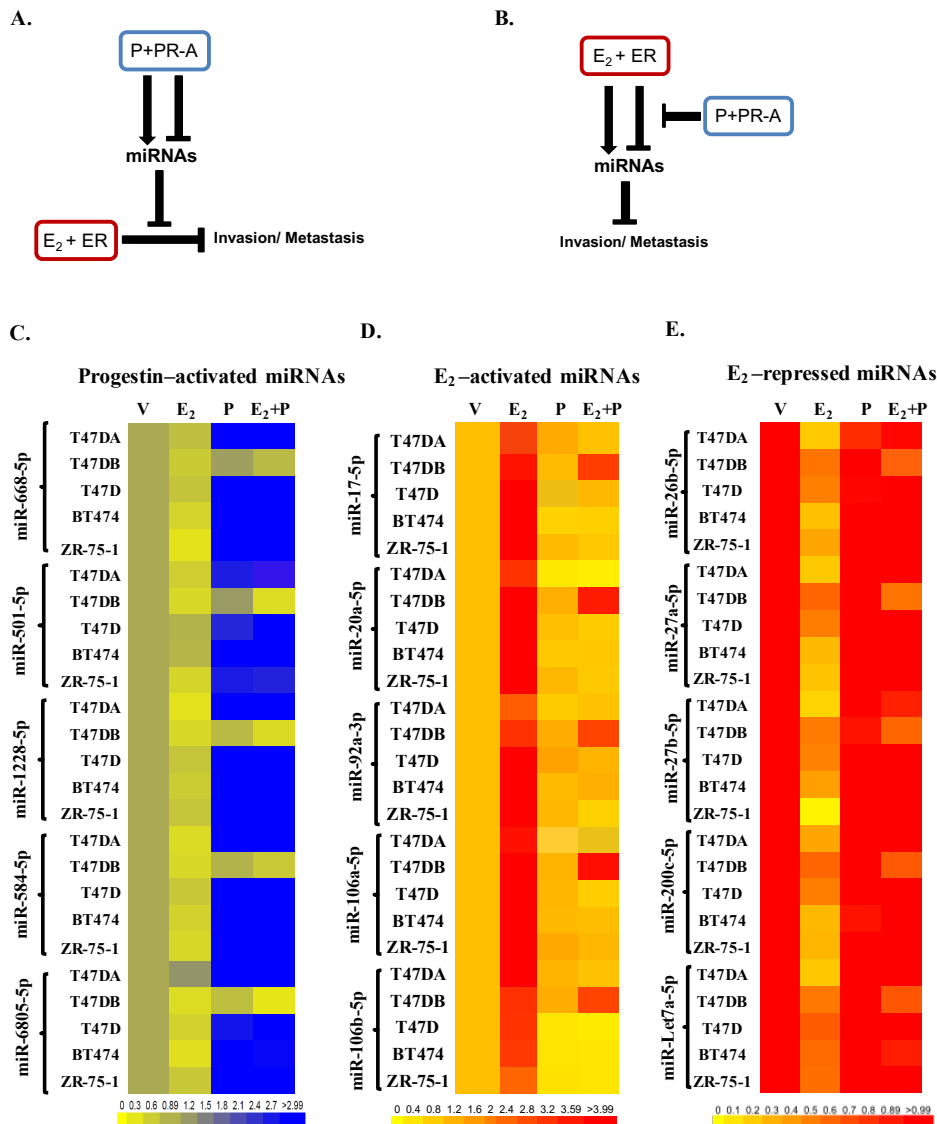
Figure 3.1: Possible pathways and candidate miRNAs that could be involved in the hormone-dependent cross talk of PR-A with ER that regulates invasiveness in breast cancer cells. Panel A and **Panel B** are schematic representations of possible pathways by which miRNAs could mediate the cross-talk of progestin (P)-bound PR-A with E_2-bound ER to influence invasiveness and metastasis of luminal breast cancer cells. In **Panels C-E**, hormone depleted T47D-A, T47D-B, T47D, BT474, and ZR-75-1 cells at 30% confluence were treated with vehicle, 1 nM E_2, 1 nM R5020 (P) or 1nM E_2 plus1nM R5020 (E_2 + P) for 48 h. Total RNA was then extracted and the relative levels of each miRNA indicated were quantified in all the samples. The miRNA profiling data is represented in the heat map in **Panel C** for miRNAs activated by progestin plus PR-A (even in the absence of E_2), in **Panel D** for E_2 activated miRNAs that were counter-regulated by progestin plus PR-A and in **Panel E** for E_2 repressed miRNAs that were counter-regulated by Progestin plus PR-A. The data represents results from experimental triplicates.

miRNA	Cell Line	Vehicle	Vehicle (STDV)	E2	E2 (STDV)	R5020	R5020 (STDV)	E2+R5020	E2+R5020 (STDV)
miR-668-5p	T47DA	1	0.137	0.759	0.179	12.787	0.456	8.995	0.888
miR-668-5p	T47DB	1	0.044	0.623	0.077	1.120	0.071	0.817	0.027
miR-668-5p	T47D	1	0.101	0.689	0.108	11.109	0.086	14.911	1.534
miR-668-5p	BT474	1	0.059	0.507	0.068	7.430	0.212	6.966	0.580
miR-668-5p	ZR-75-1	1	0.109	0.326	0.038	5.531	0.248	7.757	0.370
miR-501-5p	T47DA	1	0.115	0.570	0.011	2.665	0.014	2.131	0.195
miR-501-5p	T47DB	1	0.043	0.452	0.008	1.183	0.022	0.410	0.040
miR-501-5p	T47D	1	0.130	0.886	0.002	2.553	0.087	3.650	0.378
miR-501-5p	BT474	1	0.009	0.854	0.015	3.056	0.069	3.269	0.427
miR-501-5p	ZR-75-1	1	0.058	0.483	0.013	2.684	0.064	2.591	0.162
miR-1228-5p	T47DA	1	0.235	0.333	0.168	8.806	1.515	11.414	2.080
miR-1228-5p	T47DB	1	0.183	0.454	0.158	0.814	0.040	0.434	0.156
miR-1228-5p	T47D	1	0.731	0.671	0.100	3.463	0.047	8.467	2.631
miR-1228-5p	BT474	1	0.261	0.607	0.153	5.550	0.799	6.340	0.880
miR-1228-5p	ZR-75-1	1	0.643	0.676	0.249	14.028	1.160	13.336	2.044
miR-584-5p	T47DA	1	0.015	0.413	0.008	10.488	0.307	9.007	0.455
miR-584-5p	T47DB	1	0.023	0.454	0.012	0.884	0.019	0.634	0.026
miR-584-5p	T47D	1	0.066	0.650	0.039	9.094	0.292	7.867	0.520
miR-584-5p	BT474	1	0.058	0.527	0.006	6.498	0.077	7.806	0.787
miR-584-5p	ZR-75-1	1	0.036	0.463	0.011	11.993	0.010	11.784	0.809
miR-6805-5p	T47DA	1	0.300	1.248	0.014	3.662	0.025	5.516	0.374
miR-6805-5p	T47DB	1	0.372	0.401	0.263	0.759	0.138	0.279	0.245
miR-6805-5p	T47D	1	0.338	0.520	0.359	2.766	1.261	7.552	2.352
miR-6805-5p	BT474	1	0.794	0.365	0.364	3.895	1.628	2.909	0.458
miR-6805-5p	ZR-75-1	1	0.516	0.657	0.211	7.338	2.672	7.158	2.117

Table 3.1 Data corresponding to heat map in Figure 3.1C

miRNA	Cell Line	Vehicle	Vehicle (STDV)	E2	E2 (STDV)	R5020	R5020 (STDV)	E2+R5020	E2+R5020 (STDV)
miR-17-5p	T47DA	1	0.062	2.507	0.035	1.327	0.018	0.961	0.008
miR-17-5p	T47DB	1	0.203	3.709	0.182	1.040	0.019	3.031	0.354
miR-17-5p	T47D	1	0.009	4.162	0.178	2.035	0.050	1.098	0.056
miR-17-5p	BT474	1	0.013	6.276	0.336	0.705	0.018	0.741	0.008
miR-17-5p	ZR-75-1	1	0.023	5.567	0.051	1.066	0.040	0.858	0.039
miR-20a-5p	T47DA	1	0.013	1.703	0.014	0.353	0.003	0.262	0.006
miR-20a-5p	T47DB	1	0.195	4.368	0.286	1.253	0.086	3.572	0.222
miR-20a-5p	T47D	1	0.075	4.048	0.056	1.009	0.034	0.795	0.012
miR-20a-5p	BT474	1	0.038	7.425	0.413	0.835	0.016	0.870	0.010
miR-20a-5p	ZR-75-1	1	0.034	5.527	0.102	1.102	0.008	0.839	0.015
miR-92A-3p	T47DA	1	0.020	2.541	0.024	0.786	0.006	0.955	0.014
miR-92A-3p	T47DB	1	0.141	3.217	1.325	1.259	0.472	2.921	3.323
miR-92A-3p	T47D	1	0.046	4.651	0.123	1.445	0.072	1.168	0.022
miR-92A-3p	BT474	1	0.029	6.591	0.043	1.062	0.037	1.253	0.011
miR-92A-3p	ZR-75-1	1	0.021	5.882	0.046	1.129	0.011	0.713	0.009
miR-106A-5p	T47DA	1	0.049	3.737	0.051	2.012	0.060	1.768	0.046
miR-106A-5p	T47DB	1	0.090	4.436	0.204	1.161	0.111	3.779	0.344
miR-106A-5p	T47D	1	0.019	4.966	0.070	1.137	0.015	0.773	0.053
miR-106A-5p	BT474	1	0.038	7.581	0.291	1.075	0.015	1.026	0.020
miR-106A-5p	ZR-75-1	1	0.062	8.964	0.116	1.335	0.052	1.120	0.021
miR-106b-5p	T47DA	1	0.045	4.024	0.084	1.162	0.008	0.970	0.019
miR-106b-5p	T47DB	1	0.048	3.217	0.515	1.259	0.086	2.921	0.202
miR-106b-5p	T47D	1	0.064	1.362	0.023	0.420	0.003	0.311	0.003
miR-106b-5p	BT474	1	0.013	3.095	0.018	0.414	0.024	0.377	0.006
miR-106b-5p	ZR-75-1	1	0.022	2.404	0.010	0.470	0.013	0.408	0.002

