Exosome Mediated Purinergic Mechanisms in Breast Cancer

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry

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THE GRADUATE SCHOOL

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Abstract:

Breast cancer is the most diagnosed cancer and the second leading cause of cancer-related deaths in women. Majority of these deaths are due to metastasis, proving that there is a great demand to understanding the mechanisms underlying metastasis. One factor released by breast cancer cells called nucleoside diphosphate kinase (NDPK) has been shown to stimulate endothelial cell proliferation, migration, and tumor-mediated angiogenesis. Extracellular NDPK generates ATP in the vicinity of the breast cancer cells to activate purinergic P2Y1 receptors on adjacent endothelial cells. The P2Y1 transactivates the VEGFR-2 on the endothelial cells to promote angiogenesis. Although NDPK was detected in conditioned media of breast cancer cells and in the serum of mice bearing tumors; the appearance of extracellular NDPK in the local tumor microenvironment has remained a mystery. We proposed that NDPK travels out of the cancer cells by small extracellular vesicles called exosomes. Exosomes were purified from breast cancer cells and characterized by transmission electron microscopy, flow cytometry, western blot analysis. These signaling vectors were also examined for angiogenic response by a tubulogenesis assay with inhibitors of NDPK and P2Y1. Further development for NDPK inhibitors were tested with a drug screen utilizing the NDPK transphosphorylation assay to identify novel small molecules that could inhibit NDPK. The in vivo role of NDPK was examined with two orthotopic mouse models while tracking the growth and development of the primary tumors and metastases. Both models were also treated with inhibitors of NDPK and P2Y1. NDPK was associated with exosomes from breast
cancer cells and could stimulate tubulogenesis in endothelial cells. While ellagic acid (EA) is a known inhibitor of NDPK, several other small molecule drugs were identified as positive candidates. Treatment with EA and MRS2179, an antagonist of the P2Y1 receptor, decreased primary tumor growth and metastases in both mouse models. Exosomal NDPK supported the formation of blood vessels during tumorigenesis but may also support the growth and development of metastasis. By targeting and measuring extracellular NDPK, we could develop NDPK as a theranostic for the detection and treatment of breast cancer.
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Chapter 1: Introduction

1.1. Background on breast cancer

The chances of developing cancer in a person's lifetime are 1 in 2 in men and 1 in 3 in women. With these rates, it has become increasingly important to deepen our understanding of discovering ways to identify, treat, curing, and prevent cancer. Since the start of the war on cancer in 1971 when the National Cancer Act was established, the United States (US) government has continued to invest funds in research to end cancer. The establishments of the Cancer Moonshot Initiative in 2016, headed by then Vice President Joe Biden, and the launch of the 21st Century Cures Act (114th Congress 2015-2016) in 2017, are reflections of how we are still battling cancer. The field of cancer research has continuously advanced, and scientists have discovered many milestones that have paved our understanding of cancer today.

In the broadest classification, cancer can be divided into five groups based on the cells from which they originate: carcinoma, sarcoma, hematopoietic (lymphoma and leukemia), germinoma, and blastoma [1]. Carcinoma arises from epithelial cells, sarcomas are derived from mesenchymal cells, hematopoietic cancer arises in blood cells, germinoma emerges from cells in the germ line, and blastomas originates from blast or precursor cells. The majority of breast cancer originates in milk ducts as carcinomas. Breast cancer is classified into five subgroups based on molecular
profiling: luminal-A, luminal-B, basal-like, epidermal growth factor receptor-2 (HER-2) positive, and claudin-low [2,3]. This classification can be further characterized by the presence and absence of certain genomic and/or proteomic markers, tumor grade, lymph node status, and histology [4–6]. In addition, breast cancer types can be named by the tissue of origin, such as ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), lobular carcinoma in situ (LCIS), and others. Breast cancer is a largely heterogeneous disease, making it difficult to treat all patients with one type of standardized treatment.

Breast cancer is the most diagnosed cancer in women and the second leading cause of cancer related deaths second only to lung cancer. According to Cancer Statistics, for the 2018 year, an estimated 268,670 new cases will be diagnosed and 40,920 women will die from breast cancer that year [7]. Breast cancer represents 30% of all newly diagnosed cancer cases in women and 14% of cancer deaths in women. The major cause of cancer-related deaths in patients is due to metastatic disease [8]. Following lumpectomy, or the surgical removal of local DCIS, 25% to 30% of women can develop local recurrence within 10 years of surgery [9]. Adjuvant radiation therapy can reduce the risk of recurrence by 50% but does not prolong overall survival. This demonstrates that there is an increasing need to understand breast cancer metastasis and discover alternative methods for treating not only the primary area of cancer but also prevent its spreading as a recurrent disease. In addition to the high number of cases diagnosed, breast cancer is also one of the most well studied cancers.
Throughout medical history, effective treatments to address cancer metastasis have remained elusive. Alfred Armand Louis Marie Velpeau, a French surgeon and clinical chair of surgery at the University of Paris, wrote, “To destroy a cancerous tumor by surgical means is usually an easy matter and little dangerous in itself; but the question arises, whether such a procedure affords a chance of radically curing the patient. This proposition remains undecided. The disease always returns after removal, and operation only accelerates its growth and fatal termination [10].” He described this observation of metastasis in 1853, at a time when both ether [11] and chloroform [12] were introduced as methods of anesthesia during surgery. During this time, European surgical techniques were considered more refined than American surgeries. Henri François Le Dran, a renowned French surgeon, described cancer development as an event that originated in a localized area, but would spread through the lymphatic system and would be detrimental to the patient [13]. At the same time, Jean-Louis Petit was an advocate of total mastectomy surgery that included the removal of the axillary nodes as a preventative to recurrence [14]. This surgical approach would greatly influence breast cancer surgeries for many years. More than a century later, William Steward Halsted popularized radical surgery, which involved the removal of the breast, the axillary nodes, and the major and minor pectoralis muscles [15]. Surgeons would later add other procedures to radical surgery, such as the removal of the ovaries, adrenal glands, and pituitary gland, but these practices were later abandoned, as they were proven ineffective. Radical mastectomy should not be confused with total mastectomy, in which the pectoral muscles and axillary
lymph nodes are preserved during surgery to remove the entire breast [16]. For about fifty years, radical surgery was the accepted practice to remove breast cancer and prevent recurrence. However, as stated in Siddhartha Mukherjee’s book, *The Emperor of All Maladies: A Biography of Cancer*, “More surgery had just not translated into more effective therapy [17].” Moreover, radical surgery was disfiguring and mutilating, which led some patients to experience undue stress during the postoperative recovery period. In direct contrast to the concept of radical surgery, Geoffrey Keynes found that in cases of localized breast cancer, surgery to remove the lump (this procedure eventually became known as the lumpectomy; although at the time, it was meant to be an insult to the surgeon) and adjuvant radiation therapy significantly reduced the risk of recurrence [18,19]. In line with Keynes’ discovery, Bernard Fisher challenged Halsted’s principle of radical surgery by implementing a controlled clinical trial to show that there was no significant improvement in prognosis for patients who underwent radical surgery [20]. Although Fisher’s trial took ten years to complete, the results were staggering; it hastened the demise of the highly popular procedure. Radical mastectomy in its former manner is no longer performed today.

Pierre and Marie Curie were pioneers in X-ray and radiation. Marie Curie was awarded two Nobel Prizes (in physics and chemistry) for the discovery of radium and polonium, as well as the theory of radioactivity [21]. She would go on to implement X-rays as a medical diagnostic tool during World War I. Unbeknownst at the time, X-rays possess the ability to penetrate cells and damage nuclear DNA, leading to cell
death. Radiation would later become an instrumental tool in destroying highly proliferative cells, including cancer cells. In 1896, Emil Grubbe applied this knowledge by using radiation as a form of therapy to treat breast cancer and thus established radiotherapy [22,23].

Paul Ehrlich coined the term “chemotherapy” in the early 1900s, which was the use of chemicals to treat diseases [24–26]. Among some of the first chemicals that Ehrlich used to treat diseases were aniline dyes and alkylating reagents. During World War I, the effects of mustard gas on bone marrow depletion and decreased white blood cell count were documented [27–29]. This fostered research for the development of an antidote to mustard gas, but also sparked the idea that there may be a therapeutic potential with this chemical. This led to Alfred Goodman and Louis Gilman (authors of the original therapeutics text book in pharmacology in its 13th edition today) to use nitrogen mustard injections to treat a patient with lymphosarcoma, leading to the first developments in cancer chemotherapy [26,30]. This eventually led to the discovery of other alkylating reagents, such as chlorambucil and ultimately cyclophosphamide [31,32]. In 1948, folic deficiencies were discovered to exhibit similar bone marrow depletions and pathologies as those affected by nitrogen mustard, and eventually it was found that folic acid could accelerate the progression of leukemia [33]. This spurred the development of anti-folates or folic antagonists, of which methotrexate was one of the most well known [34]. Chemotherapy evolved as a concept to discover chemicals that induced cell death in cancer cells while circumventing healthy cells. Over time chemotherapy would
evolve into combination chemotherapy as an opportunity to treat cancer with multiple anticancer drugs. One of the earliest programs of combination chemotherapy was in the 1960s known as VAMP (the cyclically administered treatment of vincristine, amethopterin, 6-mercaptopurine, and prednisone) to treat children with leukemia with the intention to increase the time of remission [26]. The treatment of Hodgkin’s disease followed similar combination chemotherapy protocols called MOMP and MOPP (nitrogen mustard with vincristine, methotrexate/procarbazine, and prednisone). Combination chemotherapy was so successful in treating childhood leukemia and Hodgkin’s disease that by 1984 the US national mortality rate had dropped by 65%. In the 1970s, combination chemotherapy was tested in advanced breast cancer [35,36]. However, one prominent issue was where to test these reagents as adjuvants with respect to the timing of surgery. During this time, the National Cancer Institute collaborated with the Istituto Nazionale Tumori to conduct a randomized adjuvant breast cancer study using the combination chemotherapy of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) therapy versus no therapy [37]. At the same time, Bernard Fisher, while also challenging Halsted’s principle of radical mastectomy, tested L-phenylalanine mustard (L-PAM) as adjuvant chemotherapy in a similar breast cancer study [38]. Both studies were completed within 5 years, overall results were statistically significant, and opened the field to adjuvant chemotherapy.

The advent of targeted therapy has influenced the field to design drugs that could inhibit metabolic pathways or specific molecular targets in the cancer cell to
induce apoptosis. Targeted therapy can be broadly classified into two groups: small molecule drugs and antibodies. The first example of molecular targeted therapy was BCR-ABL tyrosine kinase inhibitor imatinib to treat chronic myeloid leukemia [39,40]. One of the molecular characteristics of chronic myeloid leukemia is the fusion of chromosome 9 and 22 (the Philadelphia chromosome), which transcribes a constitutively active tyrosine kinase from the merged genes of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homologue 1 (ABL; also known as ABL1). Imatinib blocks the active site of the ABL kinase and inhibits proliferation. In the field of breast cancer, anti-estrogens are an example of targeted therapy aimed at the estrogen receptor. In 1971, Elwood Jensen found that hormone ablative surgery could help patients with ER-rich breast cancer. Researchers now know that luminal-A breast cancer, which is estrogen receptor positive, is the most common diagnosed subtype [2,3] and responds well to anti-estrogen therapy. Tamoxifen was developed as a treatment and preventative therapy for breast cancer [41,42]. In addition, there was the development of trastuzumab (Herceptin), a HER2 receptor-targeted monoclonal antibody for the treatment of HER2 positive breast cancer [43]. As we increase our understanding of the molecular markers of cancer subtypes, we discover unique cancer genomic mutations that can identify patients that will respond to specific targeted therapeutics. With regard to cancer cell response and resistance, the field of targeted therapy will continue to expand on the development of drugs that focus on specific cancer mechanisms.
Immunotherapy is a growing and exciting field in the treatment of some cancers. It is characterized by the ability to harness the immune system to target the cancer through the administration of vaccines or by activation/deactivation of the immune system (also known as checkpoint inhibitors). One of the first examples of a vaccine targeted against cancer is the human papillomavirus (HPV) vaccine used to prevent cervical cancer in women. The current HPV vaccine protects against HPV types 16 and 18, which are known to cause cervical cancer [44]. Although development of a cancer vaccine using only one cancer-specific antigen has been difficult due to cellular heterogeneity of every cancer, there are some unique tumor antigens that are targeted to elicit a tumor specific immune response. Programmed cell death protein-1 (PD-1) and programmed death-ligand 1 (PD-L1) modulating drugs are currently used to treat a number of cancers, such as melanoma, non-small cell lung cancer, kidney cancer, bladder cancer, head and neck cancers, and Hodgkin lymphoma [45]. Blockade of the PD-1 receptor or competitive inhibition of PD-L1 inhibits the repressive function of the pathway and allows for activation of the adaptive immune system by regulatory CD8 T-cells [46,47]. Targeting of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), another immune inhibiting molecule, has also shown promise in combination with a PD-1 inhibitor in antitumor immunity therapy through T-cells [48,49]. Besides checkpoint inhibitor therapy, chimeric antigen receptor T-cell (CART) technology has also become a new platform in the field of immunotherapy. T-cells can be reprogrammed to express chimeric antigen receptors (CARs), such as monoclonal antibodies, to recognize CD19 on malignant
lymphocytes [50]. This technology can be adapted to treat solid tumors, in which the tumor was sequenced for unique mutations that could be used as a target for custom-made T-cells [51]. The manipulation of the immune system to target and kill tumor cells is a promising therapy for treating cancer, but there is still research needed to expand this treatment for the implementation toward additional types of cancer.

The main features of cancer cell development include cell survival, cell proliferation, and impaired cell death. The genetic component has been recognized since the 20th century as one of the most distinguishing molecular features of cancer. Alfred Velpeau wrote, "The so-called cancer cell is merely a secondary product rather than the essential element in the disease. Beneath it, there must exist some more intimate element which science would need in order to define the nature of cancer [10]." He predicted that cancer cells contained genetic mutations before Watson and Crick published the structure of DNA in 1953. The basis of cancer is the accumulation of genetic and/or epigenetic mutations, which can be caused by any of the following: abnormal chromosome numbers, chromosome translocation, accumulation of mutations, imbalanced expression of tumor suppressor genes and oncogenes, and uncontrolled growth stemming from unregulated cell cycle check points. In a recent study from Bert Vogelstein’s lab, ~65% of all cancer mutations are due to random mistakes during cell division [52]. These alterations occur in a step-wise manner during the progression of tumorigenesis. During this time, deregulation of repressive genes can promote the expression of oncogenes, such as c-Myc. According to a genetic lineage analysis by Hosseini et al., even though cancer cells may disseminate very
early on during tumor development, some cells do not possess mutations sufficient
to sustain growth as micro-metastases [53]. The cells that disseminate from the
structured primary tumor are more likely to establish growing metastatic tumors
[54]. What may seem like a disarray of incidences is actually a well-orchestrated
series of events in which oncogenes work together to transform the cell.

Apart from tumor cell survival, another defining characteristic of cancerous
solid tumors is the development of the vasculature around the tumor. Angiogenesis
is the process by which cancer cells attract and stimulate blood vessel growth. Judah
Folkman was one of the first researchers to document that a tumor cannot grow
beyond 2-3mm without a blood supply to deliver nutrients [55]. However, it was not
until the work of Napoleon Ferrara's group that purified and identified vascular
endothelial growth factor (VEGF) as the protein responsible for inducing blood vessel
development [56] that we could appreciate angiogenic signaling. Ferrara's group
showed that by blocking VEGF with an antibody, angiogenesis could be inhibited [57].

Another focus in tumorigenesis is the critical importance of the immediate
environment in which the cancer develops. In 1889, Stephen Paget proposed his seed
and soil hypothesis, stating that circulating cancer cells do not randomly distribute
themselves but instead colonize in specific organs to form secondary tumors [58].
This concept was re-explored again in 1980 by the researchers Ian Hart and Isaiah
Fidler [59]. They grafted kidney, lung, and ovarian tissues subcutaneously and in the
muscle of mice, and then injected melanoma cells into the mice to determine where
these cells would develop metastases. Radiolabelled melanoma cells metastasized
and formed secondary growths in the lung and ovary tissues, but not in the renal tissue. This shows that cancer cells possess the ability to target specific tissues and confirms that there are unique properties in the host tissues to facilitate the growth of the metastases. Perhaps the most impressive demonstration of the role of the extracellular microenvironment on tumor development lies in a set of experiments performed by Mina Bissell. She observed that when retroviral v-src, known to induce tumorigenesis in adult chickens, was injected into a developing chick embryo, the embryo did not develop cancer but continued embryogenesis suggesting that the tissue microenvironment played a role in tumorigenesis [60]. In a “normal” environment the cancer cells would adapt to a normal behavior and function in the host tissue. Bissell’s results attest to our lack of understanding regarding the communication between the normal tissues, their unique and respective microenvironments, and cancer development. By elucidating the mechanisms that control and shape the microenvironment, we may ultimately prevent its contribution to the development of the cancer cells.

Cancer stem cells (CSCs) have gained increased attention since the 1990s as potential targets for therapeutics. The concept focuses on the idea that a stem cell could be the progenitor of all tumor cells. The CSCs have the ability to regenerate the cancer cell populations and differentiate into other cell types and thereby facilitate relapse and cancer metastasis. Addressing cancer stem cells in anti-cancer therapies would target the rare stem cells that could initiate and propagate the heterogeneous growth of the solid tumor.
1.2. Current treatment

Early detection is critical to cancer treatment and management, as is the case with breast cancer. The detection of a smaller tumor size at the time of diagnosis yields a lower rate of recurrence compared to a larger tumor (>2 cm) [61]. Detection of a lump or mass is the most common symptom of breast cancer. Early detection procedures include mammograms, clinical exams, and regular self-examinations. Current treatment for breast cancer is surgery and/or adjuvant therapy, which includes radiation, chemotherapy, and/or molecular targeted therapies. Surgery includes lumpectomy, unilateral mastectomy, and bilateral mastectomy. Molecular targeted therapies include aromatase inhibitors, antibodies, anti-estrogens, kinase inhibitors, mTOR inhibitors, anti-microtubule agents, and others (see Tables 1-1 to 1-4). As described in the previous section, the evolution of breast cancer treatment is a bag of old and new approaches that is constantly changing and adapting.

Table 1-1: Current US Food and Drug Administration (FDA) approved drugs for treating breast cancer by inhibiting DNA replication. These drugs can be used in adjuvant therapy with chemotherapy and/or irradiation. Adapted from https://www.cancer.gov/about-cancer/treatment/drugs/breast. The drugs are listed according to the generic drug name.

<table>
<thead>
<tr>
<th>Generic</th>
<th>Brand</th>
<th>Mechanism of Action</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capecitabine</td>
<td>Xeloda</td>
<td>Metabolized to 5-FU to inhibit the synthesis of thymidine monophosphate</td>
<td>[62]</td>
</tr>
</tbody>
</table>
Cyclophosphamide
Cytoxan injection
Alkylating agent that crosslinks DNA to prevent replication

Doxorubicin
Adriamycin / Doxil
Anthracylene, intercalation of DNA to inhibit topoisomerase II, free radical production, and induction of histone eviction

Epirubicin
Ellence
Anthracylene, intercalation of DNA, inhibition of topoisomerase II, and free radical production

Fluorouracil
Adrucil
Inhibit the synthesis of thymidine monophosphate to block DNA replication

Gemcitabine
Gemzar
Nucleoside analog that blocks deoxyribonucleotide production and DNA replication

Methotrexate
Trexall
Anti-folate that prevents thymidine production and DNA replication

Thiotepa
Tepadina
Alkylating agent that binds alkyl groups to DNA to prevent replication

<table>
<thead>
<tr>
<th>Generic</th>
<th>Brand</th>
<th>Mechanism of Action</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docetaxel</td>
<td>Taxotere</td>
<td>Binds to microtubules and prevents depolymerization</td>
<td>[74]</td>
</tr>
<tr>
<td>Eribulin mesylate</td>
<td>Halaven</td>
<td>Binds to microtubules and induces apoptosis</td>
<td>[75]</td>
</tr>
<tr>
<td>Ixabepilone</td>
<td>Ixempra</td>
<td>Binds to microtubules and induces apoptosis</td>
<td>[76]</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Abraxane / Taxol</td>
<td>Binds to microtubules to stabilize the structure and inhibits spindle function during mitosis</td>
<td>[77]</td>
</tr>
<tr>
<td>Vinblastine</td>
<td></td>
<td>Inhibits formation of microtubules</td>
<td>[78]</td>
</tr>
</tbody>
</table>

Table 1-2: Current FDA approved drugs for treating breast cancer by inhibiting cell division. These drugs can be used in adjuvant therapy with chemotherapy and/or irradiation. Adapted from [https://www.cancer.gov/about-cancer/treatment/drugs/breast](https://www.cancer.gov/about-cancer/treatment/drugs/breast). The drugs are listed according to the generic drug name.

<table>
<thead>
<tr>
<th>Generic</th>
<th>Brand</th>
<th>Mechanism of Action</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Everolimus</td>
<td>Afinitor</td>
<td>Inhibits mTORC1 and downstreaming signaling to inhibit cell growth and proliferation</td>
<td>[79,80]</td>
</tr>
</tbody>
</table>
Lapatinib **Tykerb/Tyverb**
Inhibits the ATP-binding site of HER2 and EGFR receptor tyrosine kinases to block self-phosphorylation and downstream signaling \[81,82\]

Palbociclib **Ibrance**
Inhibition of CDK4 and CDK6 to inhibit cell cycle and slow cell growth \[83\]

**Table 1-4:** Current FDA approved drugs for treating breast cancer by receptor inhibition or hormone mediation. These drugs can be used in adjuvant therapy with chemotherapy and/or irradiation. Adapted from https://www.cancer.gov/about-cancer/treatment/drugs/breast. The drugs are listed according to the generic drug name.

<table>
<thead>
<tr>
<th>Generic</th>
<th>Brand</th>
<th>Mechanism of Action</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ado-trastuzumab emtansine</td>
<td>Kadcyla</td>
<td>Antibody-drug conjugate/chemotherapy, binds to Her2 receptor and delivers emtansine, a cytotoxic agent</td>
<td>[84]</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>Arimidex</td>
<td>Inhibits conversion of androgens to estrogens by binding to the heme group on aromatase</td>
<td>[85]</td>
</tr>
<tr>
<td>Conjugated estrogens</td>
<td>Premarin</td>
<td>Agonist of estrogen receptor</td>
<td>[86]</td>
</tr>
<tr>
<td>Estradiol</td>
<td>Estrace</td>
<td>Agonist of estrogen receptor</td>
<td>[86]</td>
</tr>
<tr>
<td>Exemestane</td>
<td>Aromasin</td>
<td>Irreversibly inhibits aromatase to block conversion of androgens to estrogens</td>
<td>[87]</td>
</tr>
<tr>
<td>Fulvestrant</td>
<td>Faslodex</td>
<td>Selective estrogen receptor degrader by destabilizing it for protein degradation</td>
<td>[88]</td>
</tr>
<tr>
<td>Goserelin</td>
<td>Zoladex</td>
<td>Gonadotropin-releasing hormone analogue, luteinizing hormone releasing hormone agonist, stimulates the production of testosterone and estrogen and disrupts the hormonal feedback system</td>
<td>[89]</td>
</tr>
<tr>
<td>Letrozole</td>
<td>Femara</td>
<td>Inhibits conversion of androgens to estrogens by binding to the heme group on aromatase</td>
<td>[90]</td>
</tr>
<tr>
<td>Megestrol acetate</td>
<td>Megace/Megace ES</td>
<td>Agonist of progesterone receptor, anti-gonadotropic and anti-androgenic activity to decrease hormone receptors</td>
<td>[86]</td>
</tr>
<tr>
<td>Pertuzumab</td>
<td>Perjeta</td>
<td>Prevents dimerization of HER2/HER3 to block downstream signaling</td>
<td>[91]</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>Evista</td>
<td>Selective regulation of estrogenic pathways depending on tissue</td>
<td>[92]</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Nolvadex/Soltamox</td>
<td>Metabolized to 4-hydroxytestosterone, estrogen receptor antagonist</td>
<td>[93]</td>
</tr>
<tr>
<td>Toremifene</td>
<td>Fareston</td>
<td>Selective estrogen receptor modulator</td>
<td>[94]</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Herceptin</td>
<td>Binds to Her2 receptor and blocks downstream signaling to reduce proliferation</td>
<td>[95]</td>
</tr>
</tbody>
</table>
According to the National Cancer Institute, the Food and Drug Administration (FDA)-approved biomarkers for breast cancer include epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER) and progesterone receptor (PR), cancer antigen (CA) 15-3, CA27-29, and cytokeratins [96–99]. HER-2 detection can be assayed by immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and fluorescence in situ hybridization (FISH) assay [100]. CA15-3 and CA27-29 are tumor markers to detect recurrence of breast cancer. HER-2, ER, and PR are biomarkers to determine treatment options to target these receptors. In addition, the first FDA approved multi-marker panels to evaluate the patient response to certain treatments and risk of recurrence are MammaPrint (a 70 gene expression panel) [101] and Oncotype Dx (a 21 gene expression panel) [102]. Along with Oncotype Dx, EndoPredict, PAM50, and Breast Cancer Index have been suggested by the 2016 American Society of Clinical Oncology (ASCO) Clinical Practice Guideline for clinical utilization of these biomarker assays for the determination of adjuvant treatment of certain breast cancer subtypes [103]. In the same paper, even though the studies met the criteria guidelines for clinical utility, specific treatments or regimens were not recommended. Even with the large increase in proteomic and genomic studies/papers in breast cancer research, there is a lack of reliable biomarkers for diagnostics. A review by Chang et al., found that out of 941 recommendations and guidelines, 45 were identified as genomic tests in cancer and only five tests had approval from the US FDA [104]. Recently, the FDA had approved a broad companion diagnostic panel for clinical assessment of solid tumors called FoundationOne CDx
This is the first comprehensive genomic profiling test of solid tumors for cancer treatment management. Biomarkers provide clues of potential mechanisms that occur in carcinogenesis and targets for therapy. Thus, it is important that the scientific community improve biomarker development for cancer identification, disease progression, metastasis detection, and treatment sensitivity.

1.3. Tumorigenesis and metastasis

Accumulation of DNA damage leading to the disruption of the cell cycle regulation and expression of proto-oncogenic genes causes uncontrolled tumor cell division and growth of the tumor [108]. Early in tumorigenesis, cancer cells disseminate and travel to distant areas in the body [53, 54, 109–111]. Current research suggests that metastatic dissemination may precede the active proliferation of the primary tumor [53]. Christoph Klein’s research group has demonstrated a mechanism of dissemination in which cell density and HER2 and PGR signaling predict early or late stage spreading. Mice with cleared mammary fat pads were transplanted with pieces of mammary glands from either early lesions or primary tumor lesions that were later surgically removed. Mice experiencing surgical removal of the early lesions were more likely to develop metastases compared to those transplanted with primary tumors. This provides evidence that cancer cell dissemination is an early event. The cells of the primary tumor are only able to grow to a certain size to sustain viability before requiring nutrients to be supplied by a capillary blood vessel. Micrometastases have a size limit of 2 mm³ before the cells require a blood supply to deliver nutrients and oxygen [55, 112, 113]. These tumor
cells remain dormant for a period of time [114], some as long as decades, before they escape quiescence and actively divide and grow. The mechanisms that trigger dormant metastatic cells to become active are not completely known.

During active growth, the tumor must obtain the ability to seize control and remodel the surrounding vasculature according to the needs of the tumor. In the process of angiogenesis, the cells promote the development of blood vessels from existing capillary vessels to provide nutrients and remove waste generated by the tumor. Cancer cells release VEGF that activates endothelial cells to form the new vessels [56]. In addition, tumor cells can be attracted toward endothelial cells as shown in a fibrin gel assay [115]. This cross talk between breast cancer and endothelial cells promotes angiogenesis [116]. As a result of the increased proliferation of tumor cells, the oxygen levels in the whole tumor are not uniform and will form hypoxic areas. This hypoxic environment creates a need for blood vessels [117], in which tumor cells express hypoxia-inducible factors 1 (HIF-1). HIF-1 is a transcription factor that can induce the expression of VEGF to promote angiogenesis [118]. In addition, tumor mediated angiogenesis creates an abnormal vasculature characterized by vascular permeability, elevated interstitial pressure, and inefficient transport.

Besides the influence of angiogenesis, tumor cells can also undergo a transformation during tumorigenesis and metastasis. This developmental switch, which typically occurs during embryogenesis, has been shown to occur in tumor cells and facilitates their dissemination. This process is known as epithelial-mesenchymal
transition (EMT), whereby the stationary cell phenotype changes to one that is migratory and invasive [119]. During EMT, the tumor cells decrease the expression of adhesion proteins, such as E-cadherin and CD44v, and increase the expression of proteins involved in remodeling and migration [120]. These proteins include matrix metalloproteases (MMPs), N-catenin, and SNAIL, among others. These cells then become circulating tumor cells (CTCs) that intravasate into the newly formed blood vessels, travel to other regions of the body to extravasate, and settle as dormant cells until they are ready to become secondary growths. Once the cells are situated, they undergo mesenchymal-epithelial transition (MET). Prior to the CTCs migrating to the distant sites, the primary tumor releases factors that condition the distant extracellular environment to form the pre-metastatic niche [121,122]. This process primes the area for the arrival of the CTCs. The formation of the pre-metastatic niche involves the non-cancerous cells developing the area that will allow future cancer cells to propagate. The host tissue stromal cells setup the foundation of the microenvironment by secretion of extracellular matrix proteins [123]. Further, bone marrow hematopoietic progenitor cells (BMHPCs) expressing VEGFR-1 have been found to localize and cluster at pre-metastatic locations before tumor cells arrive as one of the first indicators of changes to the microenvironment at the future metastatic site [124]. The BMHPCs express integrin α4β1 (VLA-4) to adhere to the pre-metastatic niche where they secrete MMP9 to break down basement membranes, and inhibitor of differentiation 3 (Id3) supports propagation of the migrating cells. As we increase
our understanding of the mechanisms that are involved with the formation of the pre-metastatic niche, additional targets for treatment and prevention can be discovered.

1.4. Tumor microenvironment

Breast cancer has a predilection to metastasize to specific areas of the body, such as to the lungs, liver, bone, or brain. This suggests that the composition of these tissues have an increased susceptibility for breast cancer metastasis. One reason may be that the extracellular environment of these tissues plays a role in attracting and supporting the pre-metastatic survival of breast cancer cells. One of the earliest concepts of the influence of the tissue environment on the metastatic potential was Stephen Paget’s “seed and soil hypothesis” made in 1889. He studied 735 cancer cases and documented the number of metastasized cancers and their respective locations [58]. Although cancer can disseminate or “seed” to many areas of the body, only certain tissue environments or “soils” have the potential to propagate those cells. This study provided evidence that some cancers were more likely to metastasize to certain tissues than others. The host tissue environment is characterized by many different cell types and the surrounding extracellular matrix (ECM). The ECM consists of various proteins, growth factors (cytokines and chemokines), receptors, and adhesion molecules that not only prepare the scaffold and basement membranes, but also possess a myriad of functions involved in maintaining structure, adhesion, communication, and signaling mechanisms. The ECM microenvironment plays many roles, including guiding embryonic development and tumor development [125]. The tumor microenvironment (TME) functionally supports tumorigenesis, the movement
and dormancy of disseminated cells, angiogenesis, and immune evasion/suppression. As the TME participates in all of these aspects of tumor development, it is also a growing target for cancer treatment [126].

The majority of the ECM is comprised of collagens, laminins, fibronectin, and other ECM proteins. These proteins organize the structure and stiffness of the ECM, thereby facilitating important protein interactions involved in signaling cascades. A balance between mechanosignaling and ECM organization is required for maintaining the tensile force exerted by daily activities. This relationship is even more pronounced in the mammary gland during lactation when contractile forces are involved in milk secretion. Increased stiffness in the ECM is thought to contribute to malignant phenotypes characterized by cell proliferation and increased tumor cell invasion by the activation of mechanosignaling pathways involving integrins [127,128]. From a physiological standpoint, elevated mammographic density correlates with greater breast cancer risks [129,130]. The same studies found that DCIS has been shown to more likely occur in dense regions. In addition, increased tumor microenvironment heterogeneity has been associated with specific genomic profiles and in certain cases, such as the correlation of a TP53 mutation, with an aggressive subtype of breast cancer [131]. The inclusion of the tumor microenvironment as a target of diagnostics would be advantageous to early detection.

Integrins play an important role in bidirectional signaling connecting the ECM and the intercellular actin cytoskeleton. Disruption of integrin signaling can disrupt
tissue structure, cellular adhesion, migration, and proliferation [132]. The interactions between integrins and ECM proteins occurs at focal adhesion sites, which recruit focal adhesion kinase and c-Src, a protein kinase, to propagate downstream signaling that promotes intra- and extracellular crosstalk. Integrins are heterodimers of α and β subunits that bind to many extracellular matrix and cell surface proteins [133]. In the mammary gland, β1 integrin has been well characterized in epithelial cells and plays a significant role in lactation [134]. ECM protein laminin-1 binds to β1 integrin to promote intracellular signaling that facilitates the hormone prolactin to bind to its receptor and activate STAT5 for expression of milk genes. Inhibition of β1 integrin increases the sensitivity of breast cancer xenograft models to radiotherapy [135]. There are several pathways that involve integrin-ECM signaling, making them an important topic to explore. A thorough understanding of the cross talk between the ECM and integrins can provide a better understanding of the mechanisms that can occur in the dysregulation of the normal cell cycle to promote tumorigenesis.

1.5. The perplexity of breast cancer

Breast cancer is not a new disease that has resulted from improved living conditions and increased lifespans in the modern world; it has been around for centuries [136]. One of the earliest documented cases of breast cancer came from a medical text from ancient Egypt (~2500 BC) describing the observed symptoms, including hard masses in the breast. As of 2015, there have been higher survival rates, likely from improved treