DEVELOPMENT OF TRANSPLANTABLE MOUSE MODELS TO ASSESS THE ROLE OF PROLACTIN INDUCIBLE PROTEIN IN BREAST TUMORIGENESIS

By

CHIDALU ARNOLD EDECHI

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Pathology

University of Manitoba

Winnipeg, Manitoba, Canada

ABSTRACT

Breast cancer (BC), a highly heterogenous disease, is the most prevalent cancer in women. The prolactin inducible protein (PIP) is expressed by over 90% of BCs to varying degrees. Higher PIP expression levels have been shown to correlate with better prognosis and patient response to chemotherapy. Previous studies suggest an immunomodulatory role, however, the role of PIP in BC pathogenesis is unknown. In addition to its role in innate immunity, our laboratory has previously shown that deficiency of PIP is associated with defective type 1 T-helper (Th1) cell immune response, which operates as a critical adaptive immune component for antitumor immunity. Therefore, it was hypothesized that PIP inhibits BC and enhances antitumor immunity. Here, the role of PIP in BC progression was investigated using syngeneic transplantable BC mouse models developed from 4T1 and E0771 mouse BC cell lines. PIP was overexpressed in both cell lines and characterized using a number of *in vitro* functional assays. Following the transplantation of these lines in syngeneic mice, the impact of PIP expression on breast tumorigenesis *in vivo* was also assessed with specific focus on tumor onset, growth, size, immune response, and metastasis. *In vitro* functional assay comparisons revealed no differences in proliferation, migration, and response to anticancer drugs in both cell lines. However, *in vivo* studies showed that the expression of PIP in 4T1 cells delayed tumor onset, reduced tumor growth, and diminished tumor size. These observations were associated with increased percentages of natural killer cells, dendritic cells and reduced frequency of $CD4^{+}IL4^{+}$ T cells in the PIP expressing 4T1 tumors. Furthermore, PIP expression in 4T1 cells resulted in elevated lung metastasis, indicating that PIP may play opposing roles in BC. Interestingly, these effects however were not observed in the *in vivo* E0771 mouse BC model thereby suggesting that PIP may have different effects on different types of BC. These studies provide initial preclinical experimental data to suggest that PIP is an important regulator

of breast tumor immunity and potentially, metastasis in mouse models, further expanding our understanding of breast cancer progression.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my advisors Drs. Yvonne Myal and Jude Uzonna for the opportunity to work under their supervision as a graduate student. This work would not have been possible without your mentorship, support and guidance in training me to become a scientist.

I would also like to extend my gratitude to my committee members Drs Sabine Hombach-Klonisch and Sam Kung for their excellent suggestions and contributions throughout this project. You have been approachable and willing to help when I came with questions about my research project.

I want to acknowledge the support of members of both the laboratories of Dr. Myal and Dr. Uzonna. I am also immensely grateful to my family, friends and Prof. Mike Eze for their support and encouragement.

ABBREVIATIONS

µl	microliter
ACK	Ammonium-Chloride-Potassium Lysing Buffer
APC	Antigen presenting cells
BCA	Bicinchoninic acid assay
CD11c+	Marker of dendritic cells
CD4	Cluster of differentiation 4
$CD4^+$ T-cell	Helper T- cells
$CD8^+$ T-cells	Cytotoxic T- cells
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GCDFP-15	Gross cystic disease fluid protein - 15
gp-17	Glycoprotein 17
HER2	Human epidermal growth factor receptor
HIV-1	Human immunodeficiency virus -1
hPIP	Human prolactin inducible protein
HRP	Horseradish peroxidase

IFN-γ	Interferon gamma
IL-12	Interleukin -12
mg	Milligram
MHC	Major Histocompatibility complex
MHC-II	Major Histocompatibility complex -II
ml	Milliliter
mPIP	Mouse prolactin inducible protein
ng	Nanogram
NK	Natural killer cells
PAGE	Polyacrylamide Gel Electrophoresis
PE	Phycoerythrin
pg	Picogram
PIP	Prolactin inducible protein
PIP KO	Prolactin inducible protein knockout mice
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMGP	Submandibular gland protein
E0771	Breast cancer cell line
E0771 EV	Empty vector control E0771
E0771 PIP	PIP expressing E0771
4T1	Breast cancer cell line
4T1 EV	Empty vector control 4T1
4T1 PIP	PIP expressing 4T1
TCR	T cell receptors

Th1	T- helper 1 subsets
Th2	T- helper 2 subsets
WT	WT mice

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1. BREAST CANCER

1.1. World and Canadian Statistics

Worldwide, the most frequently occurring cancer in females is breast cancer, and it is the second most common cancer overall (1). In 2018 alone, 2 million new cases of breast cancer were documented, representing approximately 1 in 4 cases of cancer in women, with an overall incidence rate slightly inferior to that of lung cancer (2). In the last 40 years, the rate of breast cancer occurrence was higher in women above 50 years, while women below 50 years had a decreased rate of survival (3).

In Canada, breast cancer is the third most common cancer diagnosed overall, constituting 13% of all cancers reported. Similar to world statistics, breast cancer is the most commonly diagnosed cancer in Canadian women, making up about 25% of all female cancers (4). In 2017, over 26,000 Canadian women were diagnosed and 5,000 succumbed to the disease (4,5). Despite these figures, the current overall five-year net survival rate for female breast cancer is relatively high at 87% (6). However, survival varies considerably by stage ranging from 22% survival for stage IV to nearly 100% survival for stage I. Breast cancer can often lead to significant economic burdens on affected women, their families, and society. In accordance with this concept, a US study has estimated the annual cost of breast cancer care to be $16.5 billion (7).

1.2. Breast Cancer Subtypes

Histologically, breast cancer can be broadly grouped into lobular carcinoma and ductal carcinoma categories, depending on whether the cancer affects the lobules or ducts of the breast (8). These two histological subtypes can be further divided into *in situ* carcinomas (lobular carcinoma *in situ*, LCIS, and ductal carcinoma *in situ*, DCIS) or invasive carcinomas (invasive lobular carcinoma, ILC, and invasive ductal carcinoma, IDC) (8). *In situ* carcinomas are pre-cancerous lesions that have not invaded the surrounding basement membrane, while invasive breast carcinomas have invaded the basement membrane and spread to surrounding breast tissues (8). IDCs are the most common type of invasive breast cancer. As well, there exists other rare forms of breast cancer such as inflammatory breast cancer and Paget disease (9). Inflammatory breast cancer is a highly aggressive type that can affect the lymphatic system thereby obstructing the lymphatic drainage, while Paget disease typically affects the nipples (8,9).

Breast cancer is categorized into 5 major intrinsic molecular subtypes based on gene expression profile studies (10). These subtypes are: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) over-expressing, basal-like/triple negative and normal-like tumors (10,11). However, more recently, a claudin low type has also been identified (12,13). Furthermore, a recent study conducted in conjunction with the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) group has identified at least 10 subtypes based on a combination of gene expression profiles and copy number aberrations (14).

The most commonly diagnosed breast cancer subtypes (more than 60%) are the luminal A and the luminal B (15). They are characterized by estrogen and progesterone receptor expression (ER, PR) but differ in their levels of HER2 and Ki67 (a nuclear protein, which operates as a cellular proliferation marker) expression (15). Luminal A breast cancers are HER2- and $Ki67^{low}$ (16),

whereas, the luminal B subtype may be HER2+ or HER2-, and $Ki67^{high}$ (15,17,18). Compared to luminal B, patients with luminal A breast cancers have better outcomes as it is usually of low to moderate grade with low recurrence rate. The luminal B subtype produces a worse prognosis because its tumors tend to possess mutations in the tumor suppressor gene known as p53. The expression of HER2 and/or Ki67 in breast cancer has also been reported to contribute to its rapid growth, resulting in bigger tumors and a higher tumor grade (19).

Accounting for approximately 20% of cases, the HER2+ subtype of breast cancer is aggressive and has a poor prognosis (20). The expression of HER2 protein has also been reported to contribute to the number of cancer stem cells (21), inducing angiogenesis, metastasis, and evading apoptosis. Basal-like tumors which do not express ER, PR and HER2, are characterized by the expression of cytokeratin and/or epidermal growth factor receptor (EGFR) (22). Triple negative breast cancers (TNBCs) are another class of tumors that are ER-, PR- and HER2-. Approximately 70% of TNBC's are basal-like. TNBC's constitute nearly 15% of all breast cancer cases, and at least 6 distinct subtypes with different gene expression profiles have been identified (23). It is the most aggressive subtype, with high risk and poor prognosis (24,25). Basal-like/triple negative breast cancer patients typically develop cancer at a younger age. These tumors are large and spread to distant organs (24). The normal-like subtype has been poorly defined in regard to the histological literature. However, using microarray studies, it has been shown that normal-like breast cancers express genes of tissues that are not of epithelial origin such as genes from adipose tissue (10). The normal-like breast cancer subtype accounts for 6-10% of diagnosed patients, has a good prognosis and their tumors are usually small in size (15).

1.3. Breast Tumorigenesis, Progression and Metastasis

Both genetic and epigenetic alterations in normal breast cells can result in their transformation into cancerous cells that are highly proliferative (hyperplasia) (26). These alterations subsequently lead to the formation of *in situ* breast carcinomas which are characterized by the presence of potentially malignant cells that have not yet invaded the basement membrane (27). In addition to cancer cells, the breast tumors contain stromal cells such as leucocytes, fibroblasts, and endothelial cells (26). These stromal components exhibit an important role in modulating breast tumorigenesis, progression, and metastasis. This may occur by a direct interaction with cancer cells or by the indirect release of bioactive molecules such as cytokines and growth factors. *In situ* carcinomas can become invasive when they penetrate the basement membrane and gain access to the circulatory system in a process known as intravasation. When they enter the circulation, these cancer cells are then referred to as circulating tumor cells (CTCs) which have been demonstrated to be good indicators of metastasis and relapse (28). To arrive at the metastatic site, cancer cells move out of circulation (blood vessel or lymphatics) to distant organs such as the lungs, brain, bone, and liver. The cells are deposited at these sites, forming metastatic foci (29). Metastasis accounts for about 90% of breast cancer deaths, as most primary tumors do not adversely affect patient survival if confined to the breast. The precise mechanisms of metastasis are still not well understood. However, one hypothesis is that some metastatic cells may survive at the distant organ, proliferate and induce angiogenesis to enhance their survival while evading apoptosis and antitumor immune activity (30).

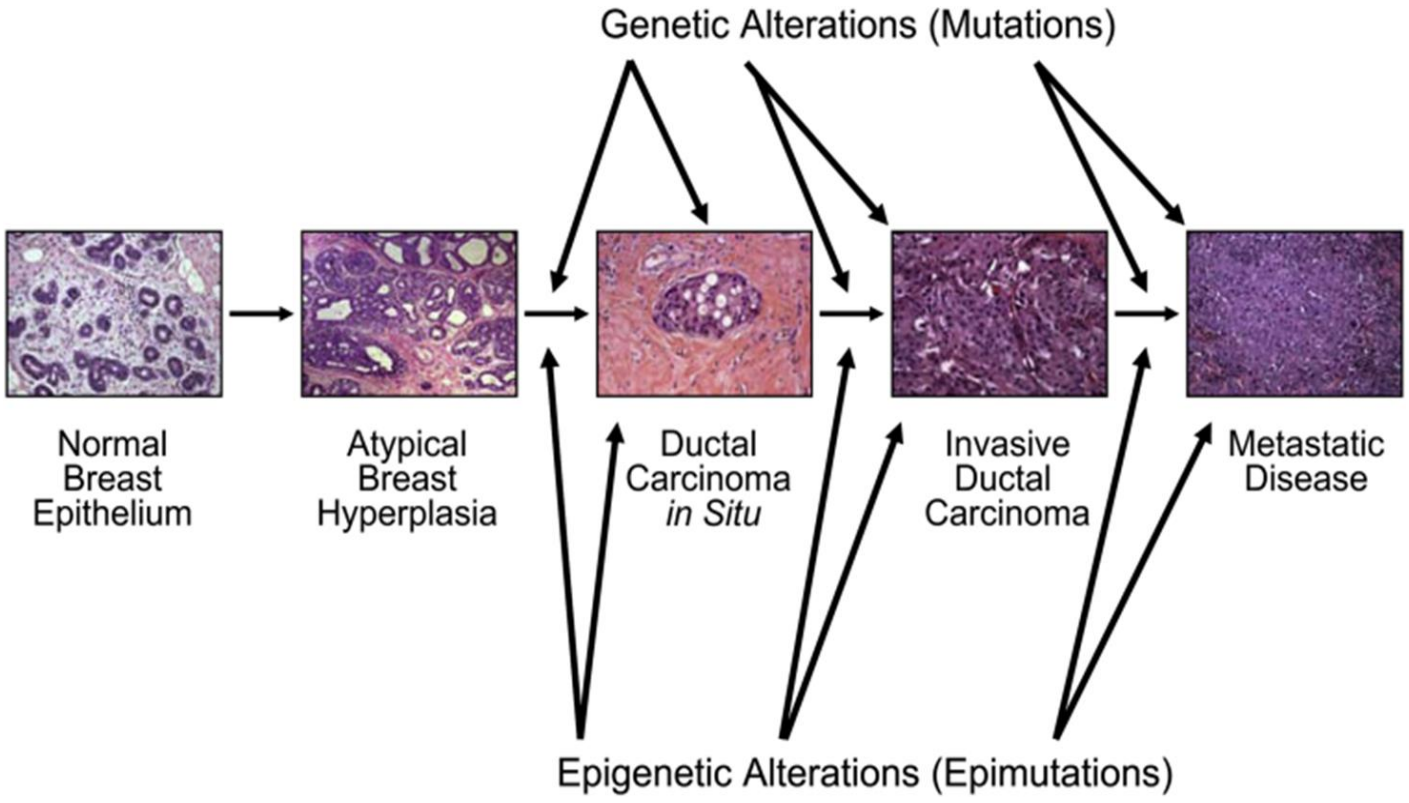

Fig 1. Proposed stages of breast cancer progression (Rivenbark, 2013)

1.4. Breast Cancer Risk Factors

Several factors can significantly contribute to the risk of breast cancer development. According to the Centre for Disease Control and Prevention (CDC), being female is a significant risk factor as the proportion of breast cancer occurrence in males is less than 1%. Other risk factors are old age, a history of breast cancer occurrence in the family, prior exposure to radiation therapy, and genetic mutations, especially in the breast cancer (*BRCA*) genes (31). Environmental and lifestyle factors are also considered to significantly contribute to breast cancer development. It is known that breastfeeding has the potential to decrease the risk of breast cancer onset (32). As well, it has been reported that in postmenopausal women, the administration of certain forms of hormone replacement therapy can elevate their risk of developing breast cancer (33). According to some studies, to reduce the risk of breast cancer development, women can engage in physical activity, limit or avoid alcohol intake, and control their weight (34).

1.5. Breast Cancer Diagnosis, Grading and Staging

Signs of breast cancer include: detection of a mass in the breast, distortion of the breast architecture or asymmetry and the detection of microcalcifications in the breast by mammography. This may require further examination using a diagnostic mammogram, core needle biopsy, ultrasound or magnetic resonance imaging (35). Specimen from core needle biopsies are analyzed by pathologists to determine the grade of the breast cancer. Grading informs on the degree of differentiation of the breast cancer cells and how likely they are to grow and spread. It is evaluated based on glandular formation, morphology of the nucleus and mitotic counts. Each criterion is given a score from 1-3. These scores are then added and grouped into three classes: grade I (low) has scores from 1-3; grade II (intermediate), 6-7; and grade III (high), 8-9 and patients with higher

grade tumors often have worse outcomes (36). In addition, breast cancer, like several other types of carcinomas, are classified into stages, which is a measure of the size of the tumors and the degree of metastasis. Breast cancer can be staged according to the tumor, node, metastasis (TNM) system which considers tumor size (T), whether it has spread to lymph nodes within the vicinity (N) or if it has spread to distant organs (metastasis) (37). Although larger tumor sizes are correlated with worse prognosis, some smaller tumors may be more aggressive than larger ones. In addition, occurrence of more lymph node metastasis is associated with more advanced breast cancers. Based on the TNM system, breast cancer is categorized into stages 0-IV, with subcategories within (38) and the disease severity increases alongside the stage number. Stage 0 is a breast carcinoma *in situ*; stages I-III are invasive carcinomas with increasing size, degree of spread and severity; and stage IV is established when the breast cancer has metastasized to distant organs (37).

1.6. Role of Hormones

Almost 70% of breast cancers are considered to be hormone dependent (39). It has been shown that hormones produced by the ovaries and pituitary glands contribute significantly to the normal growth and development of the breast and has been implicated as well in breast tumorigenesis (39). The hormone, estrogen, produced by the ovaries, is important for normal breast development. It interacts with the estrogen receptor, leading to a series of signalling events that result in the transcription of a wide array of genes which regulate the cell cycle, DNA replication, apoptosis and cellular differentiation (40,41). There are two estrogen receptors, ER-α and ER-β. ER-α was previously thought to be the primary receptor for estrogens until ER-β was discovered (42,43). They are both members of the steroid/thyroid/retinoid family of receptors and share similar structure and function (44,45). Furthermore, several ER-β isoforms have been identified, where ER-β1 is present as the major form. Some studies suggest that higher expression of ER-β1

is associated with enhanced response to anti-estrogen treatment as well as a more favourable prognosis (46–48). Estrogens are derived from androgens such as androstenedione and testosterone through the action of the aromatase enzyme (41).

Progesterone is another important hormone involved in normal breast development as well as in breast cancer (49) and its receptor is usually expressed along with the estrogen receptor. The progesterone receptor is often expressed more in the luminal A subtype and is correlated with better outcome (50). Although it has been reported that pituitary hormones such as prolactin and growth hormone influence the development of breast cancer, their mechanisms of action are still poorly understood (39). A recent study (51) reported that higher levels of prolactin in the plasma is linked to an increase in the risk breast cancer development. As well, it has been shown that there is an association between prolactin and metastasis to the bone (52). Growth hormone on the other hand, has been reported to contribute to angiogenesis, cancer stem cell formation and resistance to chemotherapy in breast cancer (39).

1.7. Breast Cancer Treatment

Common therapies for the management of breast cancer include surgery, radiotherapy, and systemic therapies such as targeted therapy (including hormonal therapy) and chemotherapy (53). These therapies may be used alone or in combination. Furthermore, the treatment option(s) is determined based on the subtype or stage of the breast cancer and can be surgery alone, or in combination with neoadjuvant and/or adjuvant therapy such as chemotherapy, radiotherapy and hormone therapy (53). Neoadjuvant therapy refers to therapies administered prior to surgical procedures. On the other hand, adjuvant therapy is provided after the surgical procedure. Common surgical interventions against breast cancer include lumpectomy and mastectomy (53). While

lumpectomy refers to the removal of the breast tumor only and nearby tissues, mastectomy involves the removal of some portion of, or the complete breast(s) affected (53).

A major breakthrough in breast cancer treatment was the development of selective estrogen receptor modulators (SERMS) such as tamoxifen and fulvestrant, which target the estrogen receptor (54). Since more than 60% of breast cancer patients have ER+ and PR+ tumors, this is of tremendous benefit for many breast cancer patients (54). Tamoxifen, an estrogen receptor antagonist, has a relatively low affinity for the estrogen receptor on its own compared to estradiol (an estrogen). However, upon consumption, it is metabolised by liver enzymes to the active forms known as afimoxifene (4-hydroxytamoxifen) and endoxifen (N-desmethyl-4-hydroxytamoxifen), which have a higher affinity for the estrogen receptor and subsequently control the growth of breast cancer (55,56). Tamoxifen is currently one of the most commonly available drugs for breast cancer treatment across the globe. It has saved countless lives, and is on the WHO list of essential medicines due to its role as the first line of treatment for patients with ER+ breast cancer and even for prevention (57). There is yet another class of drugs known as aromatase inhibitors which target the aromatase enzyme (responsible for producing estrogens from androgens) especially in post menopausal women with breast cancer. Examples of aromatase inhibitors include letrozole, anastrozole and exemestane.

The monoclonal antibody, trastuzumab, is often used in the treatment of patients with HER2+ breast tumors (58). Trastuzumab, also known as Herceptin, binds to the HER2 protein thereby blocking its action and controlling breast cancer growth. To a lesser degree, other targeted therapies including inhibitors of mechanistic target of rapamycin, mTOR; cyclin dependent kinase, CDK 4/6; phosphoinositide 3-kinase, PI3K and poly-ADP ribose polymerase, PARP, are used for treating breast cancer patients (59). For chemotherapy, cyclophosphamide, methotrexate and 5-

fluorouracil (CMF) were used but epirubicin and cyclophosphamide and paclitaxel (EC-paclitaxel) is the current standard of treatment. These regimens are used alone or in combination with other modes of treatment (60).

2. MOUSE MODELS USED IN BREAST CANCER RESEARCH

Mouse models are commonly used in preclinical breast cancer research because of the biological similarities between mice and humans. They are used to address the underlying mechanisms of breast tumorigenesis, progression and metastasis *in vivo*. In addition, they are useful for studying the tumor microenvironment, including tumor-immune system and tumor-stromal cells interaction, as well as for preclinical testing of anticancer drugs (61). These models can be grouped into four categories:

2.1. Cell Line Xenograft Models

These mouse models of breast cancer are generated by transplanting human derived breast cancer cell lines into immunocompromised mice. Cell line xenograft mouse models are commonly used in breast cancer research for preclinical drug studies (61). In addition, because several immortalized human breast cancer cell lines are well established and mimic many molecular subtypes, cell line xenograft models have been extensively used to delineate the mechanisms of breast cancer progression and metastasis (61). The advantages of using cell line xenograft models include simplicity, low maintenance cost and ease of deployment for studying breast cancer in the context of different subtypes (61). However, there are limitations. Firstly, in cell line xenografts, since an immunodeficient mouse host is used, the effect of the immune system on cancer development and response to therapy cannot be addressed. Secondly, tumor cells are implanted into mice by subcutaneous injections and do not accurately model the tumor microenvironment.

Thirdly, the human derived cell lines are from different species and the tumors formed do not properly reflect the heterogeneity observed in a clinical human breast cancer setting. Fourthly, immortalized cell lines used in this model are propagated through multiple passages during which they would have undergone significant changes that differentiate the daughter clones from parental cells (62). Altogether, the cell line xenograft model may not adequately mimic the original cancer biology of the patient, and these limitations of cell line xenograft models are often implicated as a reason for failure especially when preclinical drug testing and clinical results are incompatible (63). However, cell line xenografts still play an important role as the advantages outweigh the liabilities (64).

2.2. Patient-Derived Xenograft (PDX) Models

PDX or patient derived xenograft models are developed from patient-derived tumors that are surgically implanted into mice. Although the development of PDX models of breast cancer has been difficult (65), PDX models that mimic several subtypes of breast cancer have been successfully generated (66). PDX models have several advantages including genetic diversity and its heterogenous nature which better mimic breast cancer in humans. It is capable of modeling many subtypes of human breast cancer, stromal components and metastasis (61). Other advantages include maintenance of the biological properties of the tumor cells because they are directly implanted unlike immortalized cell lines that go through several passages. Importantly, it has been observed that it is possible to maintain the characteristics of the patient derived xenografts by "passaging in mice" for several generations, and they retain clinical responses to several therapeutic agents, making them suitable for co-clinical studies (61). However, they have several limitations including: a) use of a severely immunodeficient murine host; b) highly technical and invasive surgical procedure required for implanting tumors into mice (67); c) differences in

species between implanted tumors and host; d) long time required to generate these models (68); e) relatively expensive infrastructure and personnel required for developing this model.

2.3. Genetically Engineered Mouse Models (GEMMs)

These mice represent the most advanced *in vivo* models used to mimic human cancer. They are also able to model the series of events that result in cancer formation. Mammary tumors can be formed in GEMMs by specifically inducing the expression of an oncogene in the mouse breast epithelium (61). Hence, this model is clinically relevant and demonstrates the pathogenesis and progression of breast cancer. Another important feature is that the host stromal environment and immune system are intact. The genetic modifications that control the expression of the oncogene can be permanent or reversible as well as global or tissue-specific (64). GEMMs may lack tumor suppressor genes such as p53 or possess oncogenes such as HER2, as observed in human breast cancers. In the past several years, a wide variety of oncogenes have been used to generate transgenic models of specific molecular subtypes of breast cancer (69). As well, in GEMMs, the tumor develops in the appropriate tissue with the immune system and the appropriate tumor-stromal interactions present, thus making it a better model. However, the use of GEMMs is limited by the considerable time, cost, and resources needed to generate and maintain them. In addition, although there are similarities between mouse and human luminal breast cancer, few GEMMs which develop breast cancer are ER+. Another important difference is that mouse breast cancers usually metastasize to the lungs through the blood, whereas human breast cancers can also spread through the lymphatic system (61).

2.4. Syngeneic Mouse Models

In order to adequately model cancer immunology, it is important that the cancer is developed in an immunocompetent host. For breast cancer, this typically involves the implantation of mouse breast tumors or cell lines into syngeneic mice which possess an intact immune system (i.e. immunocompetent). Since the cell lines and mouse strains are syngeneic (compatible), there is no tissue rejection. One important advantage of syngeneic models is that breast cancer biology can be studied in the presence of a competent immune system. A major drawback is that such mouse tumor cell lines are limited. In addition, some are not well characterised (61). Two examples of syngeneic mouse models are the 4T1 and E0771 models (61).

2.4.1. The 4T1 Mouse Breast Cancer Model

2.4.1.1 4T1 Cell Line

Originally isolated from a spontaneous breast tumor (called 410.4) derived from the MMTV+ BALB/c mouse which was nursed by C3H foster mother (BALB/BfC3H) (70), the 4T1 cell line is 1 of 4 sublines obtained from this 410.4 tumor. 4T1 is a transplantable mouse breast cancer cell line that can be grown *in vitro* as well as in BALB/c mice (70,71). It is a highly aggressive triple negative breast cancer cell line and is capable of spontaneous metastasis to several distant organs such as the lungs, blood, lymph nodes, liver, bone and brain. 4T1 cells are resistant to the drug, 6-thioguanine, thus, it is possible to detect micro-metastases in distant organs more accurately than most other cancer models (72).

2.4.1.2. The 4T1 Model of Breast Cancer

In order to generate the 4T1 transplantable mouse model, the 4T1 cells are injected into the mouse mammary gland. These implanted cells are very tumorigenic and model stage IV of human

breast cancer (72). In addition, they are minimally immunogenic therefore the occurrence of inflammation upon tumor cell injection is restricted. 4T1 mouse breast cancer cells also grow into a primary tumor that becomes palpable after a few days after implantation. The progression of the disease may be monitored with relative ease by obtaining measurements of the primary tumor dimensions using calipers. The primary tumor metastasizes in a similar way to that of human breast cancer (72). Collectively, these features of the 4T1 mouse model make it suitable for studying breast cancer.

2.4.2. The E0771 Model of Breast Cancer

2.4.2.1. E0771 Cell Line

E0771 medullary mammary carcinoma cells were originally isolated from a spontaneous tumor and are syngeneic to C57BL/6 mice (73). These cells grow readily when injected subcutaneously and studies indicate that they are estrogen receptor positive (74).

2.4.2.2. The E0771 Mouse Model

The E0771 model mimics early stage human breast cancer. It is also less aggressive and less metastatic compared to the 4T1 model. It has been shown that this model is poorly immunogenic and may even be immunosuppressive (74). Mice injected with E0771 cells developed tumors which had histological features similar to human medullary breast cancer (74). These features include recognizable borders, undifferentiated cells and necrotic centers surrounded by leucocytes. Also, by evaluating the growth rate over the linear portion of the tumor growth curve, the estimated doubling time for E0771 tumors is 7 days (74). Like the 4T1 model, this model has been well studied and tumor growth and progression are relatively easy to monitor.

3. THE IMMUNE SYSTEM

The immune system is made up of many organ systems, cells and the soluble bioactive molecules they produce, that recognize and defend the host against foreign proteins or antigens (75). The immune system can be grouped into two main arms: the innate, which is immediate and non-specific; and the adaptive, which is specific and long lasting (75).

3.1. The Innate Immune System

The innate immune system confers the first line of protection against invading organisms (76). It can distinguish between "self" and "non-self" via toll-like receptors (TLRs) that can detect specific pathogen or danger associated molecular patterns (PAMPs or DAMPs) (75). In addition, the innate immune system exerts its abilities through proteins of the complement system as well as through cytokines (75). Cytokines modulate different effects depending on which cells secrete them, where they are secreted, where their receptors are located, and the signaling pathways that are activated following their binding to the receptor (77). The complement proteins are activated by three major signaling pathways: the classical, alternative, and lectin pathways. Upon activation, these proteins mediate several effector functions such as opsonization, recruiting other immune cells, and killing cells/pathogens by forming a membrane attack complex (MAC) for lysis (78). Phagocytes and natural killer (NK) cells are important cellular components of the innate immune system. The phagocytes such as monocytes, neutrophils and macrophages are able to engulf cells expressing foreign or abnormal self antigens and kill them in a process called phagocytosis (79). Natural killer cells secrete perforin and granzyme which kill cells that possess an abnormal major histocompatibility complex (MHC) class I or human leukocyte antigen (HLA) as a result of pathogens or oncogenic mutations (79). Mast cells, basophils and eosinophils release molecules

that mediate inflammation such as chemotactic leukotrienes which attract immune cells to the inflammation or injury site (75). There is yet another class of cells referred to as NKT cells that have features of NK cells and T cells (79) .

3.2. The Adaptive Immune System

The adaptive immune system has high specificity and leads to the development of immunological memory (75). This form of immune response is prolonged because upon encounter with the appropriate antigens, naïve B and T lymphocytes need time to differentiate and mature into plasma cells (antibody producing B cells) or effector T cells respectively (75). T cells can be classified into $\alpha\beta$ T cells and $\gamma\delta$ T cells depending on the type of receptor they possess. (80). $\alpha\beta$ T cells, like $CD4^+$ T cells and $CD8^+$ T cells, require MHC mediated antigen presentation (80) whereas $\gamma\delta$ T cells are able to recognize "non-self" antigens by pattern recognition (81). Maturation of naive $CD4^+$ T cells into effector cells requires co-stimulation between the T cell receptor and MHC class II found on antigen-presenting cells like dendritic cells, B cells and macrophages (82,83). $CD4^+$ T cells are able to differentiate into different effector subsets such as T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) or regulatory T cells (Tregs). This process depends on the transcription factors and cytokines that are present (82). These $CD4^+$ T cell subsets produce and secrete immunomodulatory cytokines (82). Unlike $CD4^+$ T cells which rely on MHC class II, naïve $CD8^+$ T cells depend on MHC class I in order to mature into effector cytotoxic T lymphocytes (83). The binding of activated $CD8^+$ T cells via their receptors to the MHC class I-antigen complexes on target cells leads to the release of granzymes and perforin from the $CD8^+$ T cells which kills the target cells (83). B cells secrete several antibodies which are highly specific (75) and act through various mechanisms such as neutralization of antigens, induction of complement proteins and antibody-dependent cell cytotoxicity (ADCC) (84).

3.3. Regulation of Immunity during Cancer Development

The immune system has been shown to be important during the initiation and progression of cancer (85). In the initial stages of cancer development, cancerous cells are detected and eliminated by the innate immune system through a mechanism called immunosurveillance (85). The abnormal growth of cancer cells can activate neighbouring cells which secrete tissue damage signals such as IFN-γ which in turn activate and recruit NK cells, the primary drivers of immunosurveillance (85). It has been proposed that these processes control the appearance of cancers. Furthermore, through cancer immunosurveillance, the host immune system exerts pressure on a developing tumor, often eradicating cancerous cells before a tumor is established (86). However, this same immune pressure is believed to influence tumor development and select for certain mutations thereby creating an immune-evasive cancer.

The interaction between the immune system and cancer cells proceeds in three phases referred to as the "three Es" of cancer immunoediting. These phases include elimination, equilibrium and escape (Fig. 2). First, the immune system may initially succeed in destroying all tumor cells. This is the elimination phase. If this does not occur, it may still be possible to control tumor growth but not completely eradicate it. This constitutes the equilibrium phase. Finally, in the escape phase, selection pressure from the immune system can lead to the development of resistance by some cancer cells such that they "escape" detection and/or elimination by the immune system, resulting in a failure of immune-mediated cancer control (87). Cancers develop resistance by expressing reduced levels of MHC 1 and costimulatory molecules. They can produce factors that supress the immune system which enables them to avoid immune recognition (88). Paradoxically, these immunosuppressive mechanisms may be required for the mammary glands to develop and function normally (89,90). However, cancer cells can also utilize these same

mechanisms to avoid detection by the innate immune system and promote tumorigenesis. Thus, the tumor microenvironment becomes immunosuppressive and incapable of stimulating a potent adaptive immune response (91).

The current notion of the opposing roles of immune cells in tumours is that $CD8^+$ T cells, $CD4^+$ Th1 cells, NK cells, type 1 NK T-cells, M1 macrophages and mature DCs contribute to tumor elimination. In contrast, immature DCs, $CD4^+$ Th2 cells, type 2 NKT cells, $CD4^+$ T regulatory (Tregs) cells, MDSC, alternatively activated (M2) macrophages are pro-tumorigenic (92–95). Generally, patients who have tumors with a Th2 cytokine profile have worse prognosis than patients with a Th1 or cytotoxic T lymphocyte (CTL) cytokine profile (96).

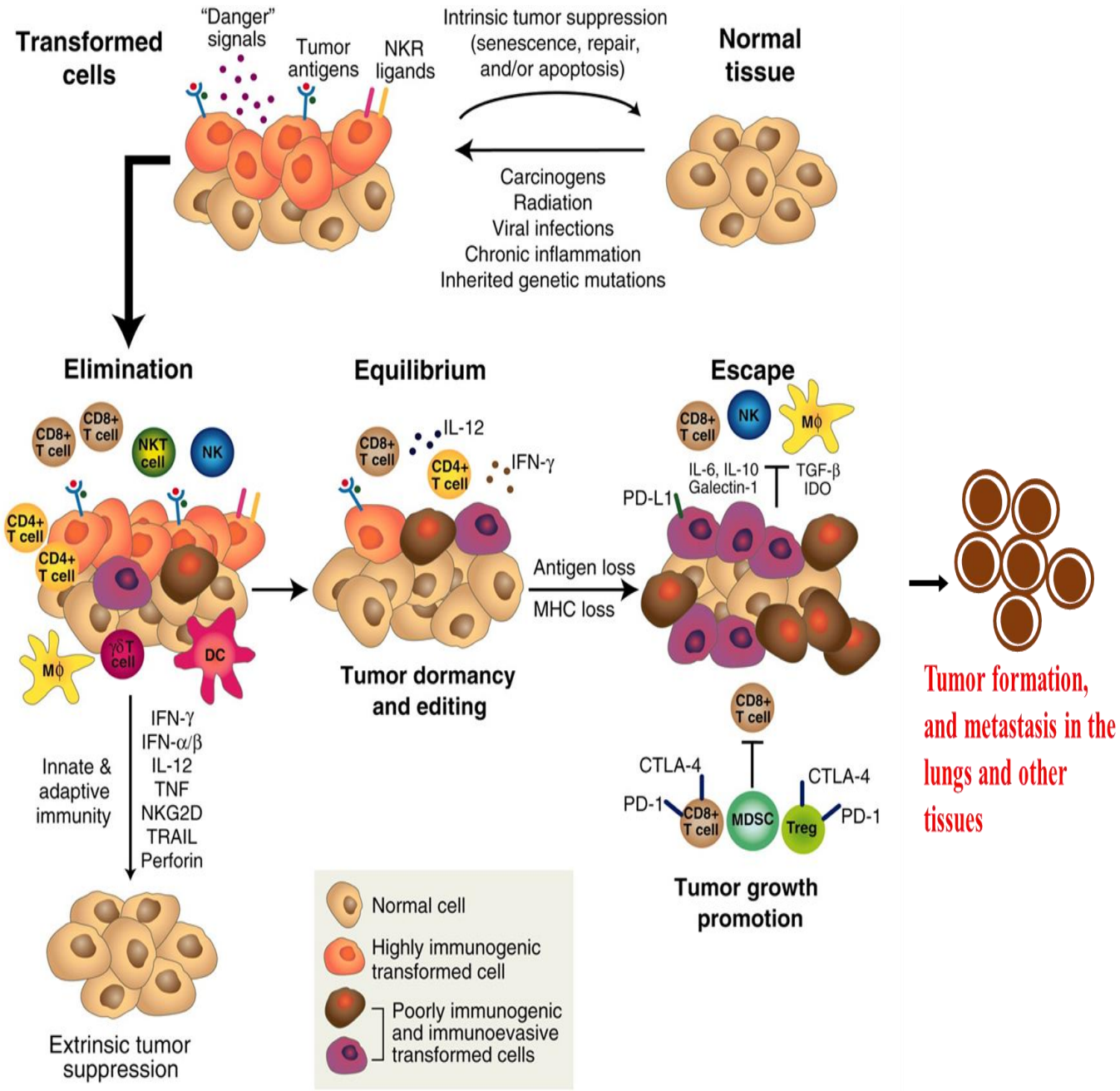

Fig 2. Cancer immunoediting (adapted from Schreiber *et al*. 2011)

4. IMMUNOTHERAPY

The activation of the immune system for therapeutic benefits, termed immunotherapy, is effective in treating several cancers especially under experimental conditions. This concept relies on the fundamental discovery that cancer cells express specific tumor-associated antigens, that may elicit measurable and occasionally protective humoral and/or cellular immune responses. The overall concept and key steps in the development of cancer immunotherapeutic strategies are focused at recruiting the host's immune cells and tailoring their ability to identify and destroy tumor cells in an antigen-specific manner.

The ability to stimulate tumor immunogenicity by converting a low tumor-infiltrating lymphocyte (TIL) tumor to a high TIL tumor is an active area of research in cancer immunology. Changes in TIL levels and composition have been monitored in mouse models and samples from patients after chemotherapy (97,98). This increased TIL infiltration is proposed to be stimulated by immunogenic cell death or apoptosis, which leads to the release of tumour neoantigens thereby resulting in enhanced antigen uptake and presentation by dendritic cells (99,100). These observations have led to studies on the development of therapeutic strategies where conventional anticancer chemotherapies are combined with a class of immunotherapeutic agents called checkpoint inhibitors (101).

4.1 Types of Immunotherapy

4.1.1. Checkpoint Inhibition

The immune system has evolved to possess several immune suppressive mechanisms such as immune checkpoints, which protect against autoimmunity by promoting tolerance to self antigens. Tumors exploit these immunosuppressive mechanisms to weaken antitumor responses

thereby escaping detection and elimination by the immune system (102). Checkpoint inhibitors are antibodies that bind to checkpoint molecules leading to their deactivation. Ipilimumab (YervoyR, Bristol-Myers Squibb, NY, USA) was the first immune checkpoint inhibitor to undergo clinical trials. Ipilimumab targets a checkpoint molecule known as cytotoxic T lymphocyte associated protein-4 (CTLA-4) and gained approval for the management of melanoma (103). CTLA-4 is an inhibitory receptor expressed on T cells which interacts with its ligand, suppresses T cell activation, and hence immune response (102). In breast cancer, anti-CTLA-4 treatment was used for the first time to treat advanced ER+ breast cancer patients. In this study, the researchers combined anti-CTLA-4 immunotherapy with tremelimumab and exemestane and observed an elevation in the levels of peripheral $CD4^+$ and $CD8^+$ T cells which express inducible co-stimulatory molecules (ICOS) thereby suggesting a role for anti-CTLA-4 inhibitors in breast cancer treatment.

Expression of another check-point molecule, programmed death ligand (PD-L1) on breast tumors cells and stromal cells has been studied in depth using pathological specimens (95). PD-L1 interacts with its receptor, PD-1, expressed on T cells, and causes inactivation or exhaustion of the T cells. Many early phase clinical trials using anti-PD1 and anti-PD-L1 monotherapy have indeed shown therapeutic potential (101).

4.1.2. Combination Therapy

Due to the relatively low response rate for checkpoint blockade monotherapy, it became necessary to enhance the response by combining treatments that increase immunogenicity with those that relieve mechanisms of immune escape. Several promising clinical trials are in progress where conventional anticancer treatments are combined with anti-PD1/PD-L1 checkpoint blockade (101). Recently, trastuzumab is used with checkpoint blockade to treat HER2+ breast cancer. Additionally, the administration of T-DM1 (Trastuzumab emtansine) and checkpoint

blockade was shown to lead to improved therapeutic efficacy in an animal model with primary resistance to immunotherapy (104). Checkpoint blockade may also have synergistic effects when combined with kinase inhibitors in targeted treatments. MEK (MAPK/ERK Kinase; MAPK, Mitogen Activated Protein Kinase; ERK, Extracellular Signal-Regulated Kinases) inhibition has been shown to reduce immune suppression in animal models of TNBC which exhibit mutations in the Ras-MAPK pathway (105). Therefore, MEK inhibitors may be combined with anti-PD1/PD-L1 checkpoint inhibition in patients with TNBC, displaying similar features as the animal model (101).

4.1.3. Emerging Immunotherapies

4.1.3.1. Adoptive cell transfer

While checkpoint inhibitors are used to re-vitalise exhausted immune responses that already exist, techniques such as adoptive cell transfer are treatment strategies used for expanding tumour-specific T cell populations. In a ground-breaking study conducted at the National Institutes of Health (NIH), a human patient with metastatic breast cancer was screened for cancer specific mutations (106). The TILs specific for these mutations were isolated, expanded *ex vivo*, and adoptively transferred to the patient together with IL-2 and pembrolizumab (a checkpoint inhibitor). Interestingly, this treatment led to complete and durable disease regression in the patient with metastatic breast cancer (106). In another study, chimeric antigen receptor (CAR)-T cell therapy which targets MUC1, a tumor antigen, was shown to be effective against breast cancer in mouse models (107).

4.1.3.2. Vaccination

Another emerging immunotherapy is vaccination. Several cancer vaccines have been developed from different immunogenic sources such as DNA, RNA, viruses, lysates from the tumor and tumor antigenic peptides. These vaccines may be used in combination with immunoadjuvants like granulocyte monocyte colony stimulating factor (GM-CSF). Reports from clinical trials with vaccines targeted against HER2+ breast cancer and other highly expressed antigens like hTERT (human telomerase reverse transcriptase) have been encouraging (108).

5. BIOMARKERS OF BREAST CANCER

ER, PR, HER2 are major biomarkers of breast cancer and have prognostic, predictive as well as therapeutic significance (109). ER and PR have played an essential role to select breast cancer patients who will benefit from endocrine therapy such as tamoxifen (110). HER2 has been demonstrated to be a prognostic indicator as well as a predictor of response to the anti-HER2 antibody, trastuzumab (109). Ki67 is applied in the determination of prognosis and neoadjuvant therapy (109). Other biomarkers include cyclin D1, cyclin E, ERβ (109), cytokeratin, mucin 1, small breast epithelial mucin (SBEM), carcinoembryonic antigen (CEA), mammaglobin and the prolactin inducible protein (111). Not all these markers are specific to breast cancer alone, thus there is a need to identify markers that are more breast cancer specific, detect early breast cancer and determine the best course of treatment. Therefore, with the introduction of high-throughput technologies, a wide variety of signatures containing multiple genes have been developed which identify genes that can be targeted in combination with traditional markers such as ER, PR and HER2. For instance, advances in genomic technologies led to the development of genomic profiling of breast tumors depending on a set of genes which serve as additional biomarkers for certain breast tumors. Examples of such tests include Oncotype DX, MammaPrint and genome

grade index (109) and these tests have been shown to be useful in predicting patient outcome and choosing appropriate therapies (110).

5.1. The Prolactin Inducible Protein (PIP)

PIP is also a biomarker that has been associated with benign and malignant breast diseases (112). Previous work done by Shiu and Iwasiow (113) identified PIP as a glycoprotein produced by T47D, a human breast cancer cell line, in response to prolactin stimulation (114,115). PIP was also independently identified by Haagensen et al. (116) as gross cystic disease fluid protein 15 (GCDFP-15) which is a highly abundant acidic protein found in the fluid of gross cystic disease of the breast. It is currently used in the clinic to determine whether a metastatic cancer is of breast origin (117–119).

5.1.1. PIP in Breast Cancer

PIP expression is very low or undetectable in normal breast but elevated in breast cancer (114,117,120,121). Furthermore, RT-PCR studies showed that PIP mRNA was detected in 92% of primary breast tumors which also correlated with PIP detection by immunohistochemistry (122). The highest amounts of PIP mRNA were found in the luminal A subtype, intermediate amounts were found in HER2-enriched and normal-like subtypes, and the lowest expression was observed in basal-like subtype (123,124). PIP expression was found to correlate with low grade breast cancers (125). Further clinical evidence showed that breast cancer cases with high PIP expression were characterized by longer overall (125) and disease-free (125–127) survival. Collectively, these observations suggest that high expression of PIP is associated with good prognosis (122,128–130).

Additionally, recent studies (131) showed that high PIP expression (both at mRNA and protein levels) is positively correlated with positive response to standard adjuvant chemotherapy

(doxorubicin and cyclophosphamide) in a cohort of 120 invasive ductal carcinoma (IDC) cases. It was also reported that PIP expression was highest in ER+ breast cancer and lowest in TNBC (131). As well, it has been reported that the levels of PIP protein and mRNA reduces as the tumor becomes more malignant or high grade (131,132), and increased levels of PIP was observed in the peripheral plasma of breast cancer patients when compared to controls (114,116).

In another recent study, the authors reported that PIP mRNA levels are higher in early stage breast cancer samples compared to the late stage, and a downregulation of PIP mRNA in normal breast tissues compared to early stage breast cancer (133). In agreement with previous studies, this study also showed that PIP expression is lower in TNBCs compared to luminal subtypes (133). This study further supports the hypothesis that PIP expression is associated with the early stage, less aggressive breast cancers with better prognosis. Collectively, these studies suggest that PIP may be protective against breast cancer, especially at the early stages. PIP could also potentially enhance response to chemotherapy through direct or indirect mechanisms. Therefore, a better understanding of the precise effects of PIP expression on breast tumorigenesis and its influence on chemotherapy response is warranted.

Notably, the molecular apocrine subtype of breast cancers, a rare subtype of invasive ductal carcinoma that frequently shows an upregulation of the androgen receptor, have been shown to commonly express PIP (134–136). In this subtype of breast cancers, there is a positive feedback loop between PIP and the androgen receptor (137). It was observed as well that there is a decrease in PIP expression in the lymph node-positive and large apocrine tumors. As a result, it has been suggested that the expression of PIP is a temporary feature of these cancers, which is then lost during tumor growth (135).

5.1.2. Expression and Regulation

PIP is found in human submandibular/and sublingual saliva where it is called the extra parotid glycoprotein (138,139) and in human seminal fluid where it is known as glycoprotein 17, an actin binding protein (140). The PIP gene is 7kb long and has 4 exons (141). PIP is highly expressed in the lacrimal, salivary and sweat glands (112,142). PIP is a secreted protein and consequently found in tears, saliva, and sweat (114). PIP has also been found in the blood, fluids of the ear canal, amniotic fluid and breast milk (114,116,140,143). Cytokines and hormones regulate the expression of the PIP gene both in normal and pathological conditions of the breast. Specifically, androgens, prolactin, glucocorticoids, and progesterone upregulate PIP expression (113). In addition, PIP expression is influenced by IL-4 and IL-13 in breast tumors (144).

Several homologous sequences of human PIP have been identified in other mammalian species. Pioneering studies by our group identified PIP sequences in other species such as monkey, dog, cow, rabbit, chicken, and yeast using Southern blot analysis (143). It has also been reported that the human PIP gene sequence is highly similar to that of other primates such as chimpanzee, 97% (145); gorilla, 94% (145); orangutan, 93% (145); gibbon 90% (145); syndactylus 89% (145); and the Japanese monkey, 71% (146). Sequence similarities with other mammals are 52% with cattle (146), 61% with guinea pig (146), 55% with rabbit (146), 38% with rat (147) and 51% with mouse (148).

5.1.2.1. Mouse Prolactin Inducible Protein (mPIP)

PIP is also expressed in mice where it is known as mouse PIP or mouse submaxillary gland protein (mSMGP). It has a molecular weight of approximately 17kDa (148,149) and like human PIP, it is strategically expressed in tissues such as the skin, eye and ears, that serve as a means of access by pathogens into the body. The locus of the *PIP* gene is on chromosome 6q34 and it shares

a common structure with four other genes including seminal vesicle auto antigen (SVA) and SVAL (SVA–like) 1-3 (146). As well, mPIP displays high similarity in tissue specific expression with human PIP (hPIP). Furthermore, both human and mouse PIP function similarly (150), thus indicating that the mouse model is suitable for studying the role of PIP and may be translatable to humans.

5.1.3. Function

Several studies suggest that the role of PIP is multifaceted. Our group reported that PIP can bind in a specific manner to many bacteria in humans and mice, such as bacteria of the genus *Streptococcus*, which are found in the mouth (151). The binding promotes bacterial aggregation thereby potentially inhibiting further proliferation and colonization of the oral cavity (151). In addition, our group found that PIP is expressed in the mouse submaxillary gland at the early stages of embryonic development, suggesting that PIP may influence submaxillary gland development (151).

PIP is an aspartyl protease. It has an aspartate residue at position 22 (Asp22), which shows homology to the aspartate residue 32 of other aspartyl proteases, such as cathepsin D, pepsin or renin (152). It was also reported that PIP degrades fibronectin due to its aspartyl proteinase activity, thereby suggesting that PIP contributes to extracellular matrix degradation and breast cancer progression. PIP also interacts with several proteins including actin, fibrinogen, β-tubulin, serum albumin, zinc α2-glycoprotein and Fc fragment of immunoglobulin G (124,138,153–157). However, in most cases the biological role of such interactions is poorly understood (132).

In addition to the role of PIP in the inhibition of bacteria proliferation, infertility, prostate tumor progression, breast tumor progression and enamel pellicle formation (138), recent findings suggest that it is may also modulate innate and adaptive immunity (158).

5.1.4. The Role of PIP in the Immune System

5.1.4.1 PIP in Innate Immunity

PIP is found in mucosal type tissues, submucosal glands of the bronchi, saliva, apocrine glands present in the skin, and tears. This suggests that PIP may affect innate and mucosal immune response (159). *In vitro* studies have shown that PIP is capable of binding to bacteria *(Gemella, Staphylococcus* and *Streptococcus)* which colonize the oral cavity, skin and ear canal thereby inhibiting their growth (160).

5.1.4.2. PIP in Adaptive Immunity

Accumulating evidence from several studies suggests that PIP plays a critical role in adaptive immunity. Firstly, PIP has been reported to interact with the CD4 molecule on T cells. During T cell activation, CD4 molecules act as coreceptors in the interaction between the T cell receptor (TCR) and the MHC-II molecule (161). CD4 molecules are also the main viral receptors for HIV-1 (162,163) which facilitates viral entry into $CD4^+$ T cells. Furthermore, CD4 interacts with glycoprotein 120 (gp120), on the HIV-1 envelope and facilitates viral attachment and entry (164). It has also been shown that when PIP binds to the first domain of the CD4 molecule, it leads to a change in conformation of CD4 which interrupts the attachment of HIV retrovirus to $CD4^+$ T cells and subsequent events (165). It is known that HIV infection leads to the loss of $CD4^+$ T cells thereby inducing a state of immunosuppression. The interaction between PIP and the CD4 molecule on T cells has been reported to shut the forced apoptotic pathway that would have occurred on HIV infection (166). This indicates that PIP can contribute to the maintenance of the host adaptive immune response by inhibiting $CD4^+$ T cell apoptosis.

Secondly, it has been shown that in human seminal plasma, PIP binds with high affinity to a region of CD4 known as the D1-D2 region (140,165). Therefore, it may be that the immunosuppressive abilities of the human seminal plasma are due to the interaction of PIP with CD4. It has also been shown that on exposure to IL-4 and IL-13, PIP mRNA levels are upregulated in ZR-75 breast cancer cells by 5.5 and 6.0 folds respectively (144). Since PIP binds to positions on the CD4 molecule which interact with MHC-II (167), it has been hypothesized that the elevation in PIP expression due to IL-13 and IL-4 regulates infiltrating $CD4^+$ T cell activity, thereby contributing to innate and adaptive immunity (144).

5.1.4.2.1. The Role of PIP in Type 1 T -Helper Cell Immune Responses

Results from studies using PIP KO mice generated in our laboratory showed that deficiency of PIP impairs $CD4^+$ T helper 1 response (155, 168). Over time, these PIP KO mice showed some immune-related pathologies such as enlarged submandibular lymph nodes, enlarged thymic medulla, and cardiac arteritis and peri-arteritis (170). When the immune phenotype was analyzed, the percentages of $CD4^+$ T cells in the spleens of PIP KO mice were significantly decreased when compared to the wild type controls (169). Furthermore, $CD4^+$ T cells obtained from KO mice were impaired in their ability to proliferate and differentiate into interferon gamma (IFN-γ)-producing type 1 T helper (Th1) cells, suggesting that PIP is important in the Th1 differentiation pathway (169). To further investigate these observations, PIP KO mice and the wild type controls were infected with *Leishmania major*, a parasite whose clearance is mediated by the Th1 immune response. Results from these studies showed that the PIP KO mice possessed elevated susceptibility to infection by *Leishmania major*. Our group also showed that this susceptibility was due to deficiencies in innate and adaptive immunity in PIP KO mice. Deficiencies in the immune response include reduction in IFN-γ and nitric oxide (NO) production by cells of the spleens and

lymph nodes of PIP KO mice (169). Specifically, the production of NO and proinflammatory cytokines (including tumor necrosis factor-alpha, TNF-α, IL-6 and IL-12) by IFN-γ-activated macrophages obtained from PIP KO mice were reduced. Further investigations showed that this reduction in proinflammatory cytokines and NO production by PIP KO macrophages was associated with defects in the intracellular signalling pathways involving MAPKs (mitogen activated protein kinases) as well as STAT (signal transducers and activators of transcription) proteins. In addition, an increase in the levels of suppressors of cytokine signalling (SOCS) proteins was observed in PIP KO mice (171). These SOCS proteins have been reported to contribute to the suppression of cell-mediated immunity (172). Altogether, these findings show the importance of PIP in adaptive immunity since the lack of PIP led to the impairment of some critical components of the adaptive immune response.

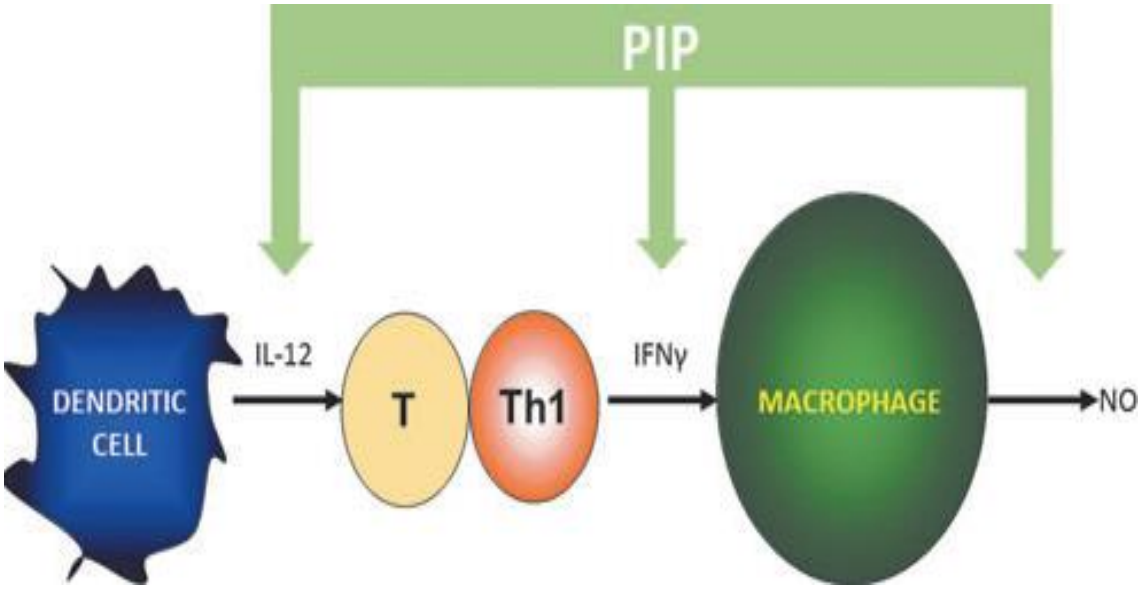

Fig 3. The role of PIP in Th1 immune response. Some steps in the Th1 immune response (indicated by the green arrow) have been shown to be impaired in PIP KO mice. Defects in the host Th1 immune response include (i) impaired polarization of naive T cells to IFN-γ-producing Th1 cells and (ii) impairment of macrophage response to IFN-γ. NO, nitric oxide; IFN-γ, interferon gamma; KO, knockout; Th1, T helper, type 1.

Revised from Ihedioha et al, 2016

II. STUDY RATIONALE

Our preliminary studies show that loss of PIP leads to defective Th1 activity (169). It has also been shown that the PIP expression in breast cancer is correlated with better prognosis (131). Since Th1 response has antitumor activity (87), PIP may be important in antitumor immunity during breast cancer development and progression

III. HYPOTHESIS

PIP expression inhibits breast tumor progression and enhances antitumor immunity

IV. STUDY OBJECTIVES

To investigate the effect of PIP on breast tumorigenesis, the following objectives were formulated:

1. To generate and characterize PIP expressing mouse breast cancer cell lines (4T1 and E0771) by lentiviral transduction techniques and conduct numerous functional assays on these cell lines.

2. Generate two transplantable mouse breast cancer models using the 4T1 and E0771 PIP expressing cell lines.

3. To assess the effect of PIP overexpression on tumor growth and development by evaluating tumor latency, growth and size, at experimental endpoint compared to control.

4. Assess the antitumor immune response and metastasis using a combination of strategies: flow cytometry, clonogenic assays and histological analysis.

V. MATERIALS AND METHODS

1. Cell Lines

The 4T1 cell line was acquired from American Type Culture Collection (ATCC, VA, USA) while the E0771 cell line was acquired from CH3 Biosystems (NY, USA). Both cell lines were cultured at 37ºC in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/l glucose, Hyclone Laboratories Inc., UT, USA) containing 2 mM glutamine, 10% fetal bovine serum (FBS), 50 μg/ml streptomycin, 50 U/ml penicillin (all obtained from Hyclone Laboratories Inc.) and 10 μg/ml bovine insulin (Sigma-Aldrich Canada, ON, Canada). The cells were passaged every 2-3 days or at 85-100% confluency. For passaging, the cells were harvested as follows: the T25 tissue culture flask (Thermofisher, NY, USA) was rinsed with 2-3ml 1x phosphate buffered saline (PBS) and treated with 1ml 0.05% Trypsin-EDTA (Gibco, Grand Island, NY, USA) for 5 minutes to detach the cells. Four ml of complete DMEM was then added. The cell suspension was pipetted 3-5 times to facilitate cell dispersion. The cells are then passaged by plating in a new T25 flask at 10% confluence.

For freezing, cells were harvested as described above and centrifuged at 700 rpm (IEC Centra-GP8R, International Equipment Company, MA, USA) for 5 minutes at room temperature. The cell pellets are then resuspended in freezing media (5% dimethyl sulfoxide, DMSO in complete DMEM) and aliquoted into cryopreservation vials (1ml/vial). These vials are placed in a Styrofoam box and kept in the -80ºC freezer for at least 3 days before long term storage in liquid nitrogen tanks.

2. Lentiviral Constructs

Lentiviral constructs for PIP and the empty vector controls were designed by Anne Blanchard in collaboration with Dr Sam Kung of the Lentiviral core facility at the University of Manitoba. In the vector design, the PIP coding region is flanked by the elongation factor 1 alpha (EF1a) promoter. Enhanced green fluorescent protein (EGFP) was used as the reporter and is flanked by the cytomegalovirus (CMV) promoter. A similar vector design without the PIP coding region was used as the empty vector control. The PIP and empty vector constructs were packaged into lentiviral particles for transduction/infection of the cell lines by the company Vector Builder (Chicago, IL, USA).

3. Lentiviral Transduction Optimization

To optimize the conditions for lentiviral transduction, different viral concentrations and incubation periods were tested. 4T1 or E0771 cells were cultured in 24 well tissue culture plates at a concentration of $5x10^4$ cells/well. Following overnight incubation, the media was removed, then 250µl of complete DMEM containing lentivirus (of different concentrations) and polybrene was added and incubated at 37ºC for 6 hours or overnight. Viral concentration was expressed as multiplicity of infection (MOI). For the optimization experiments, 1, 5, 10, 20 and 50 MOI were used. Following incubation, the virus was discarded and replaced with 1ml of fresh complete DMEM. The cells were then visualised by fluorescence microscopy 48-72 hours later to identify successfully transduced cells.

4. Quantification of Transduction Efficiency by Flow Cytometry

Since EGFP was used as the reporter, the cells were analysed by flow cytometry to quantify GFP expression. The cells were harvested and resuspended in flow cytometry buffer (1x PBS, 1% FBS and 2mM EDTA) and filtered to break cell clumps. Cells were acquired on flow activated cell sorter (FACS) Canto II (BD biosciences, CA, USA), gated on live cells and then GFP expressing cells.

5. Cell Sorting

Based on the results of the optimization experiments ("Materials and Methods" section 3 above), a lentiviral concentration of 50 MOI and overnight incubation period were chosen for 4T1 cell line, while 50 MOI and 6 hours incubation period were chosen for the E0771 cell line. These conditions were used both for PIP and empty vector transduction. For sorting, the cells were harvested, resuspended in flow cytometry buffer as described above, filtered and sorted for GFP expressing cells using the FACS Aria III (BD biosciences, CA, USA).

6. Preparation of Cell Lysates

Cells were grown to confluency in 6 well plates, rinsed with 1x PBS and lysed using 100µl of radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (Roche Diagnostics, Laval, QC, Canada). The cells were later detached using a cell scraper, transferred to a 1.5ml tube, sonicated twice for 15secs and thereafter centrifuged for 5mins (13000rpm, 4^{o}C). The supernatants were then transferred to fresh tubes and the protein concentration in the lysates was determined using the Bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford,

IL, USA) according to manufacturer's instructions. The lysates were then stored at -20^{o}C until needed.

7. Western Blot Analysis

To detect PIP in the cell lysates and culture media supernatants, Western blot analysis was employed. Cell lysates (containing 150µg protein) or supernatants (26µl) were mixed with reducing agent and loading dye (Life technologies, CA, USA) in accordance with manufacturer's instructions. These were spun for a few seconds and heated to 70^{o}C for 10 minutes. 40-50µl was loaded into each well of the polyacrylamide gel (Life technologies, CA, USA) and allowed to run at 200 Volts for 22 minutes. The proteins were transferred unto nitrocellulose membrane using the Pierce Power Blot Cassette (Thermo Scientific, USA) for 7 minutes. After the transfer step, the gel was stained with Coomasie blue for 1 hour while the nitrocellulose membrane was blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 hr. All antibodies were diluted in blocking solution. The membrane was then incubated in rabbit anti-mouse mPIP antibody (1:2,500, Alpha diagnostics, TX, USA) on a shaker, overnight at 4^{o}C. Protein bands on the gel were visualised by staining with Coomasie blue for 1 hour and then removing excess stain by incubating with a de-staining solution (35ml glacial acetic acid, 50ml methanol, 415ml double distilled water, dd$H_2$0) overnight. Following incubation with the primary antibody, the membrane was washed with TBST 3 times for 10 minutes on a shaker. The secondary antibody, goat anti-rabbit antibody, was added (1:10,000, Bio-Rad, ON, Canada) and incubated on a shaker at room temperature for 1hour. The secondary antibodies were washed off with TBST (10mins, 3 times) and developed using the enhanced chemiluminescence (ECL) kit (Immobilon, Millipore, MA, USA). ECL kit utilizes the reaction of the horseradish peroxidase (HRP) conjugated to the secondary antibody with substrate (consisting of luminal and peroxide solution) to detect antigens

immobilized onto the nitrocellulose membrane. Signal was acquired using the C-digit blot scanner (Licor, NE, USA)

8. Cell Counting by Trypan Blue Exclusion

$5x10^4$ 4T1 and E0771 (wild type, empty vector and PIP expressing) were each grown in triplicates in 12 well plates. Cells were detached using 0.05% trypsin (Hyclone Laboratories Inc.), mixed with trypan blue (1:1) and counted daily for 4 days using a TC-10 counter (Bio-Rad, ON, Canada).

9. XTT Assay

XTT cell proliferation assay kit (ATCC, VA, USA) was used to measure cell proliferation according to manufacturer's instructions. Briefly, the cells are grown for 24 hours or 4 days, then the XTT reagents are added to the cell culture and incubated for 2-4 hours after which an orange coloration was observed. Absorbance was measured at wavelengths of 475nm and 660nm using a Spectra max 190 (Molecular devices, CA, USA).

10. Wound Healing/Scratch Assay

Cells were seeded in 12 well plates in triplicates and grown until confluency. Using a 20µl pipet tip, a wound was created through the cell monolayer. Images were captured at T=0 and T=6 hours using the 10x objective of the microscope attached to a camera (ScopePhoto 3.0, ScopeTek DCM130 microscope camera). To obtain images of the same wounded areas at T=0 and at endpoint, 3 lines perpendicular to the wound were drawn at the bottom of the well with a marker. Using the lines as reference points, images were captured where the wound and line intersect so

that the same areas can be photographed later. 3 images were captured per well and analysed using the Image J program (NIH). Migration area in Pixels was measured and plotted.

11. Trans-well Migration Assay

Corning® Costar ® 8um Tran-swell® plates (Millipore Sigma, Merck, MA USA) was used for this assay. 100µl of serum free media containing $1x10^5$ cells was seeded in the top chamber/insert of the trans-well plate. 500µl media containing 30% FBS was carefully transferred to the bottom chamber. The cells were incubated at 37^{o}C for 24 hours. Using Q-tips, the inner part of the top chamber/insert was gently cleaned to remove cells that had not migrated to the outer part of the chamber. The inserts were then immersed in methanol to fix the cells. Then the cells were stained with crystal violet and photographs of at least 5 fields/ insert were taken. The number of migrated cells was counted and averaged. The number of cells that migrated to the bottom chamber was also counted.

12. Drug Sensitivity Assays

The effect of PIP expression on the sensitivity of 4T1 and E0771 to drugs was assessed by the XTT assay. $5x10^3$ cells were grown overnight in 96 well plates and treated with dimethyl sulfoxide (DMSO, vehicle control), Doxorubicin (0.5µM), Tamoxifen (10µM), Etoposide (50µM) and Cisplatin (20µM) for 48 hours (all from Sigma-Aldrich Canada). Cell viability was assessed using the XTT assay as described above. Results were normalized to DMSO control.

13. Mice

Six to eight weeks old BALB/c and C57BL/6 mice obtained from the in-house breeding colony of the University of Manitoba central animal care services, were used in this study. The

mice were maintained in specific pathogen-free environment, exposed to normal light-dark cycles, fed *ad-libitum* and kept in plastic cages containing wood chip bedding. All experiments were conducted according to the guidelines of the Canadian Council on Animal Care (CCAC).

14. Implantation of 4T1 Mouse Breast Tumor Cells

Mice were inoculated with 4T1 tumors following established protocols by Pulaski and Ostrand-Rosenberg (72). $1x10^4$ 4T1 EV or 4T1 PIP cells in 100µl PBS were injected orthotopically into the 4^{th} mammary fat pad of female BALB/c mice. Ear punches were used to identify the mice and the baseline weight for each mouse was obtained. Tumor diameter, mouse weight and other health parameters were monitored and recorded every 2-3 days. The mice were sacrificed at experimental time points defined by morbidity and loss in weight of more than 10% in the mice and/or tumor ulceration. Tumor size was measured in two dimensions using digital callipers and is calculated as follows: Mean tumor diameter (mm)= $(D \times d) \div 2$

Tumor volume (mm^3)= $(D \times d^2) \div 2$

D(mm)=largest diameter of the tumor and **d**(mm) = the tumor diameter perpendicular to D.

15. Implantation of E0771 Mouse Breast Tumor Cells

$5x10^4$ E0771 EV or E0771 PIP cells in 100µl PBS were injected orthotopically into the mammary fat pad of 6-8 weeks old female C57BL/6 mice. Mice were ear-punched for identification and an early health assessment was conducted for each mouse to obtain the baseline weight. The tumor diameter, mouse weight and other health parameters were also monitored and recorded every 2-3 days. Mice were sacrificed at experimental time points. Tumor size was also calculated as previously described.

16. Clonogenic Metastasis Assay

Lung and brain metastases in the 4T1 model were assessed using the clonogenic metastasis assay as described by Pulaski and Ostrand-Rosenberg (72). The organs were harvested from tumor bearing mice and healthy mice (negative control), minced with scissors and digested with 2ml collagenase IV solution (1.5mg/ml, Sigma) for 1 hour (for the lungs) or 2 hours (for the brain). The digested organs were passed through 40μm Falcon cell strainers to obtain single cell suspensions and washed 3 times in 5 ml complete DMEM. The resulting cells were resuspended in 10ml complete DMEM, and 6-thioguanine was added to a concentration of 60μM (10μl of 60mM 6-thioguanine was added to 10ml complete DMEM to give 60μM). The cells were then transferred to a 10mm culture dish and incubated at 37ºC for 10-14 days. Since 4T1 cells are resistant to 6-thioguanine, other cells are eliminated while the 4T1 cells survive. After culturing for 14 days, the metastatic 4T1 colonies became visible. The colonies in each culture dish were fixed with 5ml methanol, stained with 5ml 0.3% methylene blue, rinsed with 5ml water and counted.

17. Haematoxylin and Eosin (H&E) Staining of Lung Tissue

The mouse lungs were fixed for 24 hours in 10% formalin solution and subsequently embedded in paraffin. Tissue sections were made at the histology services centre, Department of Human Anatomy and Cell Sciences, University of Manitoba. H&E staining was conducted, and the slides assessed for metastasis by a pathologist at the Department of Pathology, University of Manitoba who was blinded to the experimental groups.

18. India Ink Staining of Lung Tissue

For the 4T1-Balb/c model, lung metastases were assessed by intra-tracheal injection of India ink (85% PBS, 15% India Ink). Mice were sacrificed and placed on the dorsal side. An incision was made around the neck to expose the trachea. Then a catheter was inserted into the trachea and held in place using a thread. India ink was injected into the lungs until the thoracic cavity became well inflated. The lungs were removed, washed in water to remove excess stain and transferred to Fekete's solution (30 ml 37% formaldehyde, 5 ml glacial acetic acid, 300 ml 70% ethanol) overnight. The lungs retain the dark blue of the India ink while metastatic tumor nodules appear white.

19. Tissue Immunophenotyping

At the experimental end points, mice were sacrificed and the breast tumors, spleens and lymph nodes were collected. Tumors were weighed and then minced in a culture dish using the curved end of a scissors. The minced tumors were transferred to a 15ml tube containing 2ml of collagenase IV solution and 20µl of DNase 1, then incubated in a shaker for 1 hour at 37^{o}C. After digestion, they were passed through 40µm cell strainers and washed two times in complete DMEM to obtain single cell suspensions. The cells were then counted using a haemocytometer.

Harvested spleens and lymph nodes were filtered with cell strainers to obtain single cell suspensions, then treated with 2ml ACK (Ammonium chloride-potassium) buffer for lysis of red blood cells. 5ml DMEM was added to neutralise the ACK, followed by centrifugation. The cells were resuspended in 5ml DMEM and counted. 1-10million cells were transferred to flow tubes for staining of surface markers for immune cells. The cells were resuspended in 1ml FACS buffer (2% FBS in PBS) and washed by centrifugation. Then 100µl of Fc block (anti-mouse CD16/32) was

added and incubated for 5 minutes. 1ml FACS buffer was used to wash the cells. Then the fluorochrome conjugated antibodies (anti- CD45, CD3, CD4, CD8, NK1.1, DX5, CD11c, F4/80, live/dead stain) were added and incubated for 15-30minutes on ice. Cells were acquired using a FACS Canto II (BD Biosciences, CA, USA).

20. Intracellular Cytokines Staining

Four million splenocytes (whole spleen cells), or one million cells from lymph nodes or tumours were seeded in 24 well plates in 500ml media/well. Cells were stimulated with a cocktail containing phorbol myristate acetate (PMA,), ionomycin, and brefeldin A for 4 hours at 37^{o}C. Cells were stained with antibodies for CD3, CD4 and CD8 following the procedures described above. The cells were subsequently fixed with 4% Paraformaldehyde for 15 minutes and washed with FACS buffer, then resuspended in 1ml Saponin buffer (1mg/ml) for 15-30minutes to permeabilise the cells for intracellular cytokines staining. Antibodies against the following markers CD3, CD4, CD8, IFN-γ, IL4, IL10 were added and incubated for 30minutes. Cells were washed in 1ml Saponin buffer, then in 1ml FACS buffer and finally resuspended in 0.5ml FACS buffer before acquisition using a FACS Canto II (BD Biosciences, CA, USA).

21. Isolation of Metastatic E0771 Cells from the Lungs

Lungs from tumor bearing mice were harvested, minced and separated into two portions. One portion was digested with collagenase (1.5mg/ml, 75minutes, 4^{o}C) and filtered using a 40μm cell strainer while the other portion was directly crushed and filtered. The cells were washed twice with complete DMEM and cultured in T25 flasks for about two weeks. The cells were then monitored frequently for the presence of metastatic E0771 cells. Cell morphology of metastatic

cells were assessed by microscopy and compared to parental cancer cells. As well, GFP expression was assessed by fluorescent microscopy and flow cytometry as previously described.

2.11. Statistical Analysis

Results are shown as mean ± SEM. Two-tailed Student's t-test, ANOVA or Mann Whitney test was utilised to compare the means from different groups of cells or mice. $P \leq 0.05$ was considered significant.

VI. RESULTS

1. GENERATION OF PIP EXPRESSING 4T1 CELLS

4T1 cells do not express PIP therefore lentiviral transduction was used to generate PIP expressing 4T1 cells. PIP-encoding lentiviral vectors (Fig. 4A) and the corresponding empty vector controls (Fig. 4B) possessed the coding region for enhanced green fluorescent protein (EGFP) which served as the reporter. Preliminary transduction experiments were conducted to optimize the viral titre/concentration and incubation period, and transduction efficiency was assessed by measuring the percentage of GFP expressing cells by flow cytometry. Table 1 shows different viral concentrations and incubation times used to transduce the 4T1 cells. The highest transduction efficiency was obtained at 50 MOI (multiplicity of infection) and overnight incubation (Table 1). Transduced cells were sorted by flow cytometry to obtain 4T1 cells with 96% GFP expression (Fig. 5A). The sorted cells were subsequently expanded and stored in liquid nitrogen for future use as described in “Materials and Methods”.

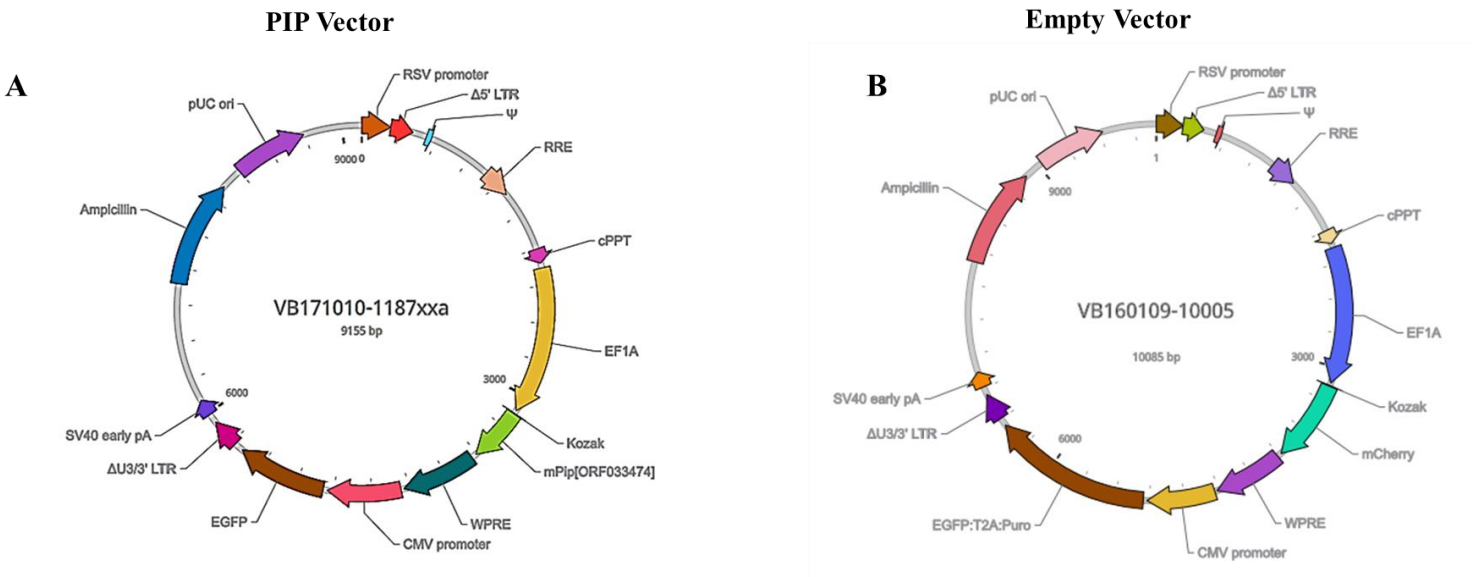

Fig. 4. **Lentiviral vectors:** (A) shows the PIP lentiviral vector with the *PIP* coding region indicated by the green arrow. (B) shows the empty vector control. *EGFP* is indicated by the brown arrow in both vectors.

Table 1: Percentages of GFP expressing 4T1 cells at different viral concentrations (MOI) as analysed by flow cytometry

MOI (multiplicity of infection)	20		50		100
Incubation time (hrs)	6	O/N	6	O/N	6
GFP +ve cells (%)	7.3	18.4	11.8	28.1	21.9

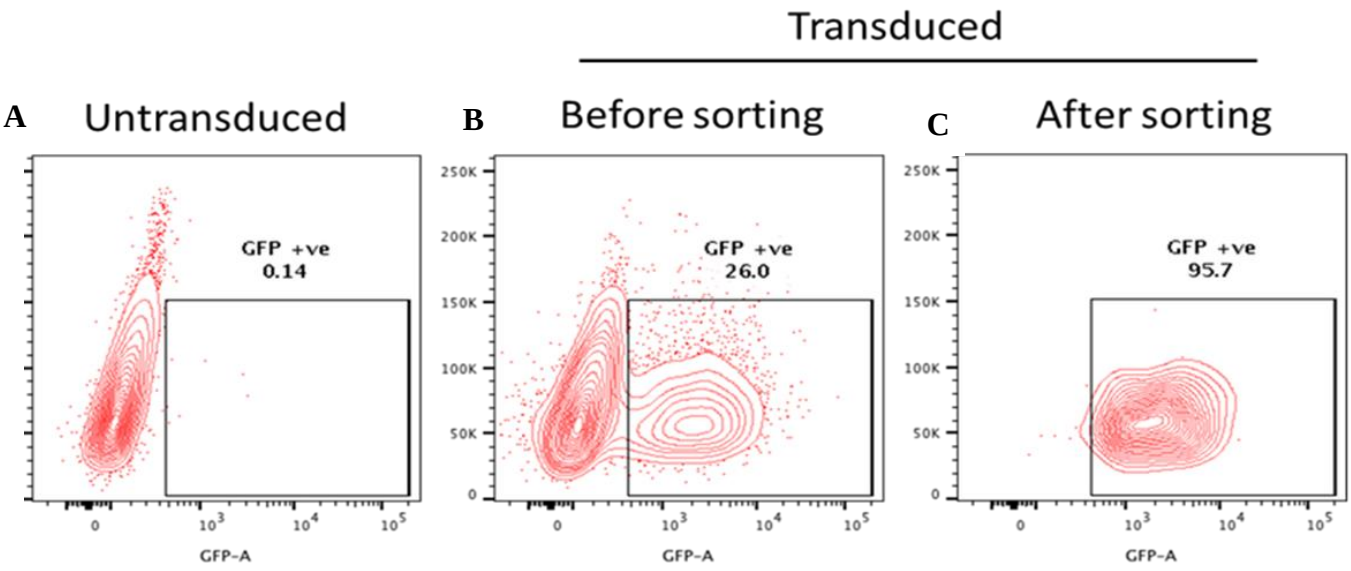

Fig. 5. Lentiviral transduction and sorting of 4T1 cells. Lentivirus encoding the *PIP* cDNA–*eGFP* construct was incubated with 4T1 cells for 6 hours and overnight (O/N). Contour plots showing the percentages of GFP expressing 4T1 cells for untransduced cells (A), and transduced cells before (B) and after sorting (C) by flow cytometry.

2. DETECTION OF PIP IN 4T1 CELLS FOLLOWING TRANSDUCTION

Western blot analysis was first undertaken in order to detect PIP in the cell lysates of transduced 4T1 cells. Cell lysates were prepared as outlined in "Materials and Methods", the protein concentrations determined by the Bicinchoninic acid (BCA) assay, and Western blot analysis was performed on protein lysates. The PIP band was identified in the transduced 4T1 but not in the corresponding empty vector control (Fig. 6A).

Since PIP is a secreted protein, cell culture media was also collected and examined for the presence of PIP by Western blot analysis. As shown in Fig. 6B, PIP was present in the media from transduced cells but not in empty vector control.

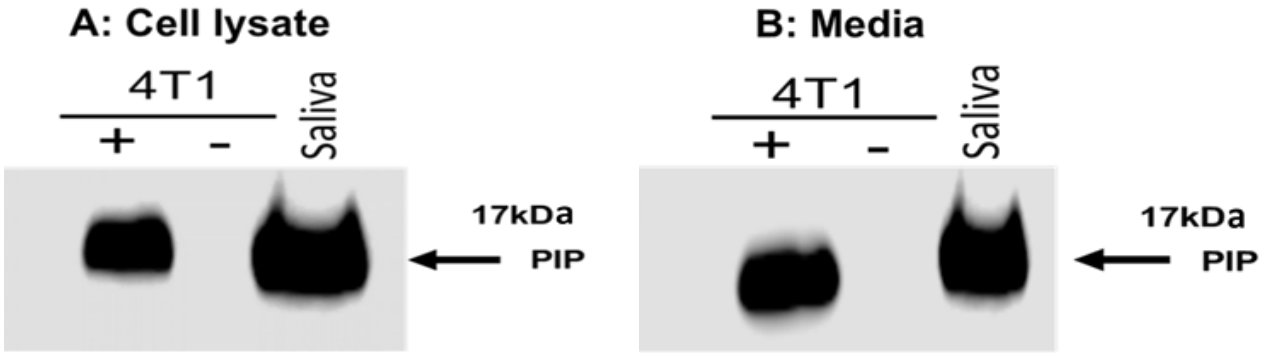

Fig. 6. Identification of PIP in 4T1 cell lysate and culture media. The PIP (17kDa) band was identified by Western blot analysis using the rabbit anti-mouse PIP antibody in 4T1 cell lysate (A) and media (B) as described in "Materials and Methods". Mouse saliva was used as positive control.

3. *IN VITRO* CHARACTERIZATION OF PIP EXPRESSING 4T1 CELLS

3.1 PIP Expression in 4T1 Cells Does Not Affect Their Proliferation

The effect of PIP expression on the proliferation of 4T1 cells was evaluated using two different approaches. The first approach was cell counting by trypan blue exclusion method. PIP expressing 4T1 cells and controls were grown for 4 days and counted daily using the Bio-Rad TC-10 counter as described in "Materials and Methods". From the results shown in Fig. 7A, the growth rates and trends were not different in PIP expressing 4T1 and the corresponding empty vector and wild type controls.

The second approach used is the XTT assay which is a metabolic assay that is an indirect measure of the proliferation of 4T1 cells. The cells were grown in 96 well plates and XTT assay performed on day 1 and day 4 (Fig. 7B and 7C). There was no difference in the proliferation rates in PIP expressing 4T1 cells and the corresponding controls. Altogether, these results show that PIP expression does not affect the proliferation of 4T1 cells.

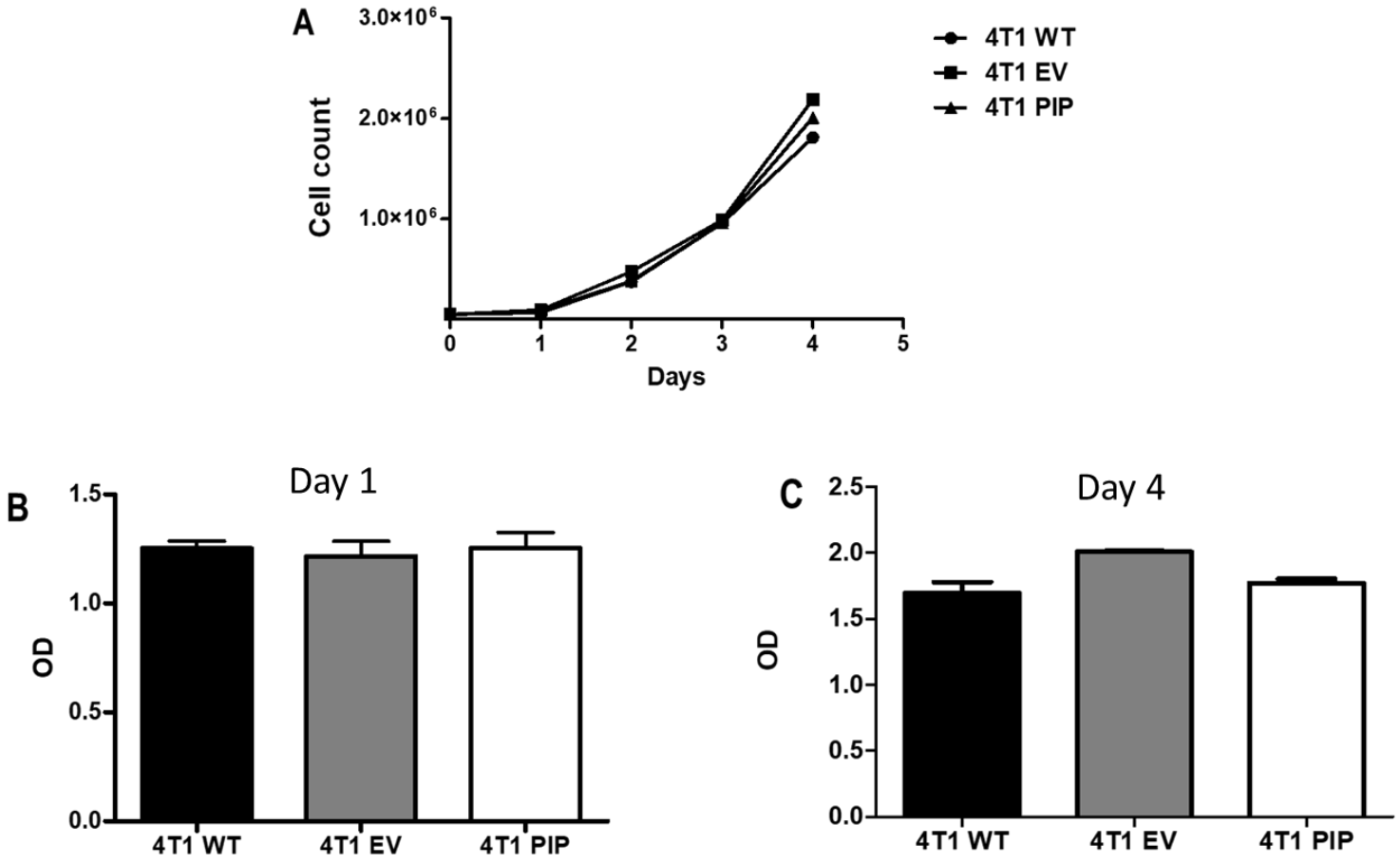

Fig. 7. PIP does not affect the proliferation of 4T1 cells. 4T1 cells were seeded in 12 well plates ($5x10^4$ cells/well) and counted daily for 4 days using the TC-10 counter. Panel A shows the growth curve for wild type (WT), empty vector (EV) transduced and PIP-expressing 4T1 cells. Panels (B) and (C) show the optical density (OD) values for 4T1 cells grown for 1 day and 4 days respectively, as assessed by the XTT assay. All experiments were done in triplicates and the results are representative of 3 different experiments with similar outcomes. Mean± SEM

3.2. PIP Does Not Affect 4T1 Cell Migration

To assess the effect of PIP expression on the migration of 4T1 cells *in vitro*, two approaches were undertaken. First, a wound healing/scratch assay was conducted as described in "Materials and Methods" using PIP expressing 4T1 cells and controls. Images were captured at time 0h and 6h after incubation and the migration area measured using the Image J software. As shown in Fig. 8A and 8B, there was no significant difference in the migration areas of PIP expressing 4T1 cells when compared to controls following 6h of incubation.

The second approach was the utilization of a trans-well migration assay which measures the migration of cells in response to a chemotactic agent (FBS, fetal bovine serum) as described in "Materials and Methods". Compared to controls, there was no difference in the number of PIP expressing 4T1 cells that migrated across the trans-well membrane in response to FBS following an incubation period of 24h (Fig. 8C, 8D). These results showed PIP does not affect the migration of 4T1 cells *in vitro.*

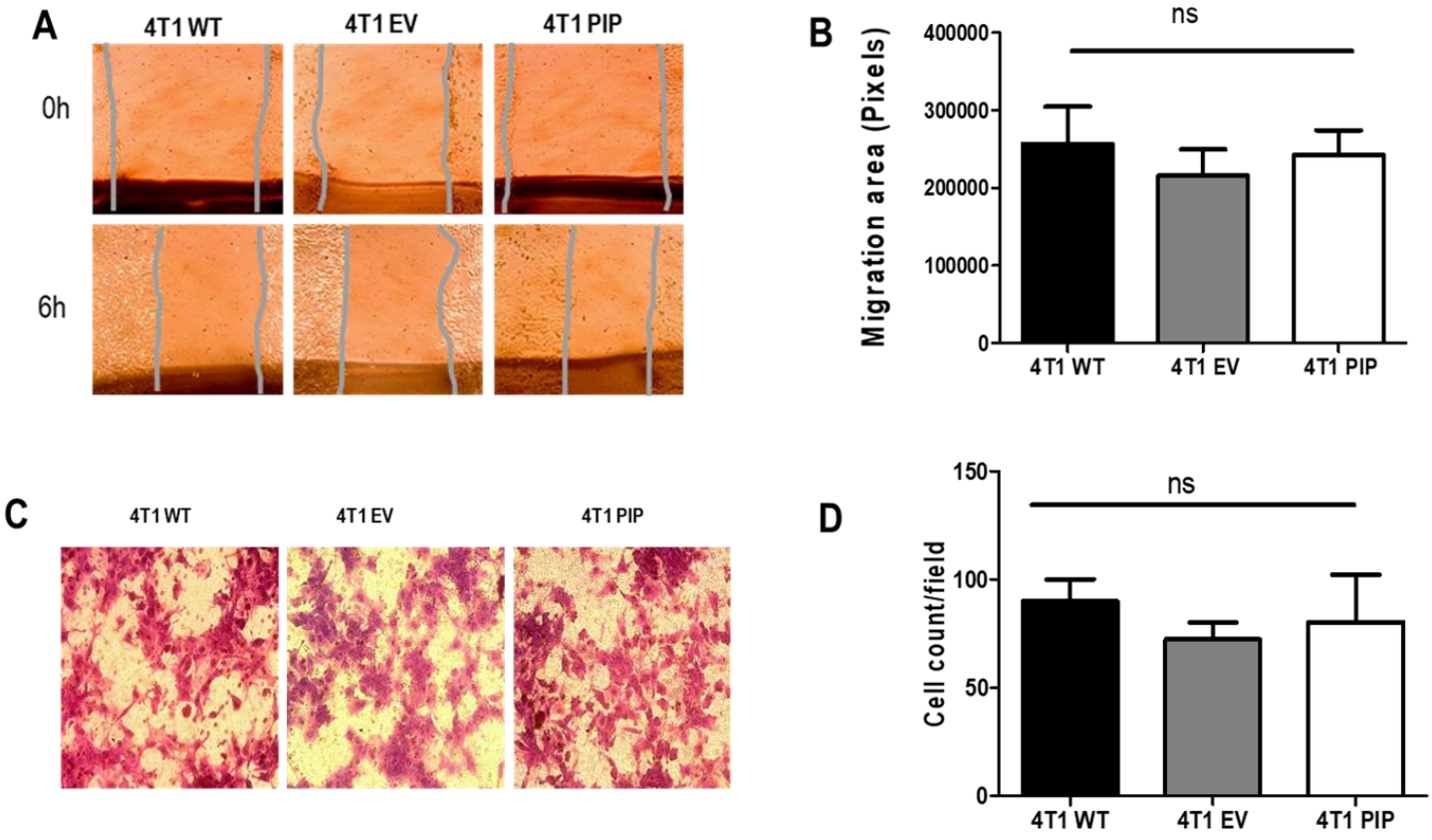

Fig. 8. PIP expression does not affect the migration of 4T1 cells *in vitro*. Cell migration was assessed by wound healing and trans-well migration assays. (A) shows representative images of wound areas at time 0 and 6h. (B) is a graph depicting the migration areas of the 4T1 cells (n=4). Panel C shows representative images of 4T1 from the trans-well migration assay. Panel D is a graph showing migrated cell count per field (n=3). All experiments were done in triplicates and repeated at least 3 times. Mean±SEM, ns, not significant; One-way analysis of variance.

3.3. PIP Does Not Affect the Response of 4T1 to Anticancer Drugs

To investigate whether PIP affects the sensitivity of 4T1 cells to anticancer drugs *in vitro,* the cells were treated with doxorubicin, cisplatin and etoposide for 48h and the viability of the surviving cells was measured by XTT assay. As well, the cells were treated with tamoxifen, a selective estrogen receptor modulator (SERM). The results show that after drug treatment, there was no difference in the viability of surviving cells between PIP expressing 4T1 cells and controls (Fig. 6).

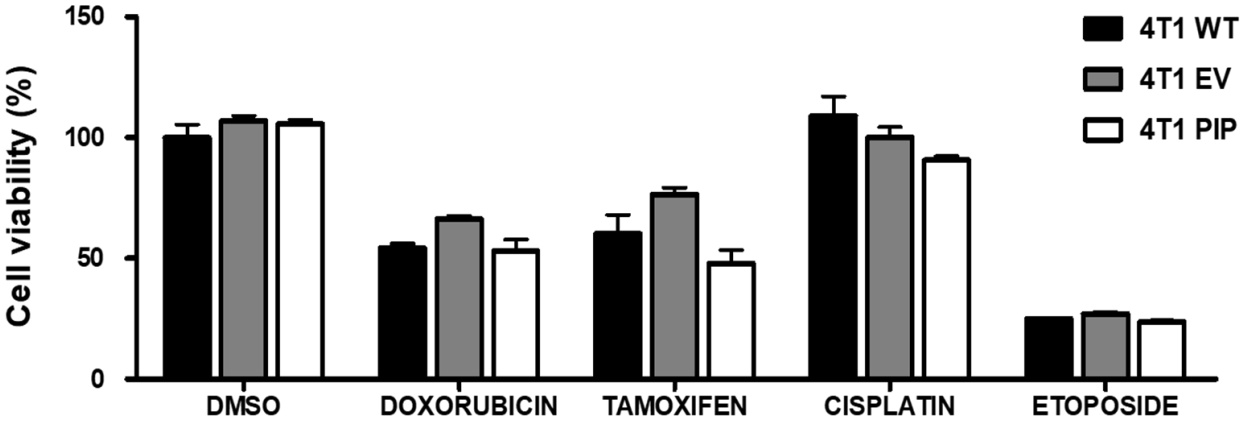

Fig. 9. PIP does not affect the sensitivity of 4T1 cells to drugs used against breast cancer. The 4T1 cells were cultured and exposed to different drugs for 48h. Cell viability following drug exposure was measured by XTT assay and normalized to DMSO control. The figure shows the relative cell viabilities for 4T1 cells following doxorubicin, cisplatin, etoposide as well as tamoxifen treatment. All experiments were done in triplicates and results are representative of 3 experiments with similar outcomes. Mean± SEM

4. *IN VIVO* STUDIES ON THE EFFECT OF PIP EXPRESSION ON BREAST TUMORIGENESIS AND IMMUNE RESPONSE

4.1. PIP Expression in 4T1 Tumor Leads to Delayed Tumor Onset, Growth and Reduced Tumor Size

To investigate the effect of PIP expression on breast tumorigenesis *in vivo*, $1x10^4$ PIP expressing 4T1 cells (4T1 PIP) and controls (4T1 EV) were injected orthotopically into the 4^{th} mammary fat pad of Balb/c mice. Tumor latency, growth and size were measured.

A delay in the onset of tumor in the PIP group compared to control group was observed. 40% of the mice in PIP group compared to 80% of mice in control group developed palpable tumor on day 9 post injection (Fig. 10A). In addition, a significant reduction in the growth of the PIP expressing 4T1 tumors was observed when compared to controls (Fig. 10B). When the mice were sacrificed, the tumors were weighed and PIP expressing 4T1 tumors were found to be smaller in size compared to the tumors derived from empty vector controls (Fig. 10C).

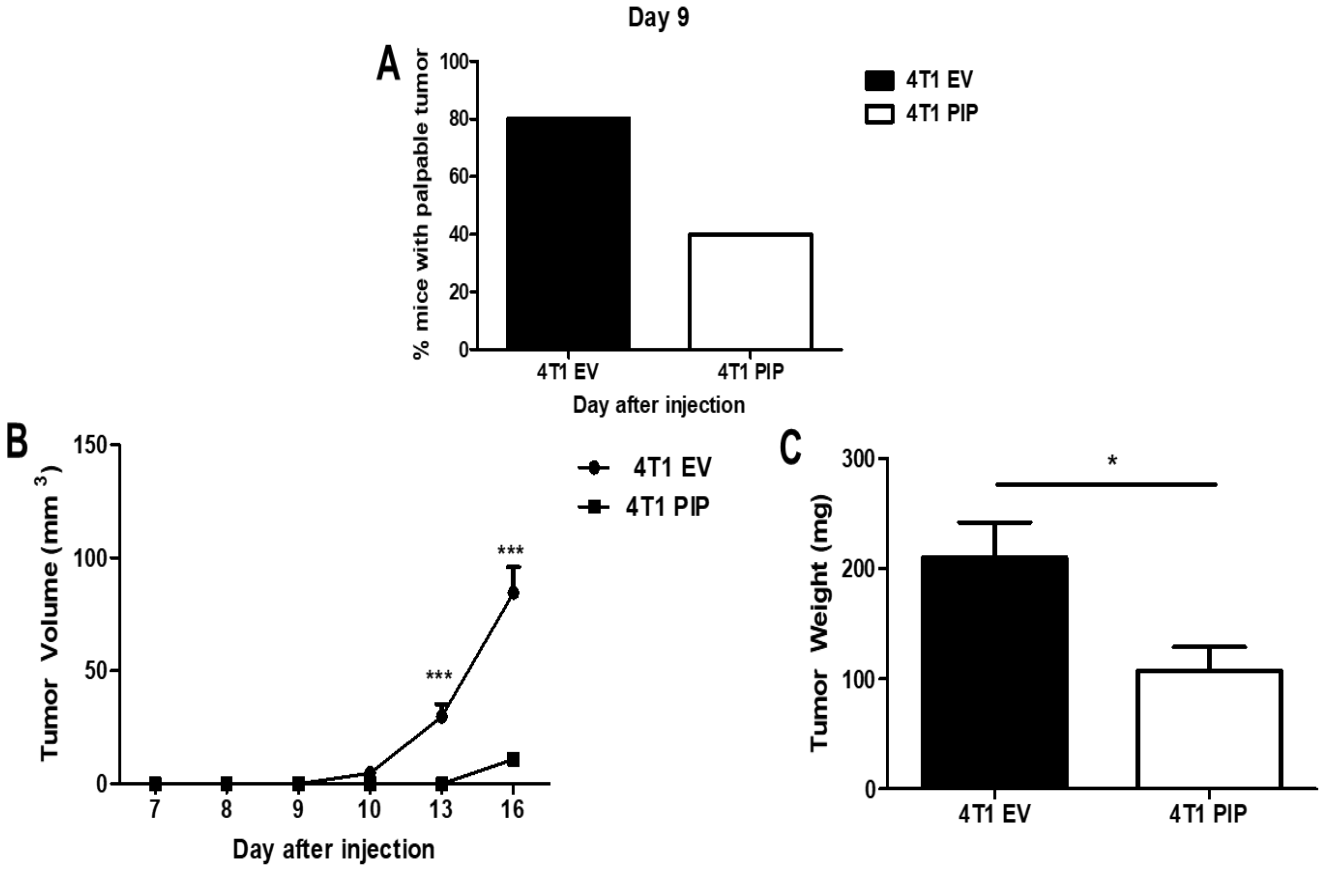

Fig. 10. PIP expression retards 4T1 tumor onset and growth: Tumor latency (A), growth curves (B) of PIP expressing 4T1 tumors and controls. Tumor growth was monitored and measured every 2-3 days using electronic calipers. Tumor sizes (C) were weighed at the end of the experiment. Results are representative of 3 different experiments. Mean ± SEM of n=3-5 mice/group. *P<0.05; ***P<0.001; 2-way ANOVA

4.2. PIP Expression in 4T1 Tumor Leads to Increase in the Percentages of Natural Killer and Dendritic Cells

To determine whether the PIP expressing 4T1 tumors impacted immunity by altering immune cell numbers, the draining lymph nodes and tumors were collected and stained for immune cell markers by flow cytometry. The results showed no significant differences in the percentages of $CD4^+$, $CD8^+$ T cells and macrophages (Fig. 11A, 11B and 11E) in the draining lymph nodes and tumors from mice injected with PIP expressing 4T1 cells, and controls. However, there was a significantly higher percentage of natural killer (NK) cells (Fig. 11C) and dendritic cells (Fig. 11D) in the PIP expressing 4T1 tumors. There was also no difference in the absolute numbers of NK cells in the PIP expressing 4T1 tumors when compared to controls (Fig. 11F).

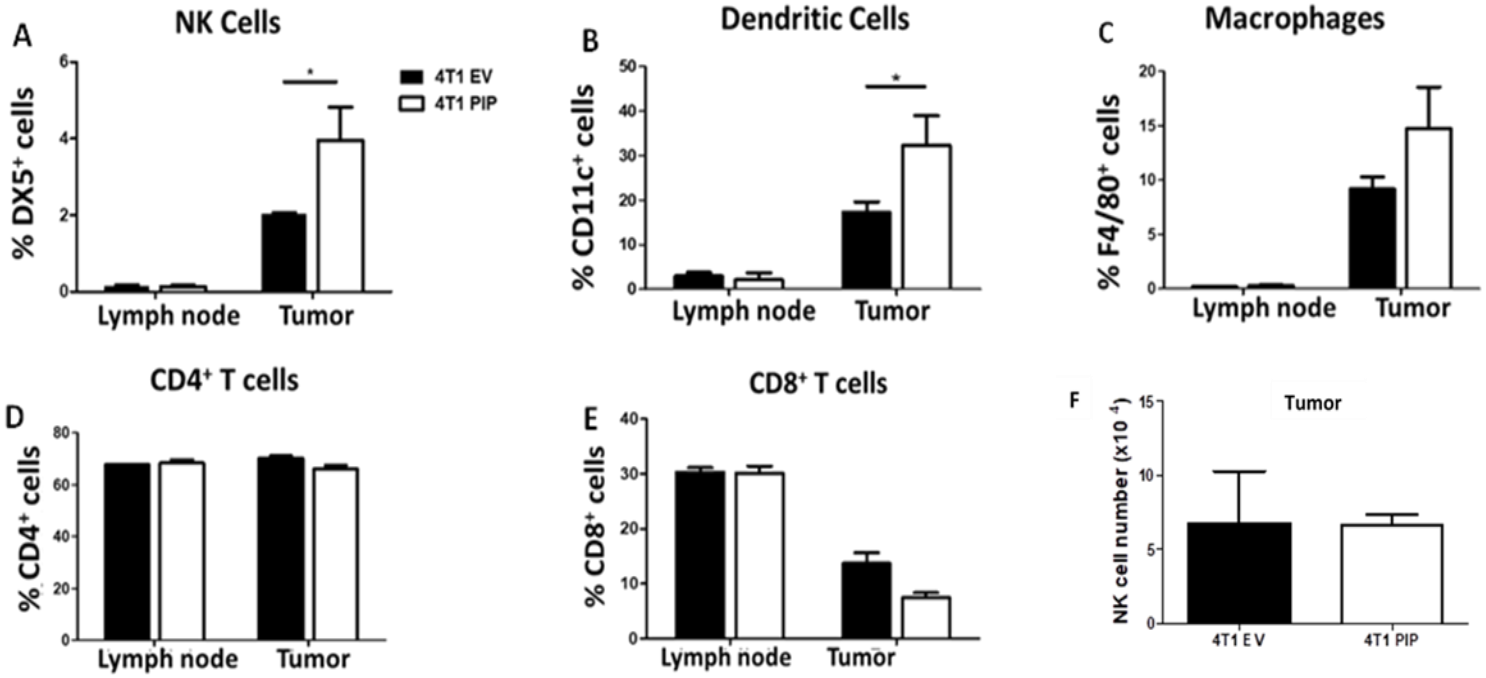

Fig. 11. PIP is associated with increased percentages of natural killer (NK) and dendritic cells in 4T1 breast tumor: Mice were injected with PIP expressing 4T1 cells and controls, and the of NK cells (A), dendritic cells (B), macrophages (C), $CD4^+$ (D) and $CD8^+$ (E) T cells, in draining lymph nodes and tumors were assessed by flow cytometry as described in "Materials and Methods". Panel F shows absolute number of NK cells in the tumors. Results are representative of 3 different experiments. Mean ± SEM of n=3-5 mice/ group. *P<0.05; 2-way ANOVA. Cell markers: DX5- NK cells, CD11c- dendritic cells, F4/80- macrophages, CD4- $CD4^+$ T cells, CD8- $CD8^+$ T cells.

4.3. PIP Expression in 4T1 Tumor Leads to Reduced Type 2 T-Helper Response

Cytokines have been reported to modulate the antitumor immune response, playing both pro-tumorigenic and antitumorigenic roles (173). Therefore, the effect of PIP expression on cytokine response was investigated. Draining lymph nodes and tumors from mice bearing PIP expressing 4T1 tumors and control mice (breast tumor lacking PIP expression) were collected and processed to obtain single cell suspensions. Cells were then stimulated with a cocktail of phorbol myristate acetate (PMA), ionomycin and brefeldin A. The percentages of IFN-γ and IL-4 producing $CD4^+$ and $CD8^+$ T cells were then assessed by flow cytometry as described in "Materials and Methods". There was a significant decrease in IL-4 producing $CD4^+$ T cells in PIP expressing 4T1 tumors compared to controls (Fig. 12A), while no differences were observed in IFN-γ producing $CD4^+$ or $CD8^+$ T cells (Fig 12B and 12C).

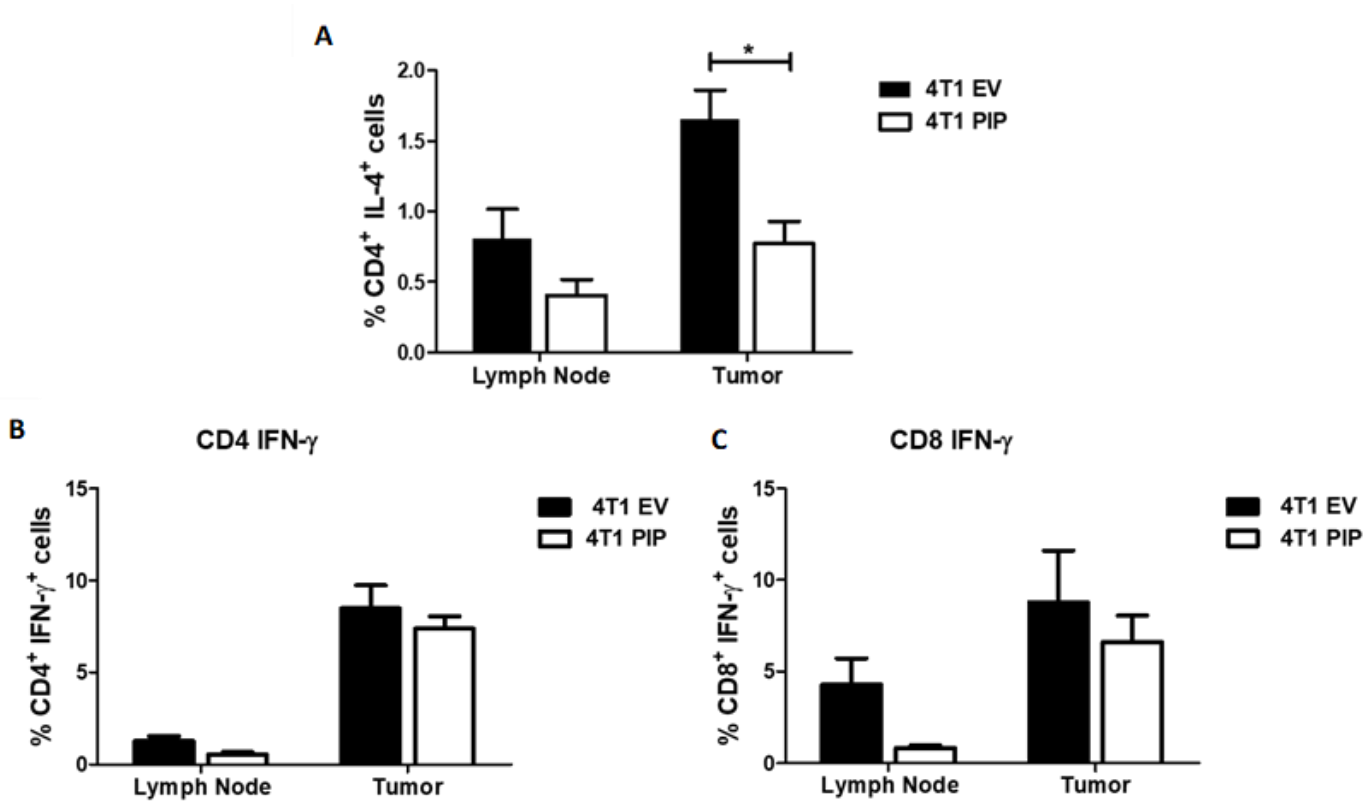

Fig. 12. PIP leads to decreased levels of $CD4^+$ $IL\text{-}4^+$ cells in 4T1 breast tumors: Mice with PIP expressing 4T1 tumors and the empty vector controls were sacrificed and the cytokine response was assessed directly *ex vivo* by flow cytometry. Panels A, B and C show the percentages of $CD4^+$ $IL\text{-}4^+$, $CD4^+$ $IFN\text{-}\gamma^+$, $CD8^+$ $IFN\text{-}\gamma^+$ T cells respectively in the draining lymph nodes and tumors**.** Mean ± SEM of 3-5 mice per group. *P<0.05; 2-way ANOVA

4.4. PIP Expression in 4T1 Tumors is Associated with Increased Metastasis to the Lungs

The 4T1 mouse breast cancer cells have been reported to metastasize from the breast to distant organs including the lungs, brain, liver and bone (72). Therefore, the rate at which tumor cells populate the lungs in mice bearing PIP expressing and control 4T1 tumors was investigated. Lung metastasis was assessed in the lungs from tumor bearing mice using the clonogenic metastasis assay as described in "Materials and Methods". A higher number of metastatic colonies in the lungs of mice bearing PIP expressing 4T1 tumors was observed when compared to lungs from control mice (Fig. 13A and 13B). The metastatic index (Fig. 13C), which is defined as the number of metastatic colonies divided by the tumor size, shows that tumor size was not a factor, as regardless of tumor size, there was greater lung metastasis in the mice from PIP group compared to controls.

Histological analysis of the lungs was also conducted. Lung sections derived from mice bearing PIP expressing 4T1 tumors displayed increased tumor infiltration compared to control lungs (Fig 13D). Collectively, results from both metastasis assays show that PIP expression in 4T1 tumors was associated with increased lung metastasis.

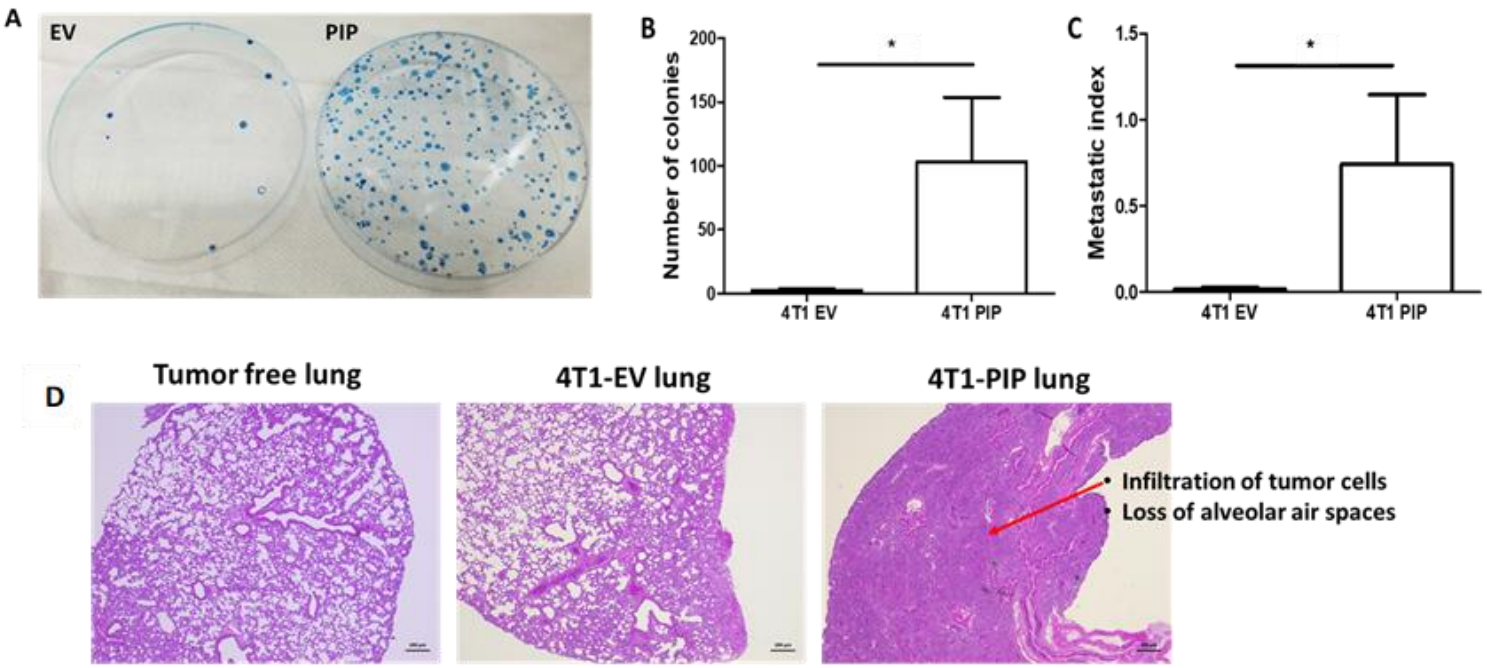

Fig. 13. PIP expression in 4T1 tumors led to increased metastasis to the lungs: Lungs were collected from mice with PIP expressing 4T1 tumors and the empty vector controls. Metastasis was assessed by clonogenic assays and histology. (A) and (B) are pictorial and graphical representations of the number of metastatic 4T1 colonies from lungs of PIP and control group mice. (C) shows the metastatic index. (D) shows H&E stained lung sections obtained from controls and PIP group mice. Results are representative of 3 different experiments. mean ± SEM of 3-5 mice per group. *P<0.05; Mann Whitney test

5. GENERATION OF PIP EXPRESSING E0771 CELLS

In contrast to the 4T1 model which mimics late stage human breast cancer, the E0771 model mimics early stage human breast cancer thereby enabling us to assess the effect of PIP at the early stage of breast cancer as well.

E0771 cells, like the 4T1 cells, do not express PIP. Therefore, lentiviral transduction was used to generate PIP expressing E0771 cells. Preliminary transduction experiments were performed to optimize the viral titre/concentration and incubation period while transduction efficiency was assessed by measuring the percentage of GFP expressing cells by flow cytometry. Table 2 shows the different viral concentrations and incubation times tested. The highest transduction efficiency was obtained at 50 MOI with overnight incubation (Table 2). Transduced cells were subsequently sorted by flow cytometry (Fig. 14B), expanded and stored in liquid nitrogen for future use as described in “Materials and Methods”.

Table 2: Percentages of GFP expressing E0771 cells at different viral concentrations (MOI) as analysed by flow cytometry.

MOI (multiplicity of infection)	1	5	10	20	50
GFP +ve cells (%)	0.6	3.0	5.5	11.3	22.7

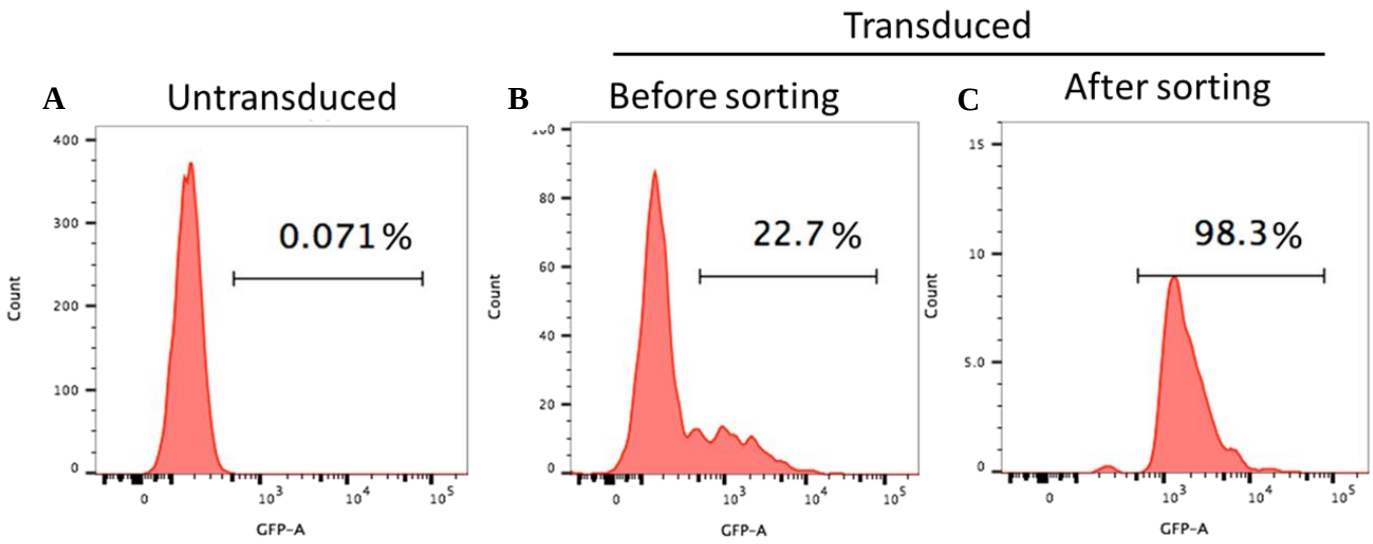

Fig. 14. Lentiviral transduction and sorting of E0771 cells: Lentivirus encoding the *PIP* cDNA–*eGFP* construct was incubated with E0771 cells for 6 hours. The cells were subsequently sorted by flow cytometry Histograms showing the percentages of GFP expressing E0771 cells for untransduced cells (A), and transduced cells before (B) and after sorting (C) by flow cytometry.

6. COFIRMATION OF PIP IN E0771 CELLS FOLLOWING LENTIVIRAL TRANSDUCTION

To confirm the presence of PIP in the transduced E0771 cell line, Western blot analysis was performed. PIP was clearly apparent in the PIP vector transduced E0771 cells but not in the corresponding empty vector control (Fig 15A). Since PIP is a secreted protein, the cell culture media from transduced E0771 cells was collected to test for the presence of PIP by Western blot using the rabbit anti-mouse PIP antibody as described in "Materials and Methods". PIP was detected in the media from PIP vector transduced cells but not in media from empty vector control (Fig 15B).

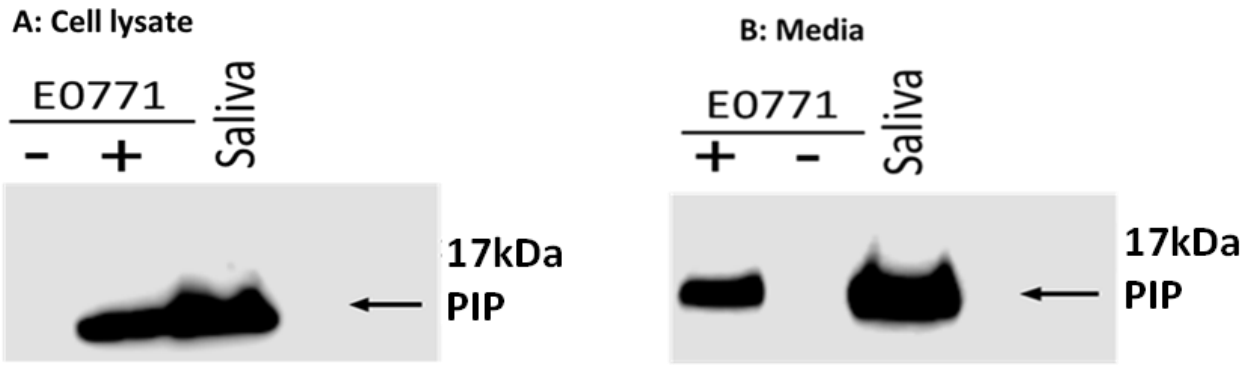

Fig. 15. Identification of PIP in E0771 cell lysate and culture media: The PIP (17kDa) band was identified by Western blot analysis using the rabbit anti-mouse PIP antibody in E0771 cell lysate (A) and media (B) as described in "Materials and Methods". Mouse saliva was used as positive control.

7. *IN VITRO* CHARACTERIZATION OF PIP EXPRESSING E0771 CELLS

7.1. PIP Expression in E0771 Cells Does Not Affect Their Proliferation

The effect of PIP expression on the proliferation of E0771 cells was evaluated using two different approaches. The first approach was cell counting by trypan blue exclusion method. PIP expressing E0771 cells and controls were grown for 4 days and counted daily using the Bio-Rad TC-10 counter as described in "Materials and Methods". From the results shown in Fig. 16A, the growth rates and trends were not different in PIP expressing E0771 when compared to the corresponding empty vector and wild type controls.

The second approach used is the XTT assay. The E0771 cells were grown in 96 well plates and XTT assay performed on day 1 and day 4 (Fig. 16B and 16C). There was no difference in the proliferation rates in PIP expressing E0771 cells and the corresponding controls. Altogether, these results show that PIP expression does not affect the proliferation of E0771 cells.

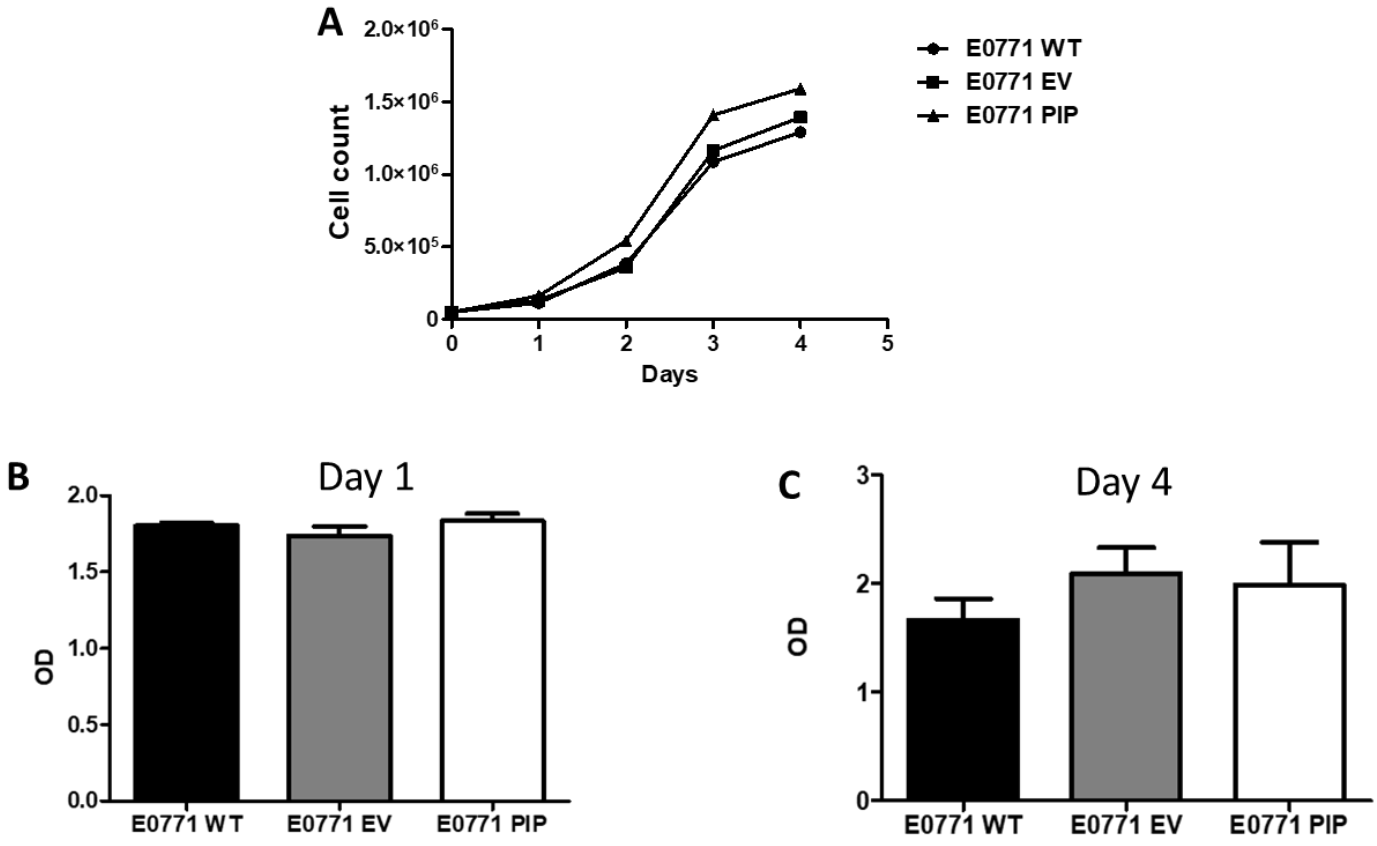

Fig. 16. PIP does not affect the proliferation of E0771 cells. E0771 cells were seeded in 12 well plates ($5x10^4$ cells/well) and counted daily for 4 days using the TC-10 counter. Panel A shows the growth curve for wild type (WT), empty vector (EV) transduced and PIP expressing E0771 cells. Panels (B) and (C) show the optical density (OD) values for E0771 cells grown for 1 day and 4 days respectively, as assessed by the XTT assay. All experiments were done in triplicates and the results are representative of 3 different experiments with similar outcomes. Mean± SEM

7.2. PIP Expression Does Not Affect the Migration of E0771 Cells

To assess the effect of PIP expression on the migration of E0771 cells, the trans-well migration assay was conducted. No significant difference in migration rates across the trans-well membrane was observed between the PIP expressing E0771 cells and controls following 24h incubation (Fig. 17A, 17B).

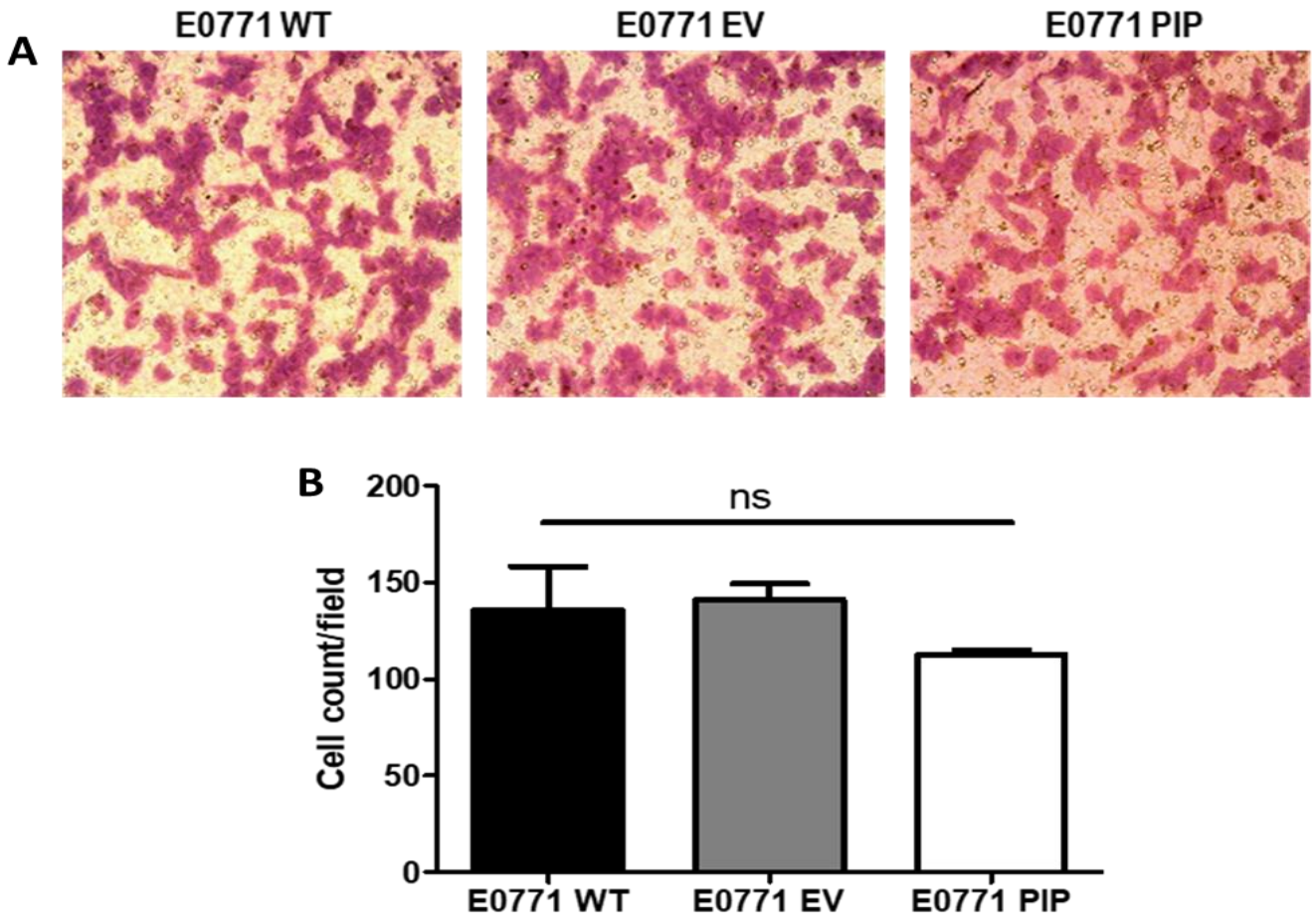

Fig. 17. PIP expression does not affect the migration of E0771 cells: Cell migration was analysed using the trans-well migration assay. (A) shows representative images of E0771 from the trans-well migration assay. (B) is a graph showing the migrated cell count per field (n=3). Results are average of 3 different experiments. Mean±SEM, ns, not significant; One-way analysis of variance.

7.3. PIP Does Not Affect E0771 Response to Anticancer Drugs

To investigate whether PIP affects the sensitivity of E0771 cells to anticancer drugs *in vitro*, the cells were treated with doxorubicin, cisplatin and etoposide for 48h and the viability of the surviving cells was measured by XTT assay. As well, the E0771 cells were treated with tamoxifen. The results show that after drug treatment, there was no difference in the viability of surviving cells between PIP expressing E0771 cells and controls (Fig. 18).

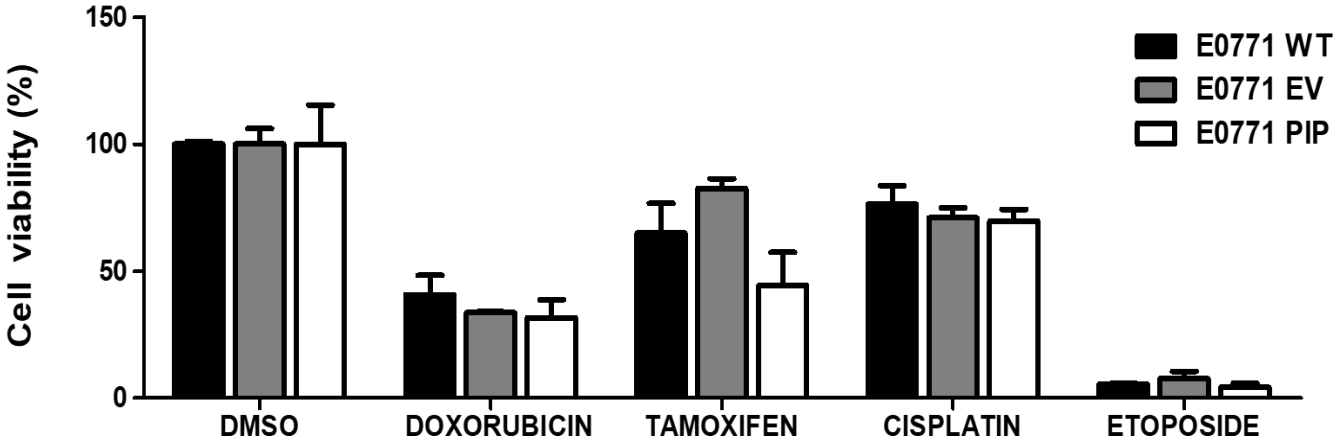

Fig. 18. PIP does not affect the sensitivity of E0771 cells to drugs used against breast cancer *in vitro*. The E0771 cells were cultured and exposed to different drugs for 48h. Cell viability following drug exposure was measured by XTT assay and normalized to DMSO control. The figure shows the relative cell viabilities for E0771 cells following doxorubicin, cisplatin, etoposide as well as tamoxifen treatment. All experiments were done in triplicates and results are representative of 3 experiments with similar outcomes. Mean± SEM

8. *IN VIVO* ASSESSMENT OF THE EFFECT OF PIP EXPRESSION IN E0771 CELLS

8.1. PIP Expression in E0771 Cells Does Not Affect Tumor Onset, Growth and Size

To assess the effect of PIP expression on breast tumorigenesis in the E0771 model, PIP expressing E0771 cells (E0771 PIP) and controls (E0771 EV) were injected orthotopically into the mammary fat pad of C57BL/6 mice. Tumor latency, growth and size were measured.

No obvious delay in the onset of tumor in the PIP group compared to control group was observed. As well, there was no difference in the tumor growth rates between PIP expressing and control E0771 tumors (Fig 19A). When mice were sacrificed at day 30 and their tumors weighed, there was no difference in the weights of the tumors between the two groups (Fig. 19B)

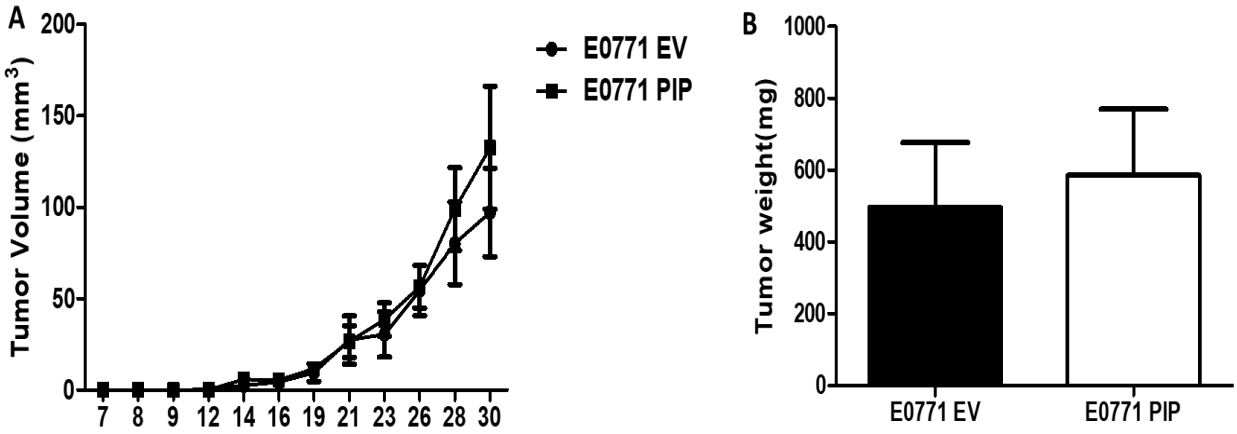

Fig. 19. PIP expression in E0771 cells does not affect tumor growth: PIP expressing E0771 cells and the corresponding empty vector controls were injected orthotopically into the mammary fat pad of 6-8 weeks old C57BL/6 mice. Tumor growth (A) and size at endpoint (B) were measured. Tumor growth was measured every 2-3 days using electronic calipers. Results are representative of 2 different experiments. Mean ± SEM of n=3-5 mice/group.

8.2. PIP Expression in E0771 Tumor Does Not Alter the Immune Phenotype

To investigate whether PIP expression in E0771 tumors alters the immune phenotype, draining lymph nodes and tumors were collected and stained for immune cell markers by flow cytometry. There was no difference in the percentages of $CD4^+$ and $CD8^+$ T cells, natural killer cells, dendritic cells and macrophages (Fig. 20A, 20B, 20C, 20D and 20E) between the PIP expressing and control E0771 tumors and their corresponding draining lymph nodes. This shows that PIP expression in E0771 tumors does not alter the immune phenotype in the draining lymph nodes and tumors.

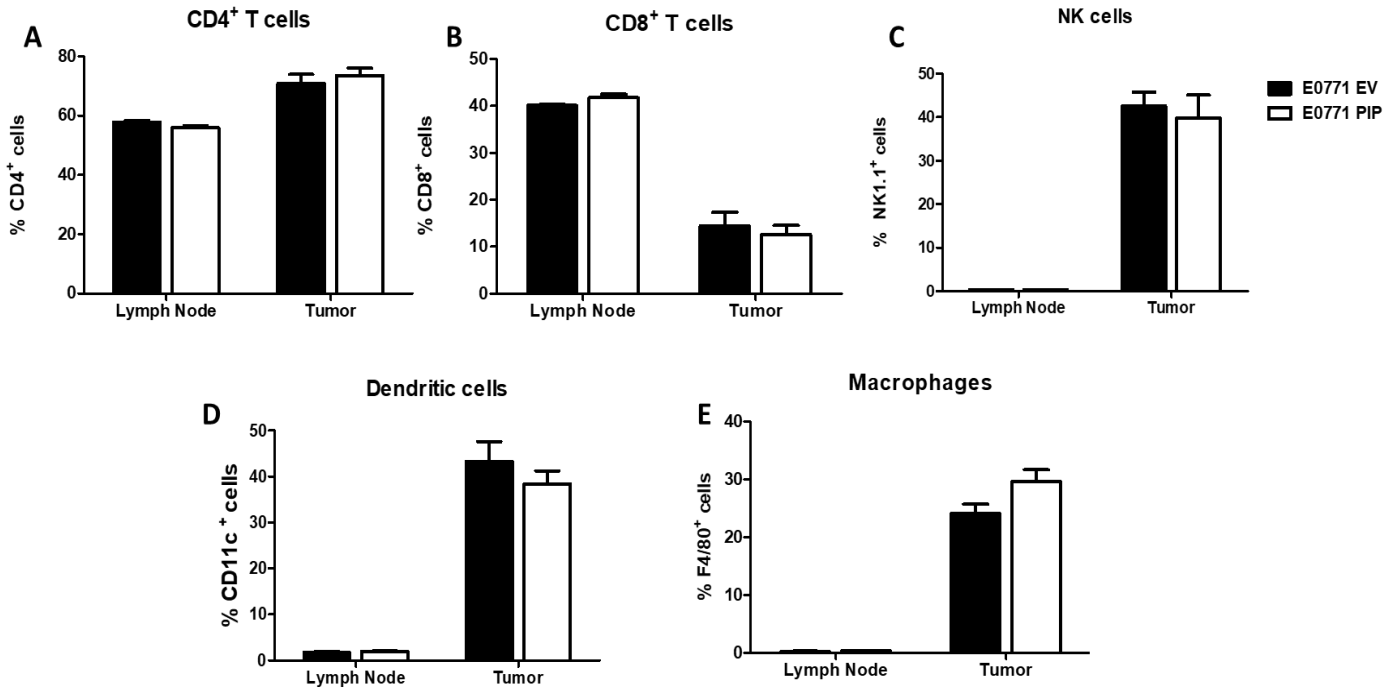

Fig. 20. PIP expression does not alter the immune phenotype in E0771 breast tumors: The frequencies of $CD4^+$ (A) and $CD8^+$ (B) T cells, NK cells (C), dendritic cells (D) and macrophages (E) in draining lymph nodes and tumors were assessed by flow cytometry. Results are representative of 2 experiments. Mean ± SEM of n=3-5 mice/ group.

8.3. PIP Expression in E0771 Tumor Does Not Affect Cytokine Response

To assess the effect of PIP expression on cytokine response, spleens, draining lymph nodes and tumors from mice injected with PIP expressing E0771 cells and controls, were harvested. The organs were processed to obtain single cell suspensions and subsequently stimulated with a cocktail of PMA, ionomycin and brefeldin A for 4 hours after which the percentages of IFN-γ and IL-4 producing $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry. There was no difference in the levels of IFN-γ and IL-4 producing $CD4^+$ or $CD8^+$ T cells (Fig 21).

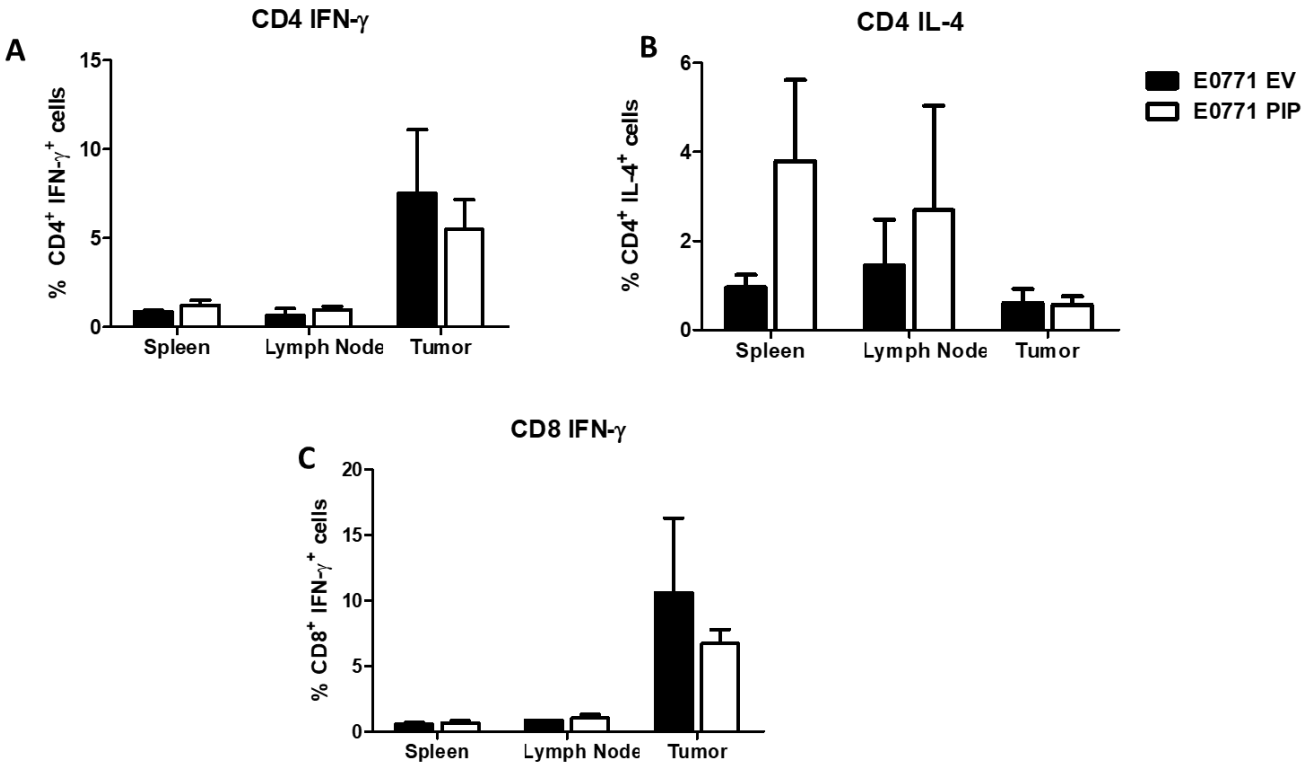

Fig. 21. PIP expression in E0771 tumors does not alter the cytokine production: Mice with PIP expressing E0771 tumors and the empty vector controls were sacrificed and the cytokine response was assessed directly *ex vivo* by flow cytometry. Panels A, B and C show the percentages of $CD4^+$ IFN-γ^+, $CD4^+$ IL-4^+, $CD8^+$ IFN-γ^+ T cells respectively, in the spleens, draining lymph nodes and tumors**.** Mean ± SEM of 3-5 mice per group.

8.4. Effect of PIP Expression in E0771 on Lung Metastasis

The lung is a prominent site for E0771 metastasis (74). To evaluate the rate at the at which tumor cells populate the lungs in mice bearing PIP expressing and control E0771 tumors, lung tissues were collected and subjected to histological analysis. By visual inspection, there appears to be more metastatic foci in the lungs of mice injected with PIP expressing E0771 cells compared to the control group. However, only 2 experiments have been conducted and further studies will be done to assess and accurately quantify the metastatic burden.

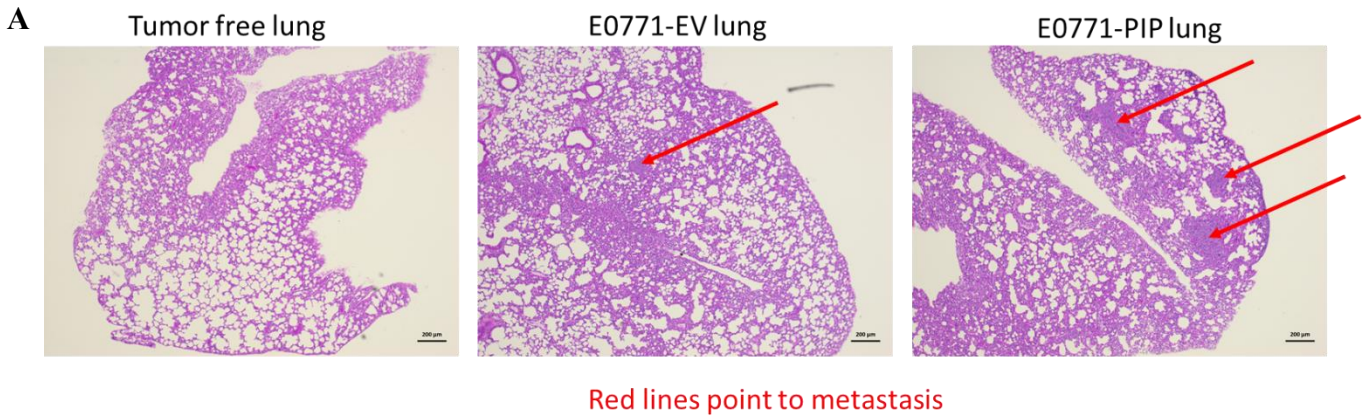

Fig. 22. Effect of PIP expression in E0771 cells on lung metastasis: Lungs were collected from mice with PIP expressing E0771 tumors and the empty vector controls. (A) shows H&E stained lung sections obtained from controls and PIP group mice. Results are representative of 2 different experiments. Mean ± SEM of 3-5 mice per group.

9. ISOLATION OF METASTATIC E0771 CELLS FROM THE LUNGS

Metastatic cells were isolated from the lungs of mice bearing PIP expressing E0771 tumors and lungs of control mice, as described in “Materials and Methods” (section 21). Cell morphology and GFP expression in the metastatic cells were assessed by microscopy and compared to wildtype (WT) E0771 cells. Results showed that the morphology of metastatic cells was similar to WT E0771 cells (Fig 23A). As well, metastatic cells from the lungs of mice injected with E0771 EV and E0771 PIP were positive for GFP expression by fluorescent microscopy (Fig. 23B) and flow cytometry analysis (Fig. 23C).

Together, these data confirm that the cells isolated from the lungs were metastatic E0771 cells and that digestion of the lungs did not affect the isolation of the metastatic cells. Further studies including functional assays and microarray analysis, will be conducted using the isolated metastatic cells to characterize them and delineate the potential role of PIP in lung metastasis.

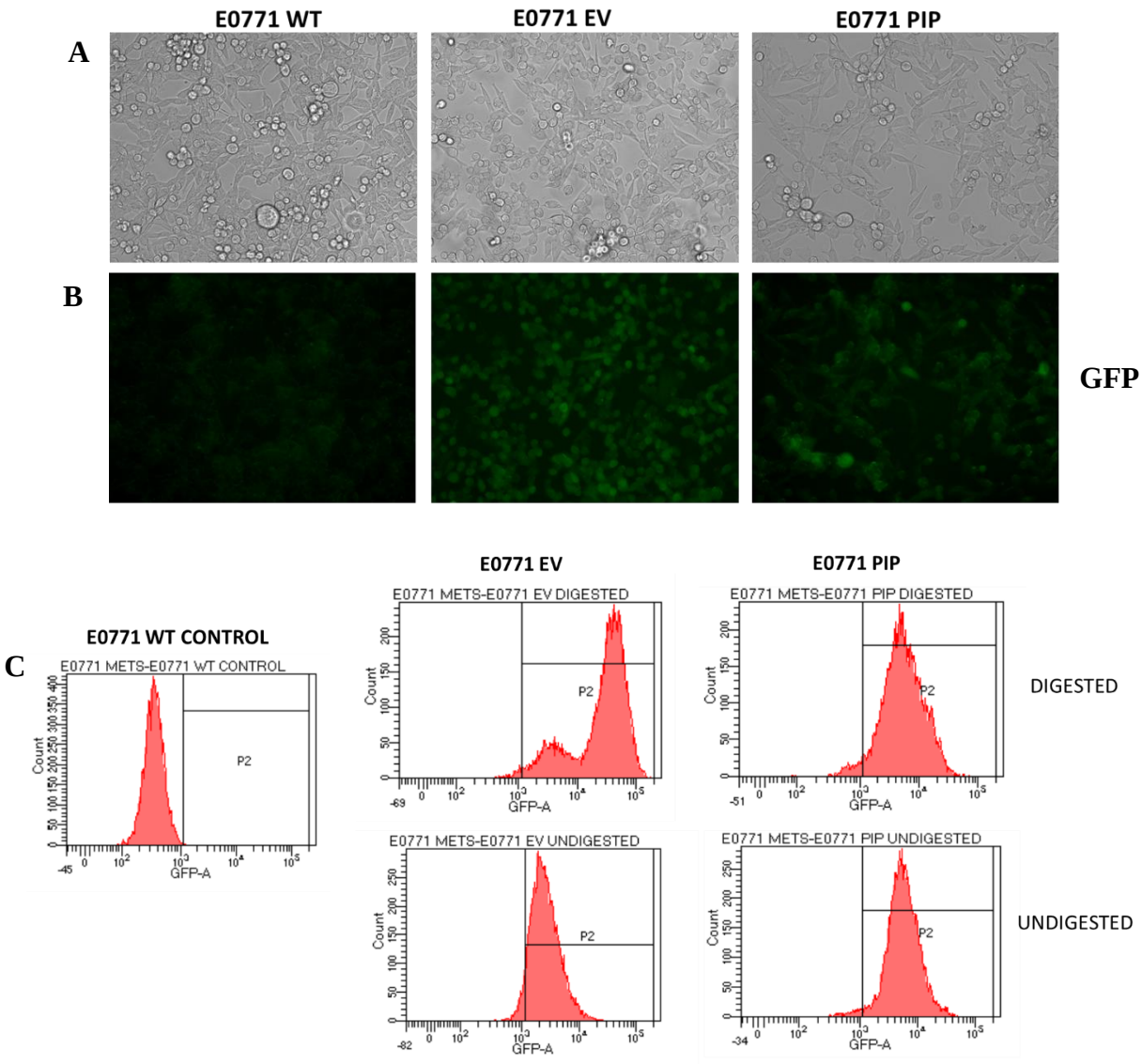

Fig 23. Isolation of metastatic E0771 EV and E0771 PIP cells from the lungs of tumor bearing mice: Lungs were collected from tumor bearing mice, processed into single cell suspensions, cultured in T25 flasks and monitored regularly. After 2 weeks in culture, isolated cells showed similar morphology (A) to WT E0771 cancer cells as well as GFP expression (B). Flow cytometry analysis shows that the majority of isolated cells were positive for GFP (C) and that this was not affected by enzymatic digestion.

VII. DISCUSSION

The role of PIP in breast cancer is not fully elucidated and current studies by our group suggest an immune regulatory role for PIP (169,171). Previous work from our laboratory showed that several components of the type 1 T helper (Th1) immune response were impaired in PIP KO mice (169). PIP KO mice displayed morphological anomalies in their secondary lymphoid organs (spleens and lymph nodes) (170). The percentages of $CD4^+$ T cells in their spleens was reduced and the differentiation of naïve $CD4^+$ T cells into IFN-γ producing $CD4^+$ T (Th1) cells was impaired (169). In addition, macrophages from PIP KO mice produced less proinflammatory cytokines and nitric oxide (171) and this was associated with decreased phosphorylation of mitogen activated protein kinase (MAPK), signal transducers and activators of transcription (STAT) 1 proteins. There was also an increase in the expression of suppressors of cytokine signalling (SOCS), indicating decreased intracellular IFN-γ signalling (171). Altogether, these findings provide supporting evidence to suggest that PIP is important in adaptive immunity, specifically with respect to the Th1 immune response, which has been shown to be critical for antitumor immunity (169).

Furthermore, studies (for review, 130) have shown that the expression of PIP is associated with better prognosis and response to chemotherapy in humans. PIP expression has also been reported to be of greater abundance in less aggressive luminal breast cancers, and of lowest presence in highly aggressive triple negative breast cancers (150). PIP levels are low or absent in normal mammary epithelium, become upregulated at the early stages of breast carcinoma development, and are subsequently downregulated as the cancer progresses to advanced stages (114,117,120,121). These observations suggest that PIP may be protective against breast cancer. They also suggest that PIP may exert its effect by modulating the antitumor immune response.

The focus of this study was to assess the role of PIP in breast tumorigenesis and immune response using transplantable mouse models. We sought to investigate whether the association of PIP expression with better prognosis in breast cancer is due to its immunomodulatory effects. To address this hypothesis that PIP inhibits breast cancer progression by enhancing antitumor immunity, I generated syngeneic immunocompetent transplantable mouse models using the 4T1 and E0771 mouse breast cancer cell lines which mimic different stages of human breast cancer when transplanted in mice.

1. The Effect of PIP in the 4T1 Mouse Model of Breast Cancer

The 4T1 cell line is a triple negative mouse breast cancer cell line which mimics stage 4 human breast cancer when injected into its syngeneic Balb/c mice and has been extensively used in preclinical mouse studies (72). However, the 4T1 cell line does not endogenously express PIP; therefore, the first step was to generate 4T1 cell lines that stably express PIP. Lentiviral transduction was the strategy of choice because it is known to induce more stable gene expression by integrating the gene of interest into the genome of the host cell. The presence of PIP was confirmed by Western blot analysis. Since PIP is a secreted protein, its presence in the cell media was also assessed and confirmed by Western blot, thereby confirming the successful generation of PIP expressing 4T1 cells.

Prior to the development of the transplantable mouse models, it was important to first characterize the effect of PIP expression on the 4T1 cells *in vitro* to determine whether the PIP expressing 4T1 cells possess significantly different features compared to the parental cells. Therefore, the effect of PIP expression on proliferation, migration and response to chemotherapeutic drugs and tamoxifen was assessed. Cell counting and XTT assays were

employed as complementary methods to evaluate the effect of PIP expression on the proliferation of 4T1 cells. Results from both assays showed comparable rates of proliferation in PIP expressing and control 4T1 cells (Fig. 4), thereby indicating that PIP does not affect the proliferation of 4T1 cells *in vitro.* In contrast, some other studies had reported a mitogenic effect of PIP on human breast cancer cell lines (137,174). Using knockdown approaches, the researchers showed that a decrease in PIP levels in T47D and MDA MB 453 cells led to a reduction in their growth, which was restored by addition of recombinant PIP to the media (137,174). The differences in these observed effects of PIP on proliferation could be attributed to the use of cell lines from different species (mouse vs human) or the different genetic approaches employed (knock down vs overexpression). Breast cancer cells, like many other cancer cells, are known to migrate from the primary tumor location to other parts of the body. Acquisition of the ability to migrate is an important step in cancer progression. Therefore, the effect of PIP expression on the migratory ability of 4T1 cells was investigated using wound healing and trans-well migration assays. These complementary approaches were utilized to strengthen the reliability and validity of the results. It was observed that PIP expression in 4T1 cells did not affect their ability to migrate at least *in vitro*. Some studies had reported that the expression of PIP was associated with better response to chemotherapy such as doxorubicin and cyclophosphamide in humans (131). Therefore, the effect of PIP expression on the sensitivity of 4T1 cells to drugs used against breast cancer was assessed. The drugs tested include doxorubicin, cisplatin and etoposide. Tamoxifen, a selective estrogen receptor modulator (SERM), was tested as well. There was no difference between the cell viability/survival of PIP expressing 4T1 cells and controls after treatment with the anticancer agents thereby suggesting that PIP expression does not directly affect the sensitivity of 4T1 cells to these drugs at least *in vitro*. When compared with the human studies (131), our *in vitro* results

suggest that PIP may be exerting an indirect influence on the sensitivity of breast cancer cells to doxorubicin. The disparities observed with the human studies could be attributed to the fact that the immune system and other stromal components are absent in the *in vitro* environment used in our study. In addition, it could also be because the human studies were retrospective, measuring the correlation between the expression of certain genes in breast cancer and response to chemotherapy, and not a direct causal effect.

To study the effect of PIP expression on breast tumorigenesis *in vivo*, PIP expressing 4T1 cells and controls were injected into the 4^{th} mammary fat pads of Balb/c mice. Tumor latency, growth and size were monitored. We observed a delay in the onset of palpable tumors in mice injected with PIP expressing 4T1 cells compared to controls and this delay was remarkable on day 9 post-injection, where about 80% of the control group mice had already developed palpable tumors compared to just 40% in the PIP group mice (Fig. 10A). In support of our hypothesis, this suggests that PIP delays the formation of tumors in the 4T1 syngeneic mouse model. The tumor growth was slower in PIP group mice compared to controls and when all mice were sacrificed, the PIP group mice also had smaller tumors compared to controls (Fig. 10B and 10C). Altogether, these data suggest that PIP expression in 4T1 tumors delayed tumor growth.

Interestingly, when metastasis to the lungs was assessed using the clonogenic assay, an increase in lung metastasis in mice injected with PIP expressing 4T1 compared to controls, was observed. The clonogenic assay data agreed with findings from histological analysis of lung sections which showed massive infiltration of tumor cells and loss of alveolar spaces in the lungs of mice bearing PIP expressing 4T1 tumors. The lung metastases did not show clear metastatic foci, but a diffuse infiltration of the lung tissue. Collectively, these display that PIP expression increases 4T1 lung metastasis and further implicates PIP as a possible regulator of breast cancer

metastasis. The reduced tumor growth observed coupled with increased lung metastasis could mean that PIP increases the metastatic potential in 4T1 tumors, which is a model of stage IV breast cancer.

The effect of PIP expression in 4T1 on the distribution of immune cells in the spleens, lymph nodes and the 4T1 tumors was assessed as PIP has been shown to modulate the immune system (168–171). Although there were no differences in the percentages of total $CD4^+$ and $CD8^+$ T cells (Fig. 11D and 11E), a significant increase in the percentages of natural killer cells and dendritic cells in the 4T1 tumors expressing PIP was observed (Fig. 11A and 11B). Additionally, there was no significant difference in the distribution of various immune cells in the spleens and lymph nodes, suggesting that PIP acts locally on the tumor. Natural killer (NK) cells have been reported to be directly cytotoxic to cancer cells and studies have shown that they are important for antitumor immunity. In a recent study, defects in NK cells were associated with increase in the occurrence of different kinds of cancers including breast carcinomas, underscoring the importance of NK cells in breast cancer development and immunity (175). Dendritic cells are professional antigen presenting cells which have also been shown to be critical in antitumor immunity. They are important modulators of the adaptive immune response and they present antigens to T cells, thereby facilitating their effector functions. Therefore, importantly, data from this study suggest that PIP expression enhances the local antitumor immune response by increasing natural killer and dendritic cells in the tumor. The effect of PIP expression on cytokine response in the 4T1 tumor microenvironment, spleens and lymph nodes was also investigated. Interestingly, a decrease in the percentage of IL-4 producing $CD4^+$ T cells (type 2 helper T cells, Th2) in the tumor environment was observed (Fig. 12A), indicating a decrease in the pro-tumorigenic Th2 response. This effect

was not replicated in the spleens and lymph nodes, suggesting a local effect as observed in the immunophenotypic analysis.

How PIP can promote metastasis and at the same time play a protective role in breast cancer is not known. One possible explanation is that PIP may act as a 'double-edged sword', enhancing antitumor immunity while enhancing cancer spread. It is possible that since our transplantable 4T1 model results in an overexpression of PIP, PIP level in these mice may be higher than normal physiological levels and thus is detrimental. Furthermore, some *in vitro* studies have suggested that PIP may be involved with invasiveness and subsequently metastasis and this was linked to the ability of PIP to degrade fibronectin due to its aspartyl protease activity (174). Fibronectin is a component of the extracellular matrix and studies have shown that it is important in regulating tumor cohesion (178) and increase in fibronectin has been linked to reduced metastasis (179). As a result, if the level of fibronectin is affected, it could lead to loss of integrity of the tumor architecture resulting in enhanced detachment of cancer cells and subsequently increased metastasis. It is also possible that PIP enhances factors that facilitate epithelial-mesenchymal transition (EMT), resulting in the promotion of metastasis. Further experiments comparing the expression of EMT markers in PIP expressing 4T1 tumors and controls will need to be conducted.

Since 4T1 cells are known to metastasize to the brain (72), the effect of PIP expression on brain metastasis was also assessed. Efforts to quantify brain metastasis using the clonogenic assay were however unsuccessful. Indeed, studies by others have shown that metastasis to the brain is reduced compared to the lungs, and could be because of the distance of the brain from the primary tumor site and difficulty in crossing the blood-brain barrier (176). In that study, the researchers were not able to detect brain metastasis on day 14 but were able to do so on day 30 (which was longer than the period for our study) (176).

2. The Role of PIP in the E0771 Mouse Model of Breast Cancer

As previously mentioned, it has been reported that the 4T1-Balb/c model mimics late human breast cancer, however, PIP is thought to be protective in early breast cancer (168). To investigate whether PIP expression during early stage breast cancer affects tumor development differentially, we utilized a PIP expressing E0771 transplantable mouse breast cancer model which mimics early stage human breast cancer. E0771 cells have been reported to be estrogen receptor positive and are less aggressive compared to the 4T1 cells (74). E0771 cells, like 4T1 cells, do not express PIP. *In vitro* characterization showed, as with the 4T1 cells, that there was no difference in the proliferation rates between PIP expressing E0771 and control cells using cell counting strategies and XTT assay. In addition, trans-well migration assay was performed to assess the effect of PIP expression on the ability of E0771 cells to migrate and the results showed that there was no difference in the migration abilities of PIP expressing E0771 compared to control cells, suggesting that PIP does not affect E0771 cell migration. The effect of PIP expression on the sensitivity of E0771 to anticancer drugs such as doxorubicin, cisplatin, etoposide and tamoxifen was also evaluated. Similar to the observations with the 4T1 cells, we observed comparable sensitivities of PIP expressing E0771 cells and control cells to the drugs tested, suggesting that PIP does not affect their sensitivity to those drugs.

We did not observe any significant differences in the tumor latency or tumor progression. We also did not observe any significant differences in the distribution of immune cells including $CD4^+$ and $CD8^+$ T cells, natural killer cells, dendritic cells and macrophages. As well, when the cytokine response was assessed, there was no significant difference in the percentages of IFN-γ producing $CD4^+$ and $CD8^+$ T cells as well as IL-4 producing $CD4^+$ T cells. Collectively, these results suggest that PIP expression does not affect breast tumorigenesis or the immune response in

the E0771 model. Although there appears to be more metastatic foci in the lungs of mice bearing PIP expressing E0771 tumors, studies will be conducted to accurately quantify these metastases.

VIII. SUMMARY AND CONCLUSIONS

In this study, the role of PIP in breast tumorigenesis, immune response and metastasis was assessed using transplantable mouse models. PIP expressing 4T1 mouse breast cancer cell line was generated by Lentiviral transduction strategies and the presence of PIP was confirmed by Western blot analysis. Characterization of the PIP expressing 4T1 cells *in vitro* showed that PIP did not affect their proliferation, migration and sensitivity to some anticancer agents. *In vivo* studies showed that PIP expression in 4T1 cells delayed tumor onset, reduced tumor growth and size compared to controls. There was increased levels of natural killer and dendritic cells in PIP expressing 4T1 tumors in addition to decreased type 2 helper response. However, PIP expression in 4T1 cells was found to be associated with increased metastasis to the lungs. The experiments were repeated using another mouse model of breast cancer (E0771 model) which mimics the early stage of the disease in humans. Here, like the 4T1 model, PIP expression did not affect proliferation, migration and drug sensitivity *in vitro*. However, there was no effect of PIP on tumor latency, size and progression. Additionally, there was no significant difference in overall immune phenotype and cytokine response in mice bearing PIP expressing E0771 tumors compared to controls.

The observed differences between the findings in the 4T1 and E0771 models could be attributed to genotypic and phenotypic differences between the cell lines and mice used. Breast cancer has been reported to be a highly heterogenous disease with different gene expression profiles, molecular characteristics as well as disease progression patterns (177). 4T1 is a triple

negative breast cancer model while the E0771 is of the luminal subtype. Furthermore, the 4T1 model is more aggressive than the E0771. Whereas 10^4 4T1 cells were sufficient to induce a palpable tumor in Balb/c mice in as little as 7 days, the same number of E0771 cells did not induce palpable tumors in C57bl/6 mice. In fact, $5x10^4$ Cells were used and tumors became palpable almost 2 weeks later, demonstrating the differences in aggressiveness between the two mouse breast cancer models. In addition, since these models represent different stages and subtypes of human breast cancer, it is possible that the effect of PIP may depend on the stage and/or subtype of breast cancer involved.

Collectively, these data suggest that PIP inhibits tumor progression and may also be a regulator of immune response and metastasis in the mouse model of late stage and triple negative breast cancer but not in the model of early stage and luminal breast cancer.

IX. LIMITATIONS OF THIS STUDY

1. An important limitation of this study is that results from mouse models do not always correlate with findings in humans due to species differences.
2. Another limitation is the use of metastatic cell lines to induce primary breast tumors. A large number of cells at one time is injected orthotopically into the mammary fat pad to initiate tumor formation, and the time from injection to end of experiment is only a few weeks. This does not mimic the natural progression of breast cancer as it is spontaneous and may be initiated by just a single cell or few cells and take much longer to progress, in addition to all the important cellular and molecular events that occur during breast tumorigenesis and progression.

X. SIGNIFICANCE

Our studies provide important preclinical data to show that PIP is an important regulator of breast tumorigenesis. In addition to data showing that PIP regulates the antitumor immune response, we have also shown that PIP affects metastasis to the lungs. This could have potential applications for the management of breast cancer because our observations suggest that PIP may enhance immune response but if overexpressed, could also enhance metastasis. Thus, it is important to monitor PIP levels in patients in order to determine the optimal level of expression which results in enhancement of antitumor immunity only, or at least predominantly. It could also be that the time of PIP expression plays a role as PIP expression may be beneficial at the initial stages of breast cancer but become detrimental later. This could have potential therapeutic implications because if the beneficial or optimal PIP expression level is known, the appropriate dose of recombinant PIP could be determined and administered to breast cancer patients at the right time.

XI. FUTURE DIRECTIONS

Metastasis is the major cause of mortality in breast cancer patients and the precise mechanisms are not fully understood. Interestingly, since we have shown that PIP enhances metastasis in addition to enhancing antitumor immunity, it becomes important to better understand the mechanisms involved. To elucidate the mechanism of PIP effect on lung metastasis, one approach is to assess fibronectin levels in the tumors in order to investigate whether the increased lung metastasis observed is due to the degradation of fibronectin by PIP. To test this hypothesis, sections of the mouse tumors from both the PIP group and controls will be made, and fibronectin

levels measured using immunohistochemistry. If our hypothesis is true, we expect to find lower fibronectin levels in the PIP expressing tumors compared to controls.

It is also possible that PIP enhances migration *in vivo* or/and the ability of the 4T1 cells to home in on the lungs and survive there. To assess this, we will inoculate mice with PIP expressing and control 4T1 cells and assess for the presence of cancer cells in a distant lymph node (such as the axillary lymph nodes). If we find similar quantities of cancer cells, then it suggests that PIP does not affect *in vivo* migration but may affect the ability of the 4T1 cells to home in on and thrive in the lungs.

Furthermore, PIP expression may lead to the upregulation of genes that are involved in epithelial- mesenchymal transition (EMT) and metastasis. To test this hypothesis, we have isolated metastatic cells from the lungs. We intend to characterize the metastatic cells *in vitro* and compare them to the primary cancer cells. We will also conduct mRNA analysis to determine whether genes related to metastasis and EMT are upregulated or downregulated.

To investigate the contribution of immune cells to the delay in tumor latency and progression, we will inoculate immune deficient mice with PIP expressing and control tumors. If we observe comparable tumor progression in both groups, it suggests that PIP may lead to reduced tumor progression through its effect on the immune response.

Since results from animal models do not always translate to humans, we intend to also carry out studies on human breast tissues from the Manitoba tumor bank to investigate the correlation between PIP, breast tumorigenesis and immune activity. Findings from our mouse studies will be compared to that of the human tissue studies to assess correlation. Finally, to investigate the role of PIP in a more clinically relevant model which mimic the natural progression

of human breast cancer, genetically engineered mice which spontaneously develop breast cancer will be employed.

XII. REFERENCES

1. Breast cancer statistics [Internet]. World Cancer Research Fund. 2018 [cited 2019 Mar 5]. Available from: https://www.wcrf.org/dietandcancer/cancer-trends/breast-cancer-statistics

2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians [Internet]. 2018 [cited 2019 Mar 5];68(6):394–424. Available from: https://onlinelibrary.wiley.com/doi/abs/10.3322/caac.21492

3. Ekwueme DU, Guy GP, Rim SH, White A, Hall IJ, Fairley TL, et al. Health and Economic Impact of Breast Cancer Mortality in Young Women, 1970–2008. Am J Prev Med [Internet]. 2014 Jan [cited 2019 Jan 23];46(1):71–9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5836720/

4. Breast cancer statistics - Canadian Cancer Society [Internet]. www.cancer.ca. [cited 2019 May 21]. Available from: https://www.cancer.ca:443/en/cancer-information/cancer-type/breast/statistics/?region=on

5. Canadian-Cancer-Statistics-2018-EN.pdf [Internet]. [cited 2019 Mar 3]. Available from: http://www.cancer.ca/~/media/cancer.ca/CW/cancer%20information/cancer%20101/Canadian%20cancer%20statistics/Canadian-Cancer-Statistics-2018-EN.pdf?la=en

6. Survival Rates for Breast Cancer [Internet]. [cited 2019 Mar 3]. Available from: https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-survival-rates.html

7. Mariotto AB, Yabroff KR, Shao Y, Feuer EJ, Brown ML. Projections of the cost of cancer care in the United States: 2010-2020. J Natl Cancer Inst. 2011 Jan 19;103(2):117–28.

8. What is breast cancer? - Canadian Cancer Society [Internet]. www.cancer.ca. [cited 2019 May 31]. Available from: https://www.cancer.ca:443/en/cancer-information/cancer-type/breast/breast-cancer/?region=on

9. Cadoo KA, McArdle O, O'Shea A-M, Power CP, Hennessy BT. Management of unusual histological types of breast cancer. Oncologist. 2012;17(9):1135–45.

10. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA. 2001 Sep 11;98(19):10869–74.

11. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000 Aug 17;406(6797):747–52.

12. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010;12(5):R68.

13. Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, et al. Identification of molecular apocrine breast tumours by microarray analysis. Oncogene. 2005 Jul 7;24(29):4660–71.

14. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature [Internet]. 2012 Apr 18 [cited 2019 Jun 8];486(7403):346–52. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3440846/

15. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006 Jun 7;295(21):2492–502.

16. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn H-J, et al. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol. 2011 Aug;22(8):1736–47.

17. Calza S, Hall P, Auer G, Bjöhle J, Klaar S, Kronenwett U, et al. Intrinsic molecular signature of breast cancer in a population-based cohort of 412 patients. Breast Cancer Res. 2006;8(4):R34.

18. Dawood S, Hu R, Homes MD, Collins LC, Schnitt SJ, Connolly J, et al. Defining breast cancer prognosis based on molecular phenotypes: results from a large cohort study. Breast Cancer Res Treat. 2011 Feb;126(1):185–92.

19. Lund MJ, Butler EN, Hair BY, Ward KC, Andrews JH, Oprea-Ilies G, et al. Age/race differences in HER2 testing and in incidence rates for breast cancer triple subtypes: a population-based study and first report. Cancer. 2010 Jun 1;116(11):2549–59.

20. Zardavas D, Bozovic-Spasojevic I, de Azambuja E. Dual human epidermal growth factor receptor 2 blockade: another step forward in treating patients with human epidermal growth factor receptor 2-positive breast cancer. Curr Opin Oncol. 2012 Nov;24(6):612–22.

21. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell. 2009 Sep 18;138(6):1083–95.

22. Prat A, Adamo B, Cheang MCU, Anders CK, Carey LA, Perou CM. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. Oncologist. 2013;18(2):123–33.

23. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 2011 Jul;121(7):2750–67.

24. Rakha EA, Chan S. Metastatic triple-negative breast cancer. Clin Oncol (R Coll Radiol). 2011 Nov;23(9):587–600.

25. Dey N, Smith BR, Leyland-Jones B. Targeting basal-like breast cancers. Curr Drug Targets. 2012 Nov;13(12):1510–24.

26. Polyak K. Breast cancer: origins and evolution. J Clin Invest [Internet]. 2007 Nov 1 [cited 2019 May 2];117(11):3155–63. Available from: https://www.jci.org/articles/view/33295

27. Lester SC, Bose S, Chen Y-Y, Connolly JL, de Baca ME, Fitzgibbons PL, et al. Protocol for the examination of specimens from patients with ductal carcinoma in situ of the breast. Arch Pathol Lab Med. 2009 Jan;133(1):15–25.

28. Weinberg RA. The biology of cancer. 2014.

29. Russo J. The Pathobiology of Breast Cancer [Internet]. Cham: Springer International Publishing; 2016 [cited 2019 May 1]. Available from: http://link.springer.com/10.1007/978-3-319-40815-6

30. Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nature Medicine [Internet]. 2000 Jan [cited 2019 May 1];6(1):100. Available from: https://www.nature.com/articles/nm0100_100

31. CDC - What Are the Risk Factors for Breast Cancer? [Internet]. 2018 [cited 2019 Apr 15]. Available from: https://www.cdc.gov/cancer/breast/basic_info/risk_factors.htm

32. Fishman A. The effects of parity, breastfeeding, and infertility treatment on the risk of hereditary breast and ovarian cancer: a review. Int J Gynecol Cancer. 2010 Oct;20(11 Suppl 2):S31-33.

33. Chlebowski RT, Anderson GL. Changing concepts: Menopausal hormone therapy and breast cancer. J Natl Cancer Inst. 2012 Apr 4;104(7):517–27.

34. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015 Mar;65(2):87–108.

35. Puglisi F, Follador A, Minisini AM, Cardellino GG, Russo S, Andreetta C, et al. Baseline staging tests after a new diagnosis of breast cancer: further evidence of their limited indications. Ann Oncol. 2005 Feb;16(2):263–6.

36. Grading breast cancer - Canadian Cancer Society [Internet]. www.cancer.ca. [cited 2019 Jun 12]. Available from: https://www.cancer.ca:443/en/cancer-information/cancer-type/breast/grading/?region=mb

37. Stages of Breast Cancer [Internet]. Memorial Sloan Kettering Cancer Center. [cited 2019 Jun 8]. Available from: https://www.mskcc.org/cancer-care/types/breast/diagnosis/stages-breast

38. Narod SA. Is breast cancer staging obsolete? Curr Oncol [Internet]. 2012 Aug [cited 2019 Apr 29];19(4):195–6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3410827/

39. Subramani R, Nandy SB, Pedroza DA, Lakshmanaswamy R. Role of Growth Hormone in Breast Cancer. Endocrinology [Internet]. 2017 Jun 1 [cited 2019 Mar 4];158(6):1543–55. Available from: https://academic.oup.com/endo/article/158/6/1543/3098660

40. Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 2006 Dec;102(1–5):89–96.

41. Brufsky AM, Dickler MN. Estrogen Receptor-Positive Breast Cancer: Exploiting Signaling Pathways Implicated in Endocrine Resistance. The Oncologist [Internet]. 2018 May 1 [cited 2019 May 23];23(5):528–39. Available from: http://theoncologist.alphamedpress.org/content/23/5/528

42. Green S, Walter P, Greene G, Krust A, Goffin C, Jensen E, et al. Cloning of the human oestrogen receptor cDNA. J Steroid Biochem. 1986 Jan;24(1):77–83.

43. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 1996 Aug 19;392(1):49–53.

44. Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988 May 13;240(4854):889–95.

45. Zhao C, Dahlman-Wright K, Gustafsson J-Å. Estrogen signaling via estrogen receptor {beta}. J Biol Chem. 2010 Dec 17;285(51):39575–9.

46. Mitter D, Ortmann O, Treeck O. [Estrogen receptor Beta isoforms -- functions and clinical relevance in breast cancer]. Zentralbl Gynakol. 2005 Aug;127(4):228–34.

47. Leygue E, Murphy LC. A bi-faceted role of estrogen receptor β in breast cancer. Endocr Relat Cancer. 2013 Jun;20(3):R127-139.

48. Wu X, Subramaniam M, Grygo SB, Sun Z, Negron V, Lingle WL, et al. Estrogen receptor-beta sensitizes breast cancer cells to the anti-estrogenic actions of endoxifen. Breast Cancer Res. 2011 Mar 10;13(2):R27.

49. Lange CA, Yee D. Progesterone and breast cancer. Womens Health (Lond). 2008 Mar;4(2):151–62.

50. Purdie CA, Quinlan P, Jordan LB, Ashfield A, Ogston S, Dewar JA, et al. Progesterone receptor expression is an independent prognostic variable in early breast cancer: a population-based study. Br J Cancer [Internet]. 2014 Feb 4 [cited 2019 Jun 12];110(3):565–72. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3915123/

51. Wang M, Wu X, Chai F, Zhang Y, Jiang J. Plasma prolactin and breast cancer risk: a meta- analysis. Sci Rep [Internet]. 2016 May 17 [cited 2019 May 23];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4869065/

52. Shemanko CS. Prolactin receptor in breast cancer: marker for metastatic risk. J Mol Endocrinol. 2016;57(4):R153–65.

53. Maughan KL, Lutterbie MA, Ham PS. Treatment of breast cancer. Am Fam Physician. 2010 Jun 1;81(11):1339–46.

54. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Davies C, Godwin J, Gray R, Clarke M, Cutter D, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 2011 27;378(9793):771–84.

55. Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive Evaluation of Tamoxifen Sequential Biotransformation by the Human Cytochrome P450 System in Vitro: Prominent Roles for CYP3A and CYP2D6. J Pharmacol Exp Ther [Internet]. 2004 Sep 1 [cited 2019 May 23];310(3):1062–75. Available from: http://jpet.aspetjournals.org/content/310/3/1062

56. Shagufta null, Ahmad I. Tamoxifen a pioneering drug: An update on the therapeutic potential of tamoxifen derivatives. Eur J Med Chem. 2018 Jan 1;143:515–31.

57. Quirke VM. Tamoxifen from Failed Contraceptive Pill to Best-Selling Breast Cancer Medicine: A Case-Study in Pharmaceutical Innovation. Front Pharmacol [Internet]. 2017 Sep 12 [cited 2019 May 23];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5600945/

58. Higgins MJ, Baselga J. Targeted therapies for breast cancer. J Clin Invest. 2011 Oct;121(10):3797–803.

59. Targeted Therapy for Breast Cancer [Internet]. [cited 2019 May 23]. Available from: https://www.cancer.org/cancer/breast-cancer/treatment/targeted-therapy-for-breast-cancer.html

60. Greene J, Hennessy B. The role of anthracyclines in the treatment of early breast cancer. J Oncol Pharm Pract. 2015 Jun;21(3):201–12.

61. Manning HC, Buck JR, Cook RS. Mouse Models of Breast Cancer: Platforms for Discovering Precision Imaging Diagnostics and Future Cancer Medicine. J Nucl Med

[Internet]. 2016 Feb 1 [cited 2019 Mar 4];57(Supplement 1):60S-68S. Available from: http://jnm.snmjournals.org/content/57/Supplement_1/60S

62. Daniel VC, Marchionni L, Hierman JS, Rhodes JT, Devereux WL, Rudin CM, et al. A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture in vitro. Cancer Res. 2009 Apr 15;69(8):3364–73.

63. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. Nat Rev Drug Discov. 2006 Sep;5(9):741–54.

64. Frese KK, Tuveson DA. Maximizing mouse cancer models. Nat Rev Cancer. 2007 Sep;7(9):645–58.

65. Tentler JJ, Tan AC, Weekes CD, Jimeno A, Leong S, Pitts TM, et al. Patient-derived tumour xenografts as models for oncology drug development. Nat Rev Clin Oncol. 2012 Apr 17;9(6):338–50.

66. DeRose YS, Wang G, Lin Y-C, Bernard PS, Buys SS, Ebbert MTW, et al. Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. Nat Med. 2011 Oct 23;17(11):1514–20.

67. DeRose YS, Gligorich KM, Wang G, Georgelas A, Bowman P, Courdy SJ, et al. Patient-derived models of human breast cancer: protocols for in vitro and in vivo applications in tumor biology and translational medicine. Curr Protoc Pharmacol. 2013 Mar;Chapter 14:Unit14.23.

68. Morton CL, Houghton PJ. Establishment of human tumor xenografts in immunodeficient mice. Nat Protoc. 2007;2(2):247–50.

69. Pfefferle AD, Herschkowitz JI, Usary J, Harrell JC, Spike BT, Adams JR, et al. Transcriptomic classification of genetically engineered mouse models of breast cancer identifies human subtype counterparts. Genome Biol. 2013 Nov 12;14(11):R125.

70. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, Heppner GH. Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res. 1978 Oct;38(10):3174–81.

71. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res. 1992 Mar 15;52(6):1399–405.

72. Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. Curr Protoc Immunol. 2001 May;Chapter 20:Unit 20.2.

73. Sugiura K, Stock CC. Studies in a tumor spectrum. I. Comparison of the action of methylbis (2-chloroethyl)amine and 3-bis(2-chloroethyl)aminomethyl-4-methoxymethyl - 5-hydroxy-6-methylpyridine on the growth of a variety of mouse and rat tumors. Cancer. 1952 Mar;5(2):382–402.

74. Ewens A, Mihich E, Ehrke MJ. Distant Metastasis from Subcutaneously Grown E0771 Medullary Breast Adenocarcinoma. ANTICANCER RESEARCH. 2005;11.

75. Murphy K, Weaver C. Janeway's immunobiology. 2017.

76. Chaplin DD. Overview of the immune response. J Allergy Clin Immunol. 2010 Feb;125(2 Suppl 2):S3-23.

77. Dinarello CA. Historical Review of Cytokines. Eur J Immunol [Internet]. 2007 Nov [cited 2019 Jan 25];37(Suppl 1):S34–45. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3140102/

78. Wills-Karp M. Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. Proc Am Thorac Soc. 2007 Jul;4(3):247–51.

79. Sun JC, Lanier LL. Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity? Eur J Immunol. 2009 Aug;39(8):2059–64.

80. Chien Y, Jores R, Crowley MP. RECOGNITION BY γ/δ T CELLS. Annu Rev Immunol [Internet]. 1996 Apr 1 [cited 2019 Jan 25];14(1):511–32. Available from: https://www.annualreviews.org/doi/10.1146/annurev.immunol.14.1.511

81. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4 [Internet]. Journal of Immunology Research. 2012 [cited 2019 Feb 1]. Available from: https://www.hindawi.com/journals/jir/2012/925135/

82. Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^+$T cells: differentiation and functions. Clin Dev Immunol. 2012;2012:925135.

83. Koretzky GA. Multiple Roles of CD4 and CD8 in T Cell Activation. The Journal of Immunology [Internet]. 2010 Sep 1 [cited 2019 Jan 25];185(5):2643–4. Available from: http://www.jimmunol.org/content/185/5/2643

84. Schroeder HW, Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010 Feb;125(2 Suppl 2):S41-52.

85. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002 Nov;3(11):991–8.

86. Tu MM, Rahim MMA, Sayed C, Mahmoud AB, Makrigiannis AP. Immunosurveillance and Immunoediting of Breast Cancer via Class I MHC Receptors. Cancer Immunol Res [Internet]. 2017 Nov 1 [cited 2019 Jan 15];5(11):1016–28. Available from: http://cancerimmunolres.aacrjournals.org/content/5/11/1016

87. Dunn GP, Old LJ, Schreiber RD. The Three Es of Cancer Immunoediting. Annual Review of Immunology [Internet]. 2004 [cited 2019 Jan 15];22(1):329–60. Available from: https://doi.org/10.1146/annurev.immunol.22.012703.104803

88. Law AMK, Lim E, Ormandy CJ, Gallego-Ortega D. The innate and adaptive infiltrating immune systems as targets for breast cancer immunotherapy. Endocrine-Related Cancer [Internet]. 2017 Apr 1 [cited 2019 Jan 14];24(4):R123–44. Available from: https://erc.bioscientifica.com/view/journals/erc/24/4/R123.xml

89. Clarkson RWE, Wayland MT, Lee J, Freeman T, Watson CJ. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. Breast Cancer Res. 2004;6(2):R92-109.

90. Stein T, Salomonis N, Nuyten DSA, van de Vijver MJ, Gusterson BA. A mouse mammary gland involution mRNA signature identifies biological pathways potentially associated with breast cancer metastasis. J Mammary Gland Biol Neoplasia. 2009 Jun;14(2):99–116.

91. Dushyanthen S, Beavis PA, Savas P, Teo ZL, Zhou C, Mansour M, et al. Relevance of tumor-infiltrating lymphocytes in breast cancer. BMC Medicine [Internet]. 2015 Aug 24 [cited 2019 Jan 25];13(1):202. Available from: https://doi.org/10.1186/s12916-015-0431-3

92. Gobert M, Treilleux I, Bendriss-Vermare N, Bachelot T, Goddard-Leon S, Arfi V, et al. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer Res. 2009 Mar 1;69(5):2000–9.

93. Ruffell B, DeNardo DG, Affara NI, Coussens LM. Lymphocytes in cancer development: polarization towards pro-tumor immunity. Cytokine Growth Factor Rev. 2010 Feb;21(1):3–10.

94. Zamarron BF, Chen W. Dual roles of immune cells and their factors in cancer development and progression. Int J Biol Sci. 2011;7(5):651–8.

95. Emens LA, Kok M, Ojalvo LS. Targeting the programmed cell death-1 pathway in breast and ovarian cancer. Curr Opin Obstet Gynecol. 2016 Apr;28(2):142–7.

96. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer discovery [Internet]. 2011 Jun [cited 2019 Jan 25];1(1):54–67. Available from: https://ohsu.pure.elsevier.com/en/publications/leukocyte-complexity-predicts-breast-cancer-survival-and-function-2

97. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity. 2013 Apr 18;38(4):729–41.

98. Demaria S, Volm MD, Shapiro RL, Yee HT, Oratz R, Formenti SC, et al. Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. Clin Cancer Res. 2001 Oct;7(10):3025–30.

99. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998 Mar 5;392(6671):86–9.

100. Fonteneau JF, Larsson M, Bhardwaj N. Interactions between dead cells and dendritic cells in the induction of antiviral CTL responses. Curr Opin Immunol. 2002 Aug;14(4):471–7.

101. Luen SJ, Savas P, Fox SB, Salgado R, Loi S. Tumour-infiltrating lymphocytes and the emerging role of immunotherapy in breast cancer. Pathology. 2017 Feb;49(2):141–55.

102. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012 Mar 22;12(4):252–64.

103. Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, et al. Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. Ann N Y Acad Sci. 2013 Jul;1291:1–13.

104. Müller P, Kreuzaler M, Khan T, Thommen DS, Martin K, Glatz K, et al. Trastuzumab emtansine (T-DM1) renders HER2+ breast cancer highly susceptible to CTLA-4/PD-1 blockade. Sci Transl Med. 2015 Nov 25;7(315):315ra188.

105. Loi S, Dushyanthen S, Beavis PA, Salgado R, Denkert C, Savas P, et al. RAS/MAPK Activation Is Associated with Reduced Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancer: Therapeutic Cooperation Between MEK and PD-1/PD-L1 Immune Checkpoint Inhibitors. Clin Cancer Res. 2016 Mar 15;22(6):1499–509.

106. Zacharakis N, Chinnasamy H, Black M, Xu H, Lu Y-C, Zheng Z, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. Nat Med. 2018 Jun;24(6):724–30.

107. Bajgain P, Tawinwung S, D'Elia L, Sukumaran S, Watanabe N, Hoyos V, et al. CAR T cell therapy for breast cancer: harnessing the tumor milieu to drive T cell activation. Journal for ImmunoTherapy of Cancer [Internet]. 2018 May 10 [cited 2019 Jan 20];6(1):34. Available from: https://doi.org/10.1186/s40425-018-0347-5

108. Benedetti R, Dell'Aversana C, Giorgio C, Astorri R, Altucci L. Breast Cancer Vaccines: New Insights. Front Endocrinol (Lausanne) [Internet]. 2017 Oct 13 [cited 2019 Jan 20];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5645504/

109. Weigel MT, Dowsett M. Current and emerging biomarkers in breast cancer: prognosis and prediction. Endocrine-Related Cancer [Internet]. 2010 Dec 1 [cited 2019 Mar 4];17(4):R245–62. Available from: https://erc.bioscientifica.com/view/journals/erc/17/4/R245.xml

110. Duffy MJ, Harbeck N, Nap M, Molina R, Nicolini A, Senkus E, et al. Clinical use of biomarkers in breast cancer: Updated guidelines from the European Group on Tumor Markers (EGTM). European Journal of Cancer [Internet]. 2017 Apr 1 [cited 2019 May 22];75:284–98. Available from: https://www.ejcancer.com/article/S0959-8049(17)30075-8/abstract

111. Lacroix M. Significance, detection and markers of disseminated breast cancer cells. Endocrine-Related Cancer [Internet]. 2006 Dec 1 [cited 2019 Jul 15];13(4):1033–67. Available from: https://erc.bioscientifica.com/view/journals/erc/13/4/0131033.xml

112. Hassan MI, Waheed A, Yadav S, Singh TP, Ahmad F. Prolactin inducible protein in cancer, fertility and immunoregulation: structure, function and its clinical implications. Cell Mol Life Sci. 2009 Feb;66(3):447–59.

113. Shiu RP, Iwasiow BM. Prolactin-inducible proteins in human breast cancer cells. J Biol Chem. 1985 Sep 15;260(20):11307–13.

114. Murphy LC, Tsuyuki D, Myal Y, Shiu RP. Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP). Regulation of PIP gene expression in the human breast cancer cell line, T-47D. J Biol Chem. 1987 Nov 5;262(31):15236–41.

115. Shiu RP, Murphy LC, Tsuyuki D, Myal Y, Lee-Wing M, Iwasiow B. Biological actions of prolactin in human breast cancer. Recent Prog Horm Res. 1987;43:277–303.

116. Haagensen DE, Mazoujian G, Dilley WG, Pedersen CE, Kister SJ, Wells SA. Breast gross cystic disease fluid analysis. I. Isolation and radioimmunoassay for a major component protein. J Natl Cancer Inst. 1979 Feb;62(2):239–47.

117. Fiel MI, Cernaianu G, Burstein DE, Batheja N. Value of GCDFP-15 (BRST-2) as a specific immunocytochemical marker for breast carcinoma in cytologic specimens. Acta Cytol. 1996 Aug;40(4):637–41.

118. Bhargava R, Beriwal S, Dabbs DJ. Mammaglobin vs GCDFP-15: an immunohistologic validation survey for sensitivity and specificity. Am J Clin Pathol. 2007 Jan;127(1):103–13.

119. Yan Z, Gidley J, Horton D, Roberson J, Eltoum IE, Chhieng DC. Diagnostic utility of mammaglobin and GCDFP-15 in the identification of metastatic breast carcinoma in fluid specimens. Diagn Cytopathol. 2009 Jul;37(7):475–8.

120. Le Doussal V, Zangerle PF, Collette J, Spyratos F, Hacene K, Briere M, et al. Immunohistochemistry of a component protein of the breast cystic disease fluid with mol. wt 15,000. Eur J Cancer Clin Oncol. 1985 Jun;21(6):715–25.

121. Wick MR, Lillemoe TJ, Copland GT, Swanson PE, Manivel JC, Kiang DT. Gross cystic disease fluid protein-15 as a marker for breast cancer: immunohistochemical analysis of 690 human neoplasms and comparison with alpha-lactalbumin. Hum Pathol. 1989 Mar;20(3):281–7.

122. Clark JW, Snell L, Shiu RP, Orr FW, Maitre N, Vary CP, et al. The potential role for prolactin-inducible protein (PIP) as a marker of human breast cancer micrometastasis. Br J Cancer. 1999 Nov;81(6):1002–8.

123. Baniwal SK, Chimge N-O, Jordan VC, Tripathy D, Frenkel B. Prolactin-induced protein (PIP) regulates proliferation of luminal A type breast cancer cells in an estrogen-independent manner. PLoS ONE. 2014;8(6):e62361.

124. Naderi A, Vanneste M. Prolactin-Induced Protein Is Required for Cell Cycle Progression in Breast Cancer. Neoplasia [Internet]. 2014 May 24 [cited 2019 Mar 9];16(4):329-342.e14. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4094838/

125. Hähnel R, Hähnel E. Expression of the PIP/GCDFP-15 gene and survival in breast cancer. Virchows Arch. 1996 Dec;429(6):365–9.

126. Pagani A, Sapino A, Eusebi V, Bergnolo P, Bussolati G. PIP/GCDFP-15 gene expression and apocrine differentiation in carcinomas of the breast. Virchows Arch. 1994;425(5):459–65.

127. Fritzsche FR, Thomas A, Winzer K-J, Beyer B, Dankof A, Bellach J, et al. Co-expression and prognostic value of gross cystic disease fluid protein 15 and mammaglobin in primary breast cancer. Histol Histopathol. 2007;22(11):1221–30.

128. Darb-Esfahani S, von Minckwitz G, Denkert C, Ataseven B, Högel B, Mehta K, et al. Gross cystic disease fluid protein 15 (GCDFP-15) expression in breast cancer subtypes. BMC Cancer [Internet]. 2014 Jul 28 [cited 2019 Mar 9];14. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4122770/

129. Satoh F, Umemura S, Osamura RY. Immunohistochemical analysis of GCDFP-15 and GCDFP-24 in mammary and non-mammary tissue. Breast Cancer. 2000 Jan;7(1):49–55.

130. Luo M-H, Huang Y-H, Ni Y-B, Tsang JYS, Chan S-K, Shao M-M, et al. Expression of mammaglobin and gross cystic disease fluid protein-15 in breast carcinomas. Hum Pathol. 2013 Jul;44(7):1241–50.

131. Jablonska K, Grzegrzolka J, Podhorska-Okolow M, Stasiolek M, Pula B, Olbromski M, et al. Prolactin-induced protein as a potential therapy response marker of adjuvant chemotherapy in breast cancer patients. Am J Cancer Res. 2016;6(5):878–93.

132. Urbaniak A, Jablonska K, Podhorska-Okolow M, Ugorski M, Dziegiel P. Prolactin-induced protein (PIP)-characterization and role in breast cancer progression. Am J Cancer Res [Internet]. 2018 Nov 1 [cited 2019 Mar 4];8(11):2150–64. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6291655/

133. Gangadharan A, Nyirenda T, Patel K, Jaimes-Delgadillo N, Coletta D, Tanaka T, et al. Prolactin Induced Protein (PIP) is a potential biomarker for early stage and malignant breast cancer. Breast. 2018 Jun;39:101–9.

134. Lehmann-Che J, Hamy A-S, Porcher R, Barritault M, Bouhidel F, Habuellelah H, et al. Molecular apocrine breast cancers are aggressive estrogen receptor negative tumors overexpressing either HER2 or GCDFP15. Breast Cancer Res. 2013 May 11;15(3):R37.

135. Honma N, Takubo K, Akiyama F, Sawabe M, Arai T, Younes M, et al. Expression of GCDFP-15 and AR decreases in larger or node-positive apocrine carcinomas of the breast. Histopathology. 2005 Aug;47(2):195–201.

136. Vranic S, Tawfik O, Palazzo J, Bilalovic N, Eyzaguirre E, Lee LM, et al. EGFR and HER-2/neu expression in invasive apocrine carcinoma of the breast. Mod Pathol. 2010 May;23(5):644–53.

137. Baniwal SK, Little GH, Chimge N-O, Frenkel B. Runx2 Controls a Feed-forward loop between Androgen and Prolactin-induced Protein (PIP) in Stimulating T47D Cell Proliferation. J Cell Physiol [Internet]. 2012 May [cited 2019 Mar 9];227(5):2276–82. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3376385/

138. Rathman WM, Van MZ, Van P den K, Bank RA, Veerman EC, Nieuw AA. Isolation and characterization of three non-mucinous human salivary proteins with affinity for hydroxyapatite. J Biol Buccale [Internet]. 1989 Sep [cited 2019 Mar 9];17(3):199–208. Available from: http://europepmc.org/abstract/med/2553688

139. Schenkels LCPM, Ligtenberg AJM, Veerman ECI, Van Nieum Amerongen A. Interaction of the Salivary Glycoprotein EP-GP with the Bacterium Streptococcus salivarius HB. J Dent Res [Internet]. 1993 Dec 1 [cited 2019 Mar 9];72(12):1559–65. Available from: https://doi.org/10.1177/00220345930720120501

140. Autiero M, Abrescia P, Guardiola J. Interaction of seminal plasma proteins with cell surface antigens: Presence of a CD4-binding glycoprotein in human seminal plasma. Experimental Cell Research [Internet]. 1991 Dec 1 [cited 2019 Mar 9];197(2):268–71. Available from: http://www.sciencedirect.com/science/article/pii/001448279190432T

141. Myal Y, Robinson DB, Iwasiow B, Tsuyuki D, Wong P, Shiu RPC. The prolactin-inducible protein (PIP/GCDFP-15) gene: Cloning, structure and regulation. Molecular and Cellular Endocrinology [Internet]. 1991 Sep 1 [cited 2019 May 23];80(1):165–75. Available from: http://www.sciencedirect.com/science/article/pii/030372079190153J

142. Mazoujian G, Pinkus GS, Davis S, Haagensen DE. Immunohistochemistry of a gross cystic disease fluid protein (GCDFP-15) of the breast. A marker of apocrine epithelium and breast carcinomas with apocrine features. Am J Pathol [Internet]. 1983 Feb [cited 2019 Mar 9];110(2):105–12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1916150/

143. Myal Y, Shiu RPC. Myal Y and Shiu RPC (2000). The physiology and pathology of an apocrine protein: the Prolactin-Inducible Protein (PIf)/Gross Cystic Disease Fluid Protein (GCDFP- 15). In: Recent Research Developments in Endocrinology. India: Transworld Research Network; 2000.

144. Blais Y, Gingras S, Haagensen DE, Labrie F, Simard J. Interleukin-4 and interleukin-13 inhibit estrogen-induced breast cancer cell proliferation and stimulate GCDFP-15 expression in human breast cancer cells. Molecular and Cellular Endocrinology [Internet].

1996 Jul 23 [cited 2019 Mar 9];121(1):11–8. Available from: http://www.sciencedirect.com/science/article/pii/0303720796038439

145. Kitano T, Tian W, Umetsu K, Yuasa I, Yamazaki K, Saitou N, et al. Origin and evolution of gene for prolactin-induced protein. Gene. 2006 Nov 15;383:64–70.

146. Osawa M, Horiuchi H, Tian W, Kaneko M. Divergent evolution of the prolactin-inducible protein gene and related genes in the mouse genome. Gene. 2004 Jan 21;325:179–86.

147. Mirels L, Hand AR, Branin HJ. Expression of gross cystic disease fluid protein-15/Prolactin-inducible protein in rat salivary glands. J Histochem Cytochem. 1998 Sep;46(9):1061–71.

148. Windass JD, Mullins JJ, Beecroft LJ, George H, Meacock PA, Williams BR, et al. Molecular cloning of cDNAs from androgen-independent mRNA species of DBA/2 mouse sub-maxillary glands. Nucleic Acids Res [Internet]. 1984 Feb 10 [cited 2019 Mar 9];12(3):1361–76. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC318582/

149. Myal Y, Iwasiow B, Yarmill A, Harrison E, Paterson JA, Shiu RP. Tissue-specific androgen-inhibited gene expression of a submaxillary gland protein, a rodent homolog of the human prolactin-inducible protein/GCDFP-15 gene. Endocrinology. 1994 Oct;135(4):1605–10.

150. Ihedioha OC. PROLACTIN-INDUCIBLE-PROTEIN (PIP) INFLUENCES HOST IMMUNITY BY REGULATING INTRACELLULAR SIGNALING PATHWAYS IN MACROPHAGES [MSc]. [Winnipeg]: University of Manitoba; 2015.

151. Lee B, Bowden GHW, Myal Y. Identification of mouse submaxillary gland protein in mouse saliva and its binding to mouse oral bacteria. Arch Oral Biol. 2002 Apr;47(4):327–32.

152. Caputo E, Manco G, Mandrich L, Guardiola J. A novel aspartyl proteinase from apocrine epithelia and breast tumors. J Biol Chem. 2000 Mar 17;275(11):7935–41.

153. Schenkels LC, Schaller J, Walgreen-Weterings E, Schadee-Eestermans IL, Veerman EC, Nieuw Amerongen AV. Identity of human extra parotid glycoprotein (EP-GP) with secretory actin binding protein (SABP) and its biological properties. Biol Chem Hoppe-Seyler. 1994 Sep;375(9):609–15.

154. Schenkels LC, Rathman WM, Veerman EC, Nieuw Amerongen AV. Detection of proteins related to a salivary glycoprotein (EP-GP). Concentrations in human secretions (saliva, sweat, tears, nasal mucus, cerumen, seminal plasma). Biol Chem Hoppe-Seyler. 1991 May;372(5):325–9.

155. Hassan MI, Kumar V, Singh TP, Yadav S. Purification and characterization of zinc alpha2-glycoprotein-prolactin inducible protein complex from human seminal plasma. J Sep Sci. 2008 Jul;31(12):2318–24.

156. Chiu WW-C, Chamley LW. Human seminal plasma prolactin-inducible protein is an immunoglobulin G-binding protein. J Reprod Immunol. 2003 Dec;60(2):97–111.

157. Kumar S, Tomar AK, Singh S, Saraswat M, Singh S, Singh TP, et al. Human serum albumin as a new interacting partner of prolactin inducible protein in human seminal plasma. Int J Biol Macromol. 2012 Mar 1;50(2):317–22.

158. Umadat V, Ihedioha O, Shiu R, Uzonna J, Myal Y. The prolactin-inducible-protein (PIP): A regulatory molecule in adaptive and innate immunity. Open Journal of Immunology [Internet]. 2013 Dec 11 [cited 2019 Mar 9];03:210. Available from: https://www.scirp.org/journal/PaperInformation.aspx?PaperID=40696&#abstract

159. Haagensen DE, Mazoujian G. Diseases of the Breast. Philadelphia: W.B. Saunders; 1986.

160. Schenkels LC, Walgreen-Weterings E, Oomen LC, Bolscher JG, Veerman EC, Nieuw Amerongen AV. In vivo binding of the salivary glycoprotein EP-GP (identical to GCDFP-15) to oral and non-oral bacteria detection and identification of EP-GP binding species. Biol Chem. 1997 Feb;378(2):83–8.

161. Zhou W, König R. T cell receptor-independent CD4 signalling: CD4-MHC class II interactions regulate intracellular calcium and cyclic AMP. Cell Signal. 2003 Aug;15(8):751–62.

162. Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, et al. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature. 1984 Jan 20;312(5996):767–8.

163. Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell. 1986 Nov 7;47(3):333–48.

164. McDougal JS, Nicholson JK, Cross GD, Cort SP, Kennedy MS, Mawle AC. Binding of the human retrovirus HTLV-III/LAV/ARV/HIV to the CD4 (T4) molecule: conformation dependence, epitope mapping, antibody inhibition, and potential for idiotypic mimicry. J Immunol. 1986 Nov 1;137(9):2937–44.

165. Autiero M, Gaubin M, Mani JC, Castejon C, Martin M, el Marhomy S, et al. Surface plasmon resonance analysis of gp17, a natural CD4 ligand from human seminal plasma inhibiting human immunodeficiency virus type-1 gp120-mediated syncytium formation. Eur J Biochem. 1997 Apr 1;245(1):208–13.

166. Gaubin M, Autiero M, Basmaciogullari S, Métivier D, Mis hal Z, Culerrier R, et al. Potent inhibition of CD4/TCR-mediated T cell apoptosis by a CD4-binding glycoprotein secreted from breast tumor and seminal vesicle cells. J Immunol. 1999 Mar 1;162(5):2631–8.

167. Autiero M, Cammarota G, Friedlein A, Zulauf M, Chiappetta G, Dragone V, et al. A 17-kDa CD4-binding glycoprotein present in human seminal plasma and in breast tumor cells. Eur J Immunol. 1995 May;25(5):1461–4.

168. Ihedioha OC, Shiu RPC, Uzonna JE, Myal Y. Prolactin-Inducible Protein: From Breast Cancer Biomarker to Immune Modulator—Novel Insights from Knockout Mice. DNA and Cell Biology [Internet]. 2016 Oct [cited 2019 Mar 4];35(10):537–41. Available from: http://www.liebertpub.com/doi/10.1089/dna.2016.3472

169. Li J, Liu D, Mou Z, Ihedioha OC, Blanchard A, Jia P, et al. Deficiency of prolactin-inducible protein leads to impaired Th1 immune response and susceptibility to Leishmania major in mice. Eur J Immunol. 2015 Apr;45(4):1082–91.

170. Blanchard A, Nistor A, Castaneda FE, Martin D, Hicks GG, Amara F, et al. Generation and initial characterization of the prolactin-inducible protein (PIP) null mouse: accompanying global changes in gene expression in the submandibular gland. Can J Physiol Pharmacol. 2009 Oct;87(10):859–72.

171. Ihedioha O, Blanchard AA, Balhara J, Okwor I, Jia P, Uzonna J, et al. The human breast cancer-associated protein, the prolactin-inducible protein (PIP), regulates intracellular signaling events and cytokine production by macrophages. Immunol Res. 2018;66(2):245–54.

172. Delgado-Ortega M, Marc D, Dupont J, Trapp S, Berri M, Meurens F. SOCS proteins in infectious diseases of mammals. Vet Immunol Immunopathol. 2013 Jan 15;151(1–2):1–19.

173. Conlon KC, Miljkovic MD, Waldmann TA. Cytokines in the Treatment of Cancer. Journal of Interferon & Cytokine Research [Internet]. 2018 Jun 11 [cited 2019 Jul 4];39(1):6–21. Available from: https://www.liebertpub.com/doi/10.1089/jir.2018.0019

174. Naderi A, Meyer M. Prolactin-induced protein mediates cell invasion and regulates integrin signaling in estrogen receptor-negative breast cancer. Breast Cancer Res. 2012 Jul 20;14(4):R111.

175. Xu X, Li J, Zou J, Feng X, Zhang C, Zheng R, et al. Inherited defects in natural killer cells shape tumor immune microenvironment, clinical outcome and immunotherapy response [Internet]. Cancer Biology; 2018 Nov [cited 2019 May 25]. Available from: http://biorxiv.org/lookup/doi/10.1101/471912

176. Liu Y, Kosaka A, Ikeura M, Kohanbash G, Fellows-Mayle W, Snyder LA, et al. Premetastatic soil and prevention of breast cancer brain metastasis. Neuro-oncology. 2013 Jul;15(7):891–903.

177. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A [Internet]. 2001 Sep 11 [cited 2019 Jan 24];98(19):10869–74. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC58566/

178. Jia D, Entersz I, Butler C, Foty RA. Fibronectin matrix-mediated cohesion suppresses invasion of prostate cancer cells. BMC Cancer. 2012 Mar 20;12:94.

179. Glasner A, Levi A, Enk J, Isaacson B, Viukov S, Orlanski S, et al. NKp46 Receptor-Mediated Interferon-γ Production by Natural Killer Cells Increases Fibronectin 1 to Alter Tumor Architecture and Control Metastasis. Immunity. 2018 16;48(1):107-119.e4.