Table 3.2 Data corresponding to the heat map Figure 3.1D

miRNA	Cell Line	Vehicle	Vehicle (STDV)	E2	E2 (STDV)	R5020	R5020 (STDV)	E2+R5020	E2+R5020 (STDV)
miR-26b-5p	T47DA	1	0.074	0.204	0.004	0.820	0.067	0.973	0.021
miR-26b-5p	T47DB	1	0.033	0.545	0.007	1.403	0.030	0.665	0.022
miR-26b-5p	T47D	1	0.056	0.501	0.018	0.978	0.469	1.031	0.067
miR-26b-5p	BT474	1	0.067	0.694	0.049	1.240	0.017	1.168	0.101
miR-26b-5p	ZR-75-1	1	0.087	0.350	0.046	1.201	0.027	1.795	0.127
miR-27a-5p	T47DA	1	0.305	0.212	0.044	3.399	0.252	2.362	0.253
miR-27a-5p	T47DB	1	0.102	0.603	0.144	0.997	0.149	0.544	0.070
miR-27a-5p	T47D	1	0.096	0.508	0.097	2.280	0.602	5.320	1.091
miR-27a-5p	BT474	1	0.258	0.267	0.238	1.004	0.284	1.440	0.336
miR-27a-5p	ZR-75-1	1	0.261	0.233	0.017	0.824	0.131	0.793	0.088
miR-27b-5p	T47DA	1	0.086	0.167	0.059	0.828	0.197	0.874	0.129
miR-27b-5p	T47DB	1	0.033	0.516	0.010	0.928	0.147	0.600	0.031
miR-27b-5p	T47D	1	0.032	0.487	0.010	1.289	0.067	1.033	0.036
miR-27b-5p	BT474	1	0.150	0.376	0.125	1.177	0.303	1.130	0.145
miR-27b-5p	ZR-75-1	1	0.073	0.046	0.012	0.714	0.015	0.683	0.034
miR-200c-5p	T47DA	1	0.265	0.343	0.069	1.495	0.087	1.188	0.070
miR-200c-5p	T47DB	1	0.085	0.600	0.084	1.583	0.153	0.756	0.023
miR-200c-5p	T47D	1	0.138	0.504	0.039	2.533	0.038	1.514	0.985
miR-200c-5p	BT474	1	0.023	0.272	0.034	0.925	0.078	1.123	0.313
miR-200c-5p	ZR-75-1	1	0.139	0.282	0.029	1.260	0.024	1.790	0.069
Let7a-5p	T47DA	1	0.055	0.216	0.002	1.519	0.012	1.258	0.056
Let7a-5p	T47DB	1	0.065	0.527	0.004	1.830	0.120	0.782	0.026
Let7a-5p	T47D	1	0.026	0.642	0.011	1.392	0.035	1.177	0.034
Let7a-5p	BT474	1	0.065	0.577	0.016	1.207	0.079	0.888	0.005
Let7a-5p	ZR-75-1	1	0.026	0.562	0.008	1.898	0.047	1.812	0.052

Table 3.3 Data corresponding to the heat map in Figure 3.1E
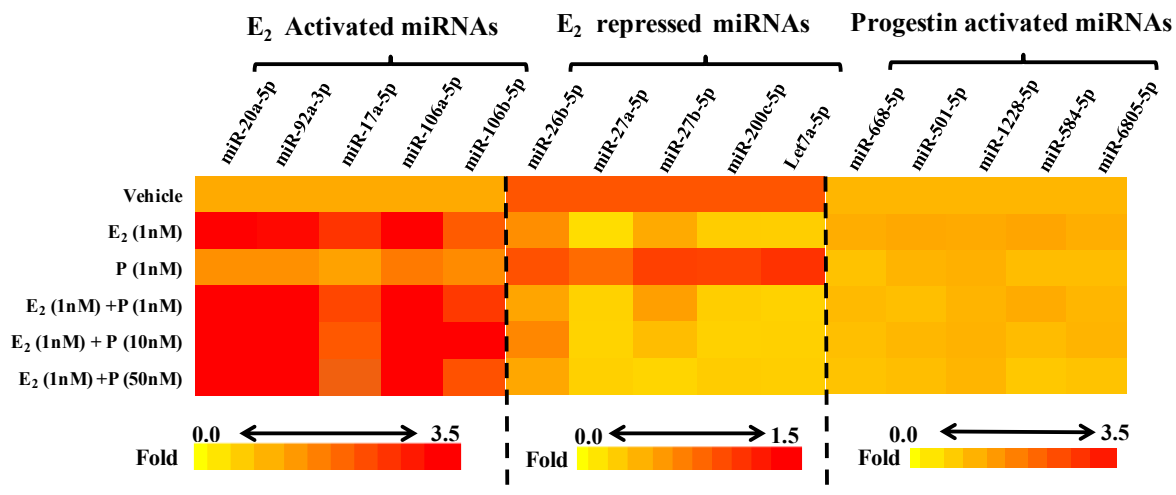
Figure 3.2. Higher doses of progestin do not enable PR-B to mimic the actions of PR-A on 15 miRNAs. Hormone depleted T47D-B cells at 30% confluence were treated with vehicle, 1 nM E_2, 1 nM R5020 (P) and 1nM E_2 plus 1 nM, 10 nM or 50 nM R5020 for 48 h. Total RNA was then extracted and the relative levels of each miRNA indicated were quantified. The miRNA profiling data is represented in the heat map.

3.3.2 Regulation of micro RNAs by PR-A is sensitized to lower doses of progestin by overexpression of PR-A to a level observed in tumors.

We have previously reported that compared to T47D cells, in the isogenic recombinant T47-D-PR-A++ cells that express a 3.6-fold higher level of PR-A (Figure 3.3E), lower doses of progesterone can rescue invasiveness from estrogen repression (Chapter 2). Among 53 primary tumor specimens examined by western blot, 13 specimens (~ 25 percent) showed levels of PR-A expression that were comparable to or higher than that in T47D-PR-A++ cells (Figure 3.3A), confirming the clinical relevance of this isogenic model. In T47DA++ cells, every one of the above 15 miRNAs was optimally regulated at or below 0.2 nM R5020 whereas the parental T47D cells required 0.5 – 1 nM R5020 for the same level of regulation (increase or decrease in the absence or presence of 1nM E_2) (Figure 3.3B-D; values in *Table 3.4*). This level of hyper-sensitization to progestin of miRNA regulation in T47D-PR-A++ cells corresponds to the previously reported progestin dose dependence of invasiveness of T47D-PR-A++ cells vs. parental T47D cells (157).

3.3.3 miR-92a-3p and miR-26b-5p are functionally linked to hormonal control of invasiveness.

We tested the ability of E_2 to repress invasiveness or for R5020 to rescue invasiveness following inhibition of each of the above 15 miRNAs in T47D and BT474 cells. Inhibition was confirmed by reduction in target miRNA 72h post-transfection, although residual miRNA-inhibitor duplexes may also be detected.
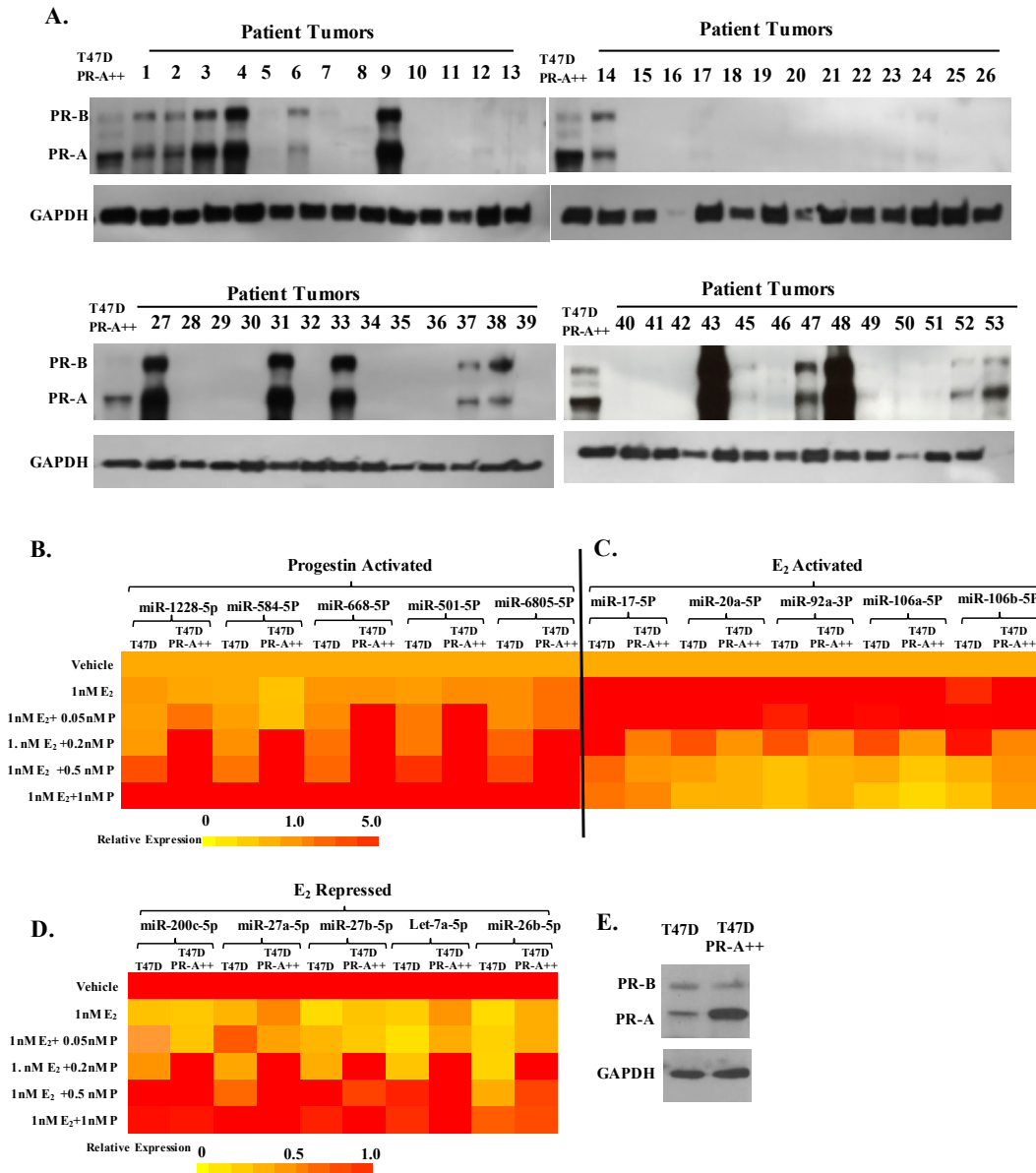
Figure 3.3: PR overexpression in primary luminal breast tumor specimens and the effect of PR-A overexpression on hormone sensitivity of miRNA regulation. Whole cell lysates from primary luminal breast tumors from 53 patients together with whole cell lysates from T47D PR-A++ cells were analyzed on western blots that were probed for PR and GAPDH (**Panel A**). Hormone-depleted T47D parental cells and T47D PR-A++ cells at 30% confluence were treated with vehicle, 1nM E_2 or 1nM E_2 plus a range (0.05nM – 1nM) of concentrations of R5020 (P) for 48h. Total RNA was then extracted and the relative levels of each miRNA indicated were quantified in all the samples. The miRNA profiling data is represented in the heat map in **Panel B** for miRNAs activated by progestin plus PR-A (even in the absence of E_2), in **Panel C** for E_2 activated miRNAs that were counter-regulated by progestin plus PR-A and in **Panel D** for E_2 repressed miRNAs that were counter-regulated by progestin plus PR-A. The data represents results from experimental triplicates. In **Panel E**, whole cell lysates from T47D cells and T47D PR-A++ cells were probed by western blot for PR and GAPDH.

Progestin Activated miRNAs Realtive Expression

Cell Line	T47D PR-A ++					T47D				
miRNA	miR-1228-5P	miR-584-5P	miR-668-5P	miR-501-5P	miR-6823-5P	miR-1228-5P	miR-584-5P	miR-668-5P	miR-501-5P	miR-6823-5P
V	1	1	1	1	1	1	1	1	1	1
1nME	1.0377	0.6988	1.2414	1.3504	1.6888	1.1959	0.9914	1.2280	1.1559	1.3404
1nM E+0.05nM P	1.6626	0.7308	3.1849	3.8049	1.6862	1.1345	1.1206	1.3647	1.5847	1.3384
1nM E+0.2nM P	5.1131	3.0360	4.4787	6.1258	3.6560	1.1678	1.2877	1.6620	1.5874	1.8280
1nM E+0.5nM P	4.5812	3.8757	5.3508	7.7104	7.2341	2.0791	1.6298	1.8000	2.4149	2.1418
1nM E+1nM P	5.5846	3.9295	10.6955	11.2391	7.3695	5.4435	4.1027	3.2645	4.2015	5.5063

Progestin Activated miRNAs Standard Deviation

Cell Line	T47D PR-A ++					T47D				
miRNA	miR-1228-5P	miR-584-5P	miR-668-5P	miR-501-5P	miR-6823-5P	miR-1228-5P	miR-584-5P	miR-668-5P	miR-501-5P	miR-6823-5P
V	0.2821	0.2511	0.2605	0.0327	0.2895	0.1909	0.3371	0.0768	0.0472	0.1406
1nME	0.0240	0.2530	1.3226	0.4523	0.3514	0.0262	0.2002	0.1574	0.0405	0.8399
1nM E+0.05nM P	1.5487	0.2511	1.0117	1.4731	0.2774	0.0200	0.1436	0.3878	0.6602	0.5661
1nM E+0.2nM P	0.7863	0.8540	1.2262	0.4526	0.1742	0.0156	0.3212	0.7843	0.5650	0.6601
1nM E+0.5nM P	1.4848	1.1721	0.7908	0.8417	1.0526	0.7158	0.7465	0.7117	0.0576	1.1079
1nM E+1nM P	0.2519	0.6110	2.4907	0.1754	1.0388	1.8445	0.8914	1.1964	0.4397	0.7762

E2 Repressed miRNAs Realtive Expression

Cell Line	T47D PR-A ++					T47D				
miRNA	miR-200c-5p	miR-27a-5p	miR-27b-5p	LET7a	miR-26b-5p	miR-200c-5p	miR-27a-5p	miR-27b-5p	LET7a-5p	miR-26b-5p
V	1	1	1	1	1	1	1	1	1	1
1nME	0.2120	0.4680	0.2290	0.4142	0.3212	0.2368	0.2891	0.1382	0.1937	0.1394
1nM E+0.05nM P	0.2213	0.3612	0.2089	0.3256	0.3197	0.5974	0.6471	0.2833	0.1142	0.1767
1nM E+0.2nM P	1.3835	1.0420	1.1593	1.7439	1.0006	0.4183	0.3477	0.3214	0.2216	0.1692
1nM E+0.5nM P	1.0297	1.5235	0.7375	1.2017	0.7361	1.3036	0.5882	1.2017	0.8695	0.3253
1nM E+1nM P	0.9201	2.0322	1.0644	1.2943	0.7032	0.9459	1.5867	0.8708	0.8190	0.6338

E2 Repressed miRNAs Standard Deviation

Cell Line	T47D PR-A ++					T47D				
miRNA	miR-200c-5p	miR-27a-5p	miR-27b-5p	LET7a	miR-26b-5p	miR-200c-5p	miR-27a-5p	miR-27b-5p	LET7a-5p	miR-26b-5p
V	0.0500	0.0329	0.1349	0.0659	0.0566	0.0789	0.1228	0.1277	0.0258	0.0516
1nME	0.0344	0.0634	0.0475	0.0102	0.0054	0.0148	0.0295	0.0379	0.0095	0.0042
1nM E+0.05nM P	0.0104	0.1058	0.0166	0.0014	0.0101	0.0652	0.0524	0.0264	0.0418	0.0409
1nM E+0.2nM P	0.0473	0.0961	0.0626	0.0255	0.0540	0.0171	0.0346	0.3291	0.0092	0.0291
1nM E+0.5nM P	0.0391	0.0789	0.1241	0.0303	0.0396	0.0562	0.0335	0.1603	0.0117	0.0212
1nM E+1nM P	0.0437	0.0866	0.1258	0.0121	0.0039	0.0446	0.0789	0.0583	0.0286	0.0557

E2 Activated miRNAs Realtive Expression

Cell Line	T47D PR-A ++					T47D				
miRNA	miR-17-5p	moR-20a-5p	miR-92a-3p	miR-106a-5p	miR-106b-5p	miR-17-5p	moR-20a-5p	miR-92a-3p	miR-106a-5p	miR-106b-5p
V	1	1	1	1	1	1	1	1	1	1
1nME	7.823	4.965	4.436	4.261	5.504	6.306	4.054	3.786	3.116	2.516
1nM E+0.05nM P	7.544	4.582	3.952	3.670	5.007	5.190	3.508	2.639	2.897	2.970
1nM E+0.2nM P	1.514	1.247	1.284	1.147	1.410	4.090	2.038	2.036	2.095	2.794
1nM E+0.5nM P	1.206	0.911	0.921	0.656	1.274	1.800	1.116	0.728	1.013	0.916
1nM E+1nM P	1.413	0.917	0.921	0.454	1.180	1.633	0.887	0.718	0.653	0.703

E2 Activated miRNAs Standard Deviation

Cell Line	T47D PR-A ++					T47D				
miRNA	miR-17-5p	moR-20a-5p	miR-92a-3p	miR-106a-5p	miR-106b-5p	miR-17-5p	moR-20a-5p	miR-92a-3p	miR-106a-5p	miR-106b-5p
V	0.1333	0.2080	0.2075	0.0180	0.0665	0.0790	0.2086	0.4110	0.0202	0.0616
1nME	0.1146	0.1938	0.5273	0.0674	0.1015	0.1690	0.3245	0.9685	0.1023	0.1320
1nM E+0.05nM P	0.0829	0.2934	0.0738	0.0692	0.1176	0.4861	0.0736	0.0759	0.1628	0.0178
1nM E+0.2nM P	0.1163	0.0626	0.1987	0.0140	0.0627	0.0697	0.3214	0.0539	0.0592	0.2230
1nM E+0.5nM P	0.0810	0.1299	0.1858	0.0136	0.0506	0.1451	0.1580	0.0087	0.0510	0.0634
1nM E+1nM P	0.1902	0.2831	0.0502	0.0136	0.0342	1.4383	0.1822	0.0112	0.0330	0.0265

Table 3.4 Data corresponding to heat maps in Figure 3.2B -D
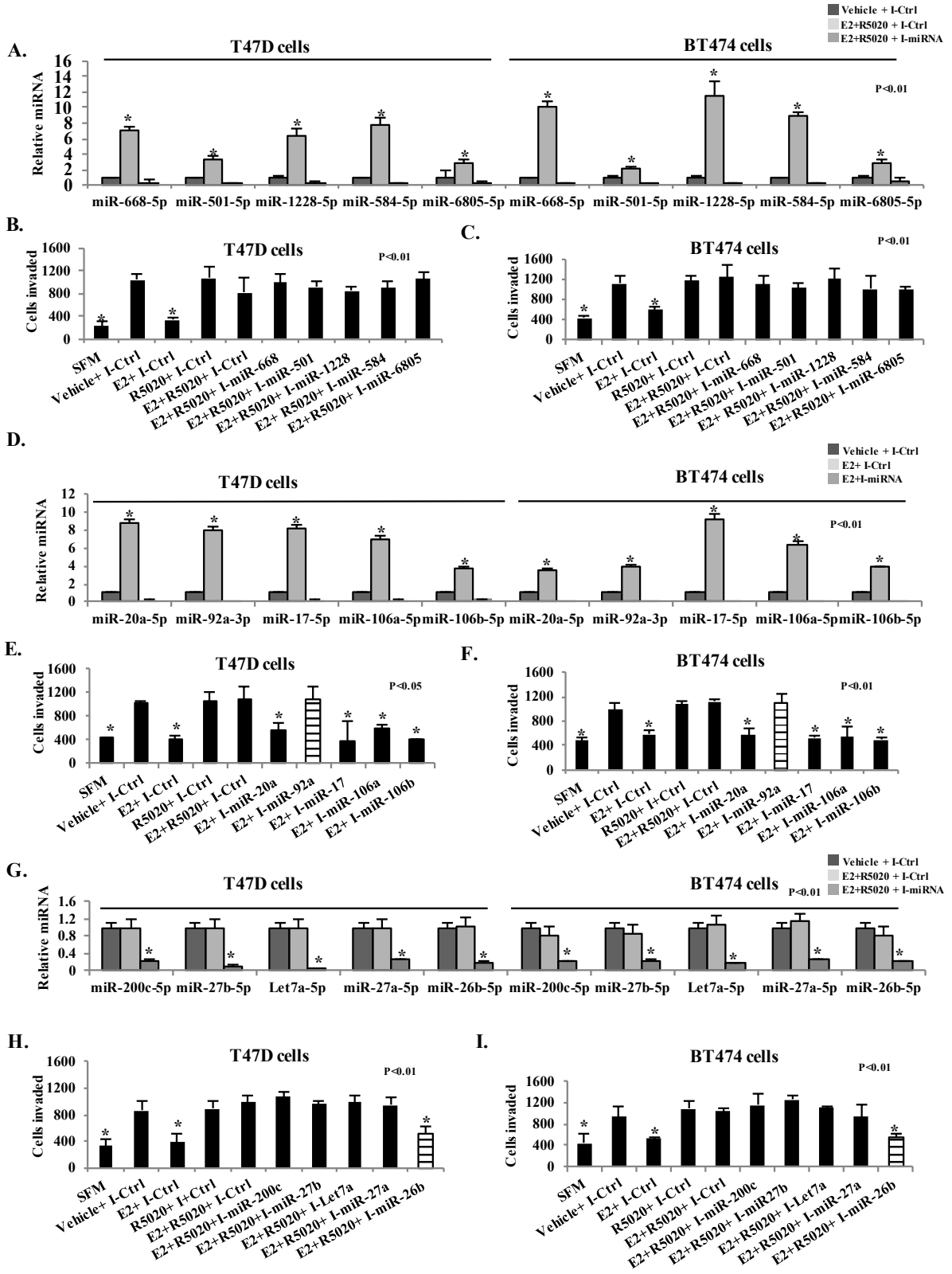
Figure 3.4: Effect of inhibiting miRNAs hormonally regulated by PR-A and ER on invasiveness of luminal breast cancer cells. Hormone-depleted T47D and BT474 cells were transfected with either control Inhibitor (I-Ctrl) or the indicated miRNA specific inhibitor. The inhibitors are indicated in the Figure by using the prefix 'I' for the corresponding target miRNA. Twenty-four hours later, cells were treated for 48 h with vehicle, E_2 (1nM), or E_2 (1nM) + R5020 (1nM) as indicated. Total RNA was then extracted and the relative levels of each miRNA indicated were quantified in all the samples. Histograms show the miRNA expression data for progestin activated miRNAs (**Panel A**), E_2 activated miRNAs **(Panel D) and** E_2 repressed miRNAs **(Panel G).** In parallel, the treated cells were subjected to the trans-well matrigel invasion assay (**Panels B, C, E, F, H and I**). One-way ANOVA with Post-hoc unpaired t-test was performed on triplicate treatment sets and *P*-values are indicated.

We first inhibited each of the progestin/PR-A activated (but not E_2-regulated) miRNAs, miR-6805-5p, miR-584-5p, miR-1228-5p, miR-501-5p and miR-668-5p (Figure 3.4A). Invasiveness was unaffected in all cases in the presence of E_2 and R5020 (Figure 3.4B and 3.4C), indicating that these miRNAs do not have a role in hormonal cross-talk between progestin/PR-A and E_2/ER that influences invasiveness.

Next, we inhibited the five miRNAs whose activation by E_2 was blocked by progestin (miR-17-5p, miR-20a-5p, miR-92a-3p, miR-106a-5p and miR-106b-5p) (Figure 3.4D). Inhibition of miR-92a-3p alone abrogated the ability of E_2 to repress invasiveness in both T47D and BT474 cells (Figure 3.4E and 3.4F). Therefore, the ability of progestin/PR-A to block activation of miR-92a-3p by E_2 must be critical for the functional cross-talk of progesterone via PR-A.

Finally, we inhibited the five miRNAs whose repression by E_2 was blocked by progestin (miR-26b-5p, miR-27a-5p, miR-27b-5p, miR-200c-5p and Let7a-5p) (Figure 3.4G). Inhibition of miR-26b-5p alone abrogated the ability of progestin to rescue invasiveness from E_2 repression in both T47D and BT474 cells (Figure 3.4H and 3.4I). Therefore, the ability of progestin/PR-A to block repression of miR-26b-5p by E_2 must also be a critical mechanism of cross-talk of progesterone via PR-A in the rescue of invasiveness.

3.3.4 miR-92a-3p and miR-26b-5p regulate genes associated with invasiveness and metastasis in luminal breast cancer cells

To further study the roles of miR-92a-3p and miR-26b-5p in relation to gene expression and metastasis, we generated two types of pooled (to avoid clonal bias) stable recombinant BT474 cells. In one case, miR-92a-3p (E_2 activated miRNA) was constitutively expressed ('miR-92a-3p-On' cells in Figure 3.5A). In the other case, miR-26b-5p (E_2 repressed miRNA) was constitutively

inhibited due to expression of a miR-26b-5p inhibitor ('miR-26b-5p-Off' cells in Figure 3.5B). Hormonal regulation of miR-26b-5p and miR-92a-3p was unaffected in miR-92a-3p-On cells and miR-26b-5p-Off cells, respectively, indicating these two miRNAs are regulated independently (Figure 3.5A and 3.5B). Both the miR-92a-3p-On cells (Figure 3.5C) and the miR-26b-5p-Off cells (Figure 3.5D) showed repressed invasiveness even in the absence of hormones, similar to the E_2 treated control cells (harboring Lenti-miR-blank plasmid). Moreover, the ability of R5020 to rescue invasiveness was lost in both the miR-92a-3p-On cells (Figure 3.5C) and in the miR-26b-5p-Off cells (Figure 3.5D), in contrast to the control cells. Next, the miR-92a-3p-On and miR-26b-5p-Off cells, treated with both E_2 and R5020, were examined for quantitative changes in the metastasis/invasion transcriptome, using a commercial pathway cDNA TaqMan PCR array. When compared to the control cells, both the recombinant cells showed broad and partially overlapping increases in the expression of cellular adhesion molecules and inhibitors of metastasis but not a remarkable effect on the expression of genes known to promote metastasis (Figure 3.5E; values in *Table 3.5*). Therefore, miR-92a-3p and miR-26b-5p are part of independently regulated but convergent pathways through which E_2 controls genes that have functional roles in metastasis. It follows that the ability of PR-A to oppose regulation of these miRNAs by E_2 enables progesterone to induce a gene regulatory pattern that supports metastasis.
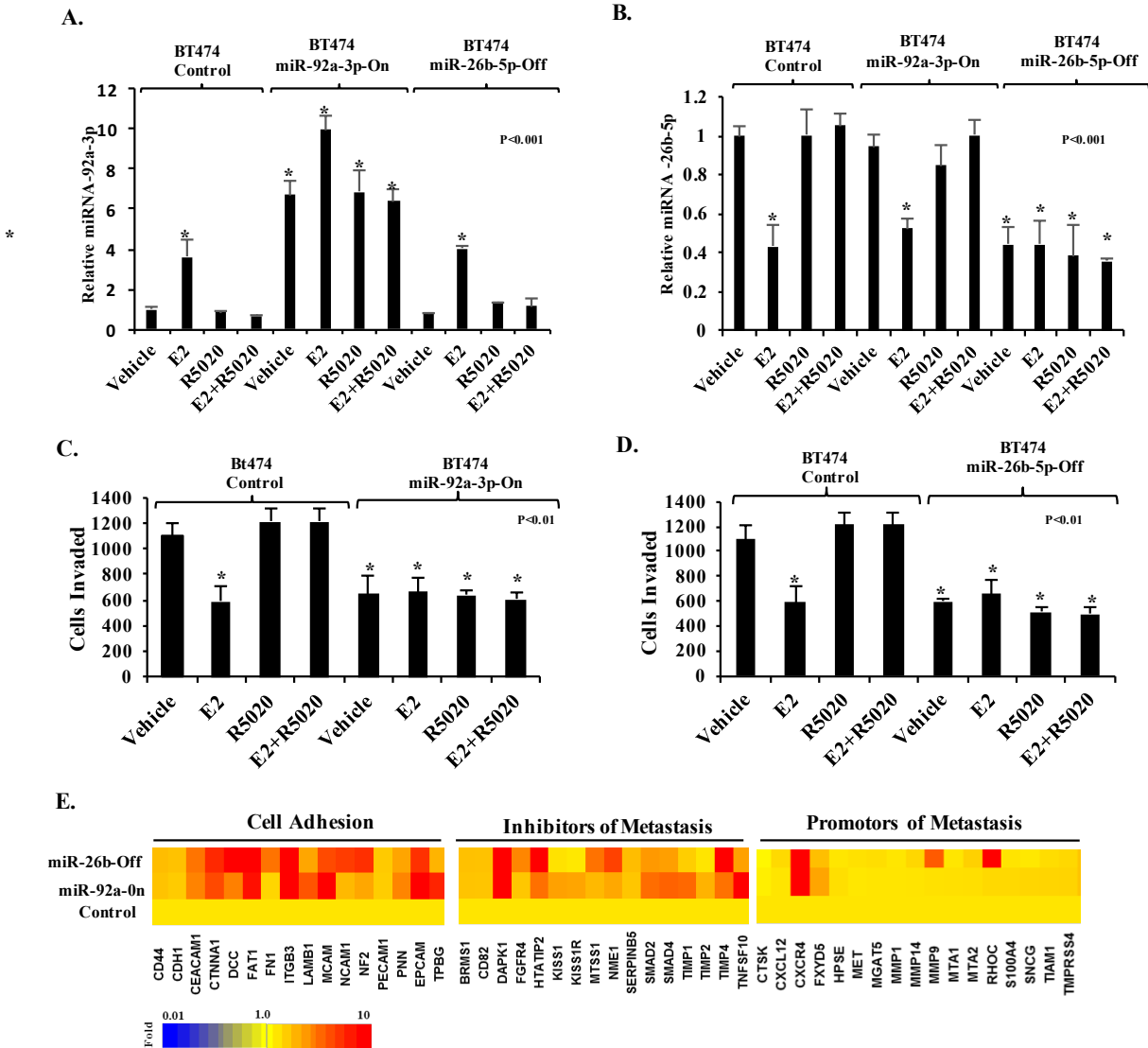
Figure 3.5: Roles of miR-92a-3p and miR-26b-5p in regulation of invasiveness and metastasis genes. Control BT474 cells (harboring Lenti-miR-Blank plasmid) and isogenic recombinant cells constitutively expressing miR-92a-3p mimic (BT474 miR-92a-3p-On cells) or stably expressing miR-26b-5p inhibitor (BT474 miR-26b-5p-Off cells) were hormone depleted and treated with vehicle or 1 nM E_2, 1 nM R5020 or the combination of E_2 and R5020 for 48 h. Total RNA was then isolated and miR-92a-3p (**Panel A**) and miR-26b-5p (**Panel B**) were quantified. In parallel, the trans-well invasion assay was performed on the treated BT474 miR-92a-On cells (**Panel C**) and BT474 miR-26b-Off cells **(Panel D)** together with the parental control cells. In **Panel E**, total RNA was isolated from BT474 control, miR-92a-On and miR-26b-Off cells treated with1 nM E_2 in combination with 1nM R5020 for 48 h and analyzed using the Taqman metastasis transcriptome array. The transcriptome expression profile is shown in the heat map.

GENE	Fold Ctrl	Fold BT474-92a-ON	Fold BT474 26B-Off
CD44	1	2.316416999	2.574727833
CDH1	1	2.025666086	2.384783942
CEACAM1	1	4.845103232	5.42203053
CTNNA1	1	7.106474336	8.400452225
DCC	1	4.958002985	44.31955593
FAT1	1	9.395387515	16.2554325
FN1	1	1.578332857	5.461599989
ITGB3	1	21.29900314	15.36544899
LAMB1	1	6.696123735	3.480976036
MCAM	1	14.83712831	7.093445645
NCAM1	1	2.723552889	7.633145435
NF2	1	3.432347888	8.139986046
PECAM1	1	1.950441098	2.064285982
PNN	1	4.776060885	3.502094472
EPCAM	1	12.85159964	8.040079562
TPBG	1	8.611349358	2.909127387
BRMS1	1	2.298133076	2.404106858
CD82	1	2.262846516	2.365688268
DAPK1	1	15.38801379	18.52161213
FGFR4	1	2.171072951	4.718021745
HTATIP2	1	5.600129858	11.83932987
KISS1	1	3.663169991	1.108748001
KISS1R	1	3.500650202	0.864848727
MTSS1	1	2.708765346	5.514740787
NME1	1	3.39929129	7.445899935
SERPINB5	1	2.196839365	2.576055727
SMAD2	1	5.694093453	3.994134299
SMAD4	1	6.143653096	3.756083873
TIMP1	1	5.899718246	1.913834685
TIMP2	1	2.966268703	0.984000086
TIMP4	1	5.502287445	10.74901235
TNFSF10	1	10.75559405	3.654028186
CTSK	1	0.621476427	0.293877228
CXCL12	1	1.01222664	1.448997555
CXCR4	1	11.3978419	13.94306207
FXYD5	1	3.52564289	2.506085504
HPSE	1	1.218603449	0.958312208
MET	1	0.916107629	1.171739803
MGAT5	1	1.2646	1.259167763
MMP1	1	1.01222664	0.975231364
MMP14	1	1.047621586	1.550015227
MMP9	1	1.170272731	6.45689996
MTA1	1	1.186129555	0.942979457
MTA2	1	1.218603449	1.799029205
RHOC	1	1.482436467	18.48372069
S100A4	1	1.488414392	1.05132146
SNCG	1	1.578332857	0.892392677
TIAM1	1	1.636131835	1.331492053
TMPRSS4	1	1.680661473	1.523832323

Table 3.5 Data corresponding to heat map in Figure 4E.

An additional relevant question was whether estrogen and/or progesterone could at least partially regulate epithelial vs mesenchymal characteristics of luminal breast cancer cells, although it has been well established that ER expression confers the epithelial phenotype. Using a commercial EMT panel antibody kit and BT474, T47D, and ZR-75-1 cell lines, we observed no change in established epithelial/mesenchymal markers when cells were treated individually or in combination with E_2 and R5020 (Figure 3.6A and 3.6B) in contrast to depletion of the ER apo-protein (Figure 3.6C). Therefore, it was irrelevant to explore EMT in the context of hormonal regulation of miR-92a-3p or miR-26b-5p.

3.3.5 miR-92a-3p and miR-26b-5p profoundly impact metastasis *in vivo*

Studies of hormonal regulation of metastasis of model luminal breast tumor xenografts in mice is technically challenging for the following reasons. First, estrogen needs to always be present for the tumors to grow, optimally administered to the mice through implanted slow release, low dose E_2 pellets; therefore, the hormone levels cannot be modulated. Second, the constant exposure to even the low dose of E_2 needed to support tumor growth causes urinary retention and cystitis in the mice, limiting the duration of the experiments to about 2-3 weeks. Finally, luminal breast tumors are inherently less aggressive and metastasize relatively slowly, so that it is not possible to histologically detect micro-metastases within the 2-3-week duration of the experiments. We overcame these problems in the current study design that investigated whether modulation of miRNAs 92a-3p and 26b-5p could govern metastasis in mice bearing luminal breast tumor xenografts.

A.

B.

C.
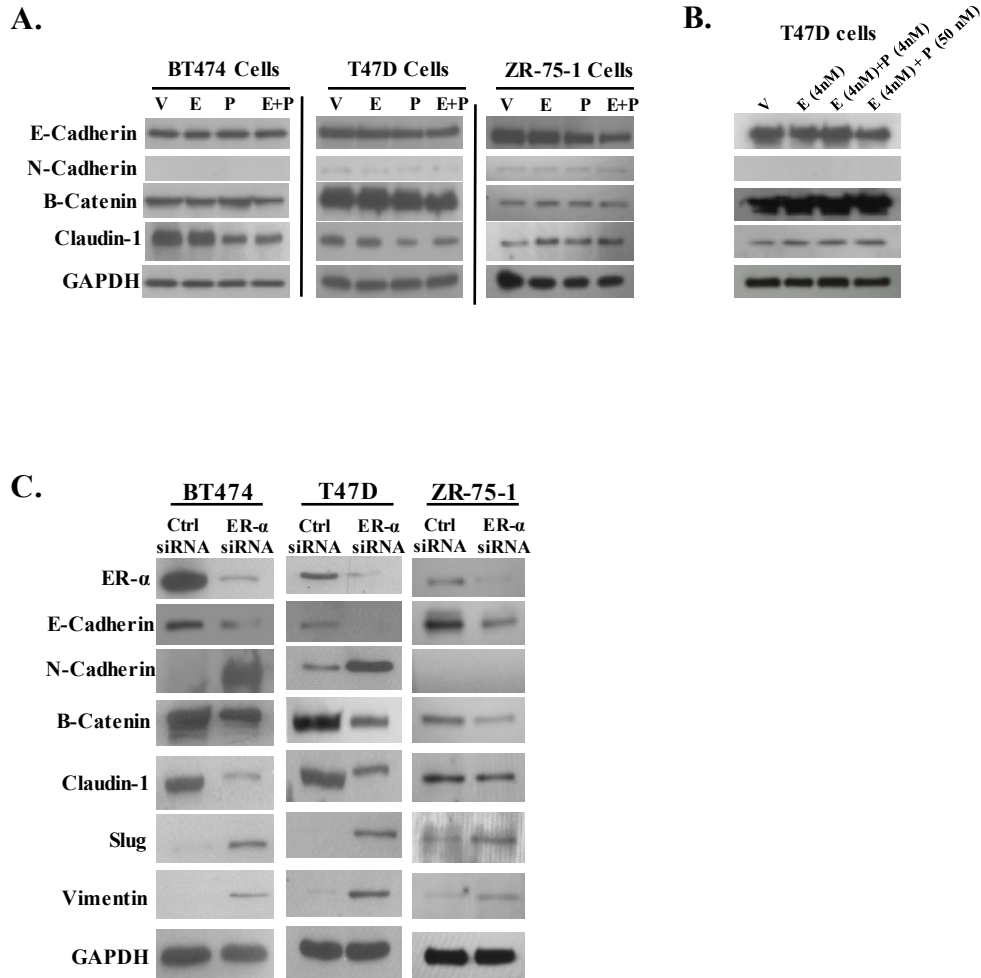
Figure 3.6. Hormonal regulation of invasiveness is not mediated by epithelial to mesenchymal transition (EMT). BT474, T47D and ZR-75-1 cells cells were treated with vehicle (V), E_2 (1nM), R5020 (P) (1nM)), E_2+R5020 (E+P) (each at 1nM) for 48 h and and total protein was analyzed by western blot for epithelial and mesenchymal markers (Panel A). A similar analysis was performed on T47D cells using hormone concentrations representing the follicular phase (4 nM E_2, 4 nM P) and luteal phase (4 nM E_2, 50 nM P) of the menstrual cycle (Panel B). In no case did hormones induce EMT. BT474, T47D, and ZR-75-1 cells were hormone-depleted, transfected with either Control siRNA or ER siRNA and cell lysates were analyzed by western blot for epithelial and mesenchymal markers 72 h post-transfection (Panel C); loss of the ER apo-protein induced EMT.
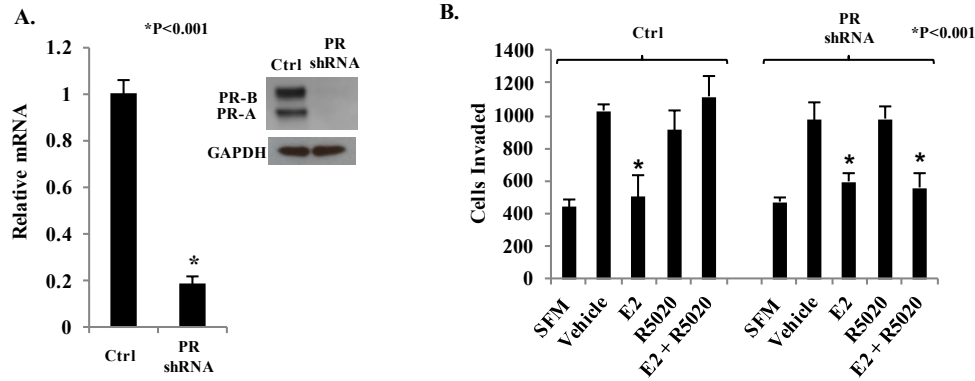
Figure 3.7 In BT474 PR-shRNA cells, progestin cannot rescue invasiveness from estrogen repression. BT474 PR-shRNA cells show depletion of PR mRNA (Panel A, left) and loss of PR protein (western blot in Panel A, right) compared with the control BT474 cells. In the trans-well invasion assay, E_2 (1 nM) suppresses invasiveness even in the presence of R5020 (1 nM) in BT474 PR-shRNA cells (Panel B, right). In contrast, in the control BT474 cells, R5020 rescues invasiveness from E_2 repression (Panel B, left).

We used as xenograft models, the recombinant BT474 cells in which cross-talk between E_2/ER and progestin/PR-A affecting invasiveness was disrupted by constitutive expression of miR-92a-3p (miR-92a-3p-On cells discussed above) or constitutive inhibition of miR-26b-5p (miR-26b-5p-Off cells discussed above) for comparison with the control cells. As a metastasis assay validation tool, we also generated and used as xenografts, pooled (to avoid clonal bias) stable recombinant BT474 cells in which PR was knocked down (BT474 PR-shRNA cells); in these cells, we confirmed that the PR knockdown resulted in loss of the ability of R5020 to rescue *in vitro* invasiveness from E_2 regulation (Figure 3.7). The previously established range of plasma estrogen in mice implanted the slow release E_2 pellets is 1.8 - 4.8 nM (353,354). The established range of plasma progesterone in mice on commercial diet is 3.2 - 5.6 nM (354). To measure metastasis of subcutaneous implantation of the xenografts in the right flank, we measured tumor cell infiltration in the ipsilateral axillary lymph nodes by the sensitive assay of measuring mRNA for human GAPDH present in the lymph nodes, 14 days after implanting the xenografts. The control BT474 cells consistently infiltrated the lymph node whereas depletion of PR in these cells showed virtually complete suppression of lymph node infiltration, validating the metastasis assay (Figure 3.8A and 3.8B). In mice bearing xenografts of miR-92a-3p-On cells (Figure 3.8C and 3.8D) or miR-26b-5p-Off cells (Figure 3.8E and 3.8F), metastasis was similarly dramatically suppressed, demonstrating that the profound effects of changing the levels of these two miRNAs on cellular invasiveness observed above extend to their effects on metastasis *in vivo*.
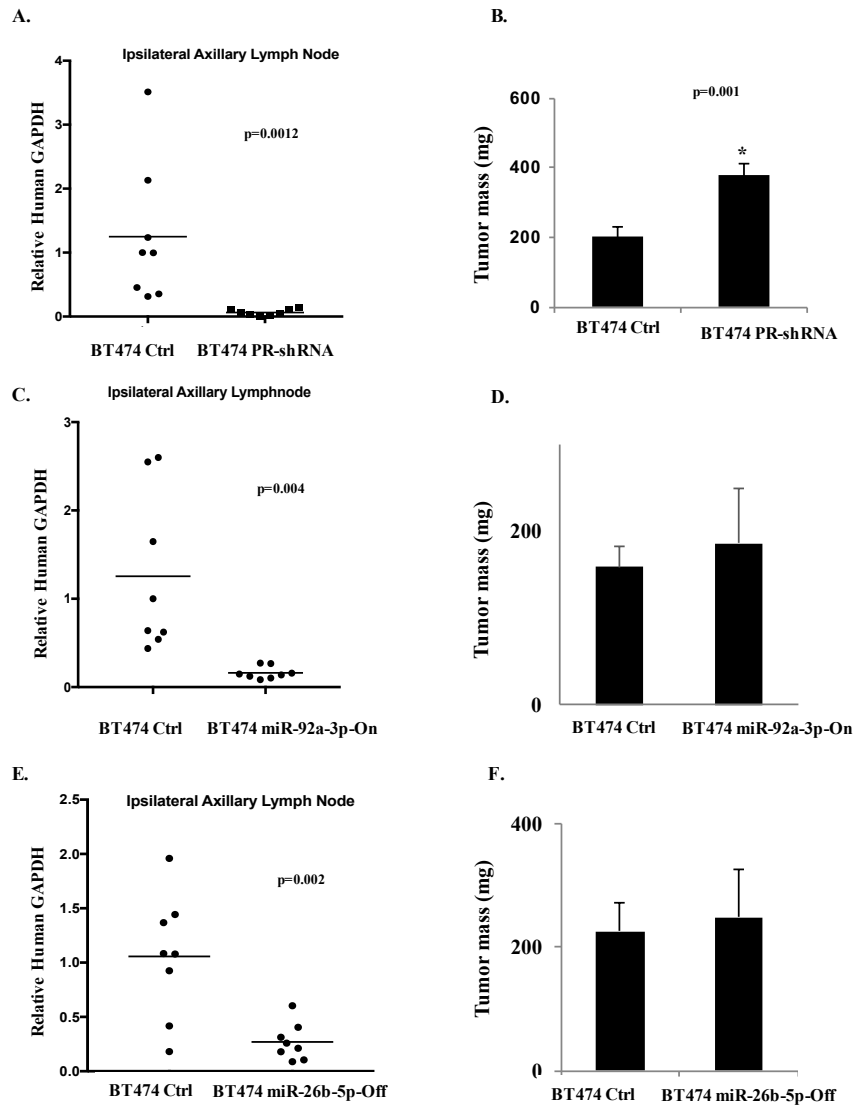
Figure 3.8: Roles of miR-92a-3p and miR-26b-5p in regulating metastasis in an *in vivo* lymph node infiltration model. BT474 control cells **(Panels A-F)**, BT474 PR-shRNA cells **(Panels A and B),** BT474 miR-92a-3p-On cells **(Panels C and D)** and BT474-miR-26b-5p-Off **(Panels E and F)** were implanted subcutaneously in the right flank to form tumor xenografts in athymic female nude mice (8 mice per group) that had been implanted with slow release, low dose estradiol pellets. Mice were sacrificed at 2 weeks, and the proximal ipsilateral lymph node (right axillary) was harvested. Total RNA was extracted from lymph nodes, and high efficiency species specific Taqman probes for human and mouse GAPDH were used to measure relative degrees of lymph node infiltration by the human tumor cells. Using the Ct values for human GAPDH as Target and mouse GAPDH as endogenous reference, the human cell infiltration into mouse lymph node was calculated by the ΔΔCt method with a calibrator ΔCt of 10.79 in all cases **(Panels A, C and E)**. Tumor mass was measured on the day of sacrifice (**Panels B, D, and F**). Statistical analysis using Student's unpaired t-test was used for two group comparisons and *P*-values are indicated.

The foregoing results strongly support the premise that positive (for miR-92a-3p) or negative (for miR-26b-5p) regulation of these two miRNAs by E_2 causes suppression of not only invasiveness but also metastasis of luminal breast cancer cells and that by extension of this reasoning, the restoration of these miRNAs to their original levels (as in the control tumors) by progesterone via PR-A must support metastasis.

3.3.6 In primary human luminal breast tumors PR-A expression correlates negatively with miR-92a-3p and positively with miR-26b-5p

Using total RNA samples extracted from 53 ER+/PR+ primary luminal breast tumor specimens, the expression profile of PR-A mRNA was obtained and compared with the expression profiles of miR-92a-3p and miR-26b-5p obtained from the same RNA preparations. Despite the inherent and variable heterogeneity among the tumor specimens due to a variable ratio of tumor cells to stroma, miR-92a-3p correlated negatively with PR-A mRNA with an r = -0.272 (p=0.004) (Figure 3.9) and miRNA-26b-5p correlated positively with PR-A mRNA with r = 0.342 (p=0.001) (Figure 6B). Moreover, as shown in the heat map in Figure 3.9C, the inverse expression trends of the two miRNAs generally occurred within the same samples. Finally, relative PR-A protein levels among the tumors generally corresponded to the relative PR-A mRNA levels (Figure 3.9D) with the caveat that the protein was measured only semi-quantitatively by non-linear densitometry from western blots (Figure 2A) and was also necessarily extracted from a different part of each tumor specimen than RNA.
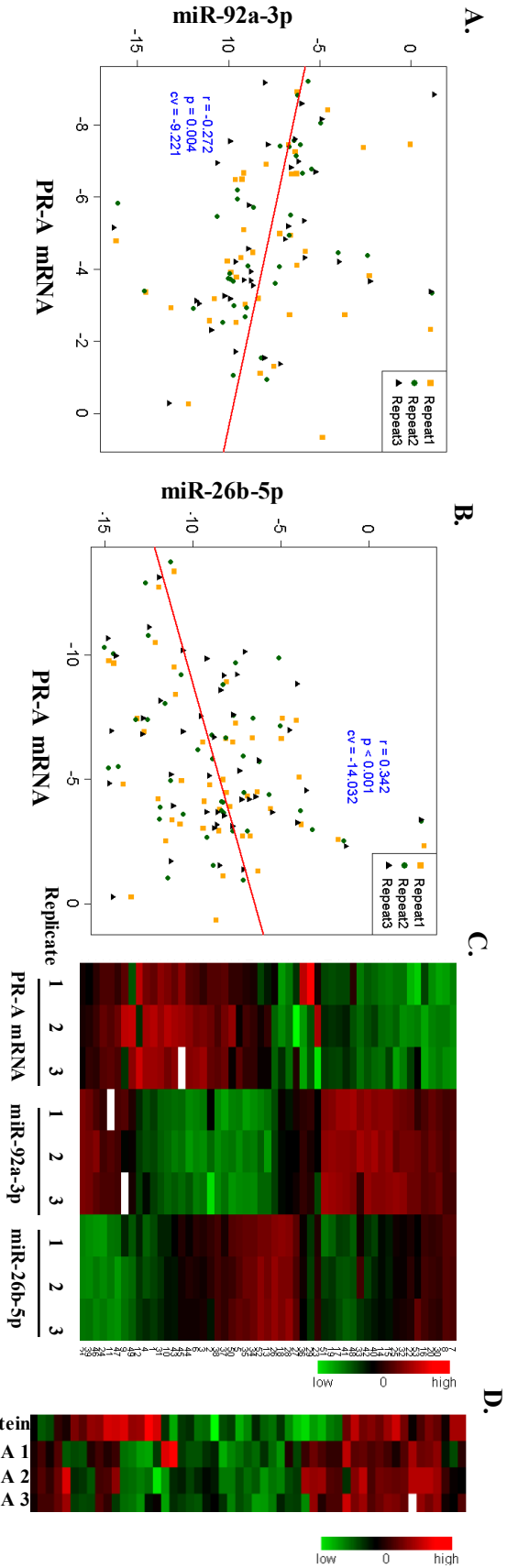
Figure 3.9. Correlation analysis of PR-A, miR-92a-3p and miR-26b-5p in primary luminal clinical breast tumor specimens. Total RNA was extracted from tumor specimens from 53 breast cancer patients. Real time RT-PCR was used to quantify PR-A mRNA, miR-92a-3p and miR-26b-5p. The measurements were repeated 3 times to generate biological replicates, resulting in 159 Ct values for each RNA. The efficiency of each primer pair (E = 2) was used to normalize the real time RT-PCR data and a relative gene expression

3.4 Discussion

In aggressive cancers including hormone receptor-negative breast cancers, at least the early stages of metastasis have likely already occurred at the time of detection of the primary tumor. In contrast, luminal breast tumors are better differentiated and relatively indolent, metastasize much slower and offer a wider window of time for prediction of metastatic potential and for effective intervention to suppress metastasis. This study has elucidated a critical role for two miRNAs in enabling progesterone to oppose specific actions of estrogen, thus promoting invasiveness and metastasis of luminal breast cancer cells. The mechanism entails a central role for the short PR isoform A in mediating this effect of progesterone. We have previously shown that PR-A induces invasiveness in the entire range of circulating hormone levels covering pre- and post-menopausal years and that overexpression of PR-A further sensitizes the cells to progesterone levels in the low end of its post-menopausal plasma range, an observation that also held true for the response of the two miRNAs to progesterone. In this study, the discovery of miRNAs that mediate the cross-talk between PR-A and ER that results in invasiveness was crucial in developing a study design that demonstrated the profound role of this cross-talk in supporting metastasis *in vivo*.

None of the five miRNAs identified in this study as being regulated by progesterone in a PR-A isoform-specific, but estrogen-independent, manner affected the ability of estrogen to suppress invasiveness. However, two of ten miRNAs that were regulated by estrogen in a manner that was opposed by progesterone, exclusively via isoform A of PR, had profound roles in invasiveness and metastasis. They are miR-92a-3p (activated by estrogen) and miR-26b-5p (repressed by estrogen). We demonstrated that regulation of these two miRNAs enabled estrogen to suppress invasiveness and blocking of this regulation by progesterone via PR-A restored invasiveness. Further, up-regulation of miR-92a-3p and down-regulation of miR-26b-5p induced

changes in invasion and metastasis pathway genes that were similar in part but both trending toward a non-metastatic phenotype and also resulted in suppression of metastasis *in vivo*. Notably miR-92a-3p and miR-26b-5p mediate independent but convergent pathways of hormonal control of invasion and metastasis. The ability of estrogen to effectively suppress invasiveness even at very low concentrations (~ 0.01 nM)(157) may therefore be explained by the combined effects of sub-optimal regulation of these two miRNAs by estrogen.

We observed relative overexpression of PR-A in about 25 percent of primary luminal breast tumors obtained from patients. When, in an isogenic cell line model, PR-A was overexpressed to such a level, the reduced progestin concentration required for optimal induction of invasiveness also optimally suppressed regulation of miR-92a-3p and miR-26b-5p by estrogen. The clinical relevance of this functional effect of PR-A overexpression is further supported by the negative correlation of miR-92a-3p expression and positive correlation of miR-26b-5p expression with PR-A expression in clinical tumors, notwithstanding the inherent heterogeneity in tumor vs. stromal content in the specimens.

Consistent with our findings, miR-92a expression is inversely correlated to tumor grade, positive lymph node status and recurrence-free survival in breast cancer (355,356). Curiously, miR-92a is part of the miR-17~92 cluster that supports oncogenesis and cancer progression in many other cell types (356,357) demonstrating cell type-specific differences in the actions of miR-92a. miR-26a and miR-26b are both repressed by estrogen via stimulation of c-MYC expression resulting in the proliferative effect of estrogen in breast cancer cells (358). However, it is only miR-26b that is of interest in our study as the repression of miR-26a was opposed by progestin through PR-B as well as PR-A. Relatively little is known about the role of miR-26b in invasiveness and metastasis of breast cancer. In mesenchymal stem cells, miR-26b induces migration by

activating focal adhesion kinase (359) whereas, in bladder cancer, miR-26b inhibits migration and invasion (360). In the case of luminal breast cancer, our studies show that the ability of progesterone plus PR-A to prevent suppression of miR-26b-5p by estrogen leads to increased invasion and metastasis, again underscoring cell type specific differences in the actions of miR-26b-5p.

In conclusion, our model systems have established narrow miRNA-mediated pathways of cross-talk in hormone-dependent signaling between ER and PR-A which may account for the variable invasive and metastatic potential of primary luminal breast tumors. The relative expression/activity of PR-A may be a particularly significant determinant of the extent of this cross-talk, especially in the context of post-menopausal plasma hormone levels. Therefore, this study may have established a fundamental physiological mechanism governing metastatic spread of luminal breast cancer. Additionally, miRNA signatures of hyperactive PR-A have the potential to serve as predictors of clinical progression of luminal breast cancer. Moreover, miRNAs identified in this study that mediate functionally relevant cross-talk between PR-A and ER may reveal target pathways for interventions to suppress progression of luminal breast cancer that would avoid disruption of hormone signaling in normal tissues.

CHAPTER 4- CONCLUSION

Our studies have been described in this thesis in two parts entitled "Role of the short isoform of the progesterone receptor in breast cancer cell invasiveness at estrogen and progesterone levels in the pre- and post-menopausal ranges" and "The Short Progesterone Receptor Isoform Supports Invasiveness and Metastasis of Luminal Breast Cancer by Suppressing Regulation of Critical Micro RNAs by Estrogen". The results of these two studies are summarized below.

Role of the short isoform of the progesterone receptor in breast cancer cell invasiveness at estrogen and progesterone levels in the pre- and post-menopausal ranges. Overexpression of PR-A is a negative prognosticator for ER+ breast cancer but *in vitro* studies have implicated PR-B in progestin-induced invasiveness. As E_2 is known to suppress invasiveness and tumor progression and as the *in vitro* studies were conducted in models that either lacked ER or excluded estrogen, we examined the role of PR isoforms in the context of estrogen signaling. E_2 (< 0.01nM) strongly suppressed invasiveness in various ER+ model cell lines. At low (< 1nM) concentrations, progestins completely abrogated inhibition of invasiveness by estrogen. It was only in a higher (5nM - 50 nM) concentration range that progestins induced invasiveness in the absence of estrogen. The ability of low dose progestins to rescue invasiveness from estrogen regulation was exclusively mediated by PR-A, whereas PR-B mediated the estrogen-independent component of progestin-induced invasiveness. Overexpression of PR-A lowered the progestin concentration needed to completely rescue invasiveness. Among estrogen-regulated genes, progestin/PR-A counter-regulated a distinctive subset, including breast tumor progression genes (e.g., HES1, PRKCH, ELF5, TM4SF1), leading to invasiveness. In this manner, at relatively low hormone concentrations (corresponding to follicular stage and post-menopausal breast tissue or plasma

levels), progesterone influences breast cancer cell invasiveness by rescuing it from E_2 regulation via PR-A, whereas at higher concentrations the hormone also induces invasiveness independent of estrogen signaling, through PR-B. The findings point to a direct functional link between PR-A and progression of luminal breast cancer in the context of the entire range of pre- and post-menopausal plasma and breast tissue hormone levels.

The Short Progesterone Receptor Isoform Supports Invasiveness and Metastasis of Luminal Breast Cancer by Suppressing Regulation of Critical Micro RNAs by Estrogen.

Distal metastasis of luminal breast cancer is frequent and incurable, yet the underlying mechanisms leading to it are poorly understood. E_2 suppresses invasiveness of luminal breast cancer cells even at post-menopausal concentrations through ER Invasive tumors overexpress PR-A. Even at low (post-menopausal) concentrations, progesterone activates PR-A, inducing invasiveness by counteracting estrogen, particularly when cells are hyper-sensitized to progesterone by PR-A overexpression. As a means to interrogating the role of this cross-talk in determining metastatic potential, we explored micro RNA mediators of selective cross-talk of PR-A with ER. We also developed a quantitative PCR-based lymph node infiltration assay in mouse xenograft models to address the slowness of tumor spread that limits studies of metastasis of luminal breast cancer. Fifteen miRNAs were regulated by progesterone via PR-A, but not PR-B, with increased progesterone sensitivity when PR-A was overexpressed. Two among them, whose induction (miR-92a-3p) or repression (miR-26b-5p) by estrogen was suppressed by progesterone plus PR-A, were critical for the cross-talk of PR-A with ER that caused a gene regulatory pattern of invasiveness and metastasis and complete rescue of invasiveness *in vitro*. The effect of expression changes of these miRNAs on *in vitro* invasiveness also manifested as metastatic potential *in vivo*. Finally, in primary breast tumors, PR-A expression correlated negatively with expression of miR-92a-3p and

positively with expression of miR-26b-5p. The studies establish hormonal cross-talk between PR-A and ER as likely a fundamental physiological mechanism that enables metastasis of luminal breast cancer. Additionally, micro RNAs, as biomarkers of hyperactive PR-A, may aid in predicting metastatic potential of luminal breast tumors. Further, miR-92a-3p and miR-26b-5p may reveal target pathways for selective intervention to suppress hormone-regulated metastasis, both pre- and post-menopause.

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ABSTRACT

MECHANISMS OF HORMONAL REGULATION OF INVASIVENESS AND METASTASIS OF LUMINAL BREAST CANCER

by

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Advisor: Dr. Manohar Ratnam

Major: Cancer Biology

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Over 20% of breast cancer cases present with distal metastasis and they are predominantly of luminal subtypes. As luminal breast cancer is relatively indolent, it is believed that progression to metastasis must occur over many years, generally well into post-menopausal years. Unfortunately, very little is known about the mechanisms by which these hormone receptor positive tumors metastasize, likely in part due to their slow metastatic rates in animal model systems as well. Moreover, the literature lacks adequate mechanistic understanding of cross talk between estradiol (E_2) and progesterone, particularly in the context of breast cancer invasion and metastasis. In this thesis, we sought to investigate the roles of estrogen and progesterone and their nuclear receptors to better understand hormonal regulation of metastasis at physiologically relevant hormone levels both pre- and post-menopause. The novelty of our experimental approach and study design is three-fold: 1. exploration of the isoform-specific actions of the progesterone receptor; 2. investigation of selective micro RNA mediated pathways of cross talk between estrogen and progesterone and 3. development of a quantitative lymph node infiltration assay to monitor metastasis of luminal breast cancer in xenograft models.

AUTOBIOGRAPHICAL STATEMENT

Thomas McFall was born in Kalamazoo Michigan in 1985. Graduated from Northern Michigan University with a B.S. in Biology/Human Physiology in 2008 and an M.S. in Biology in 2012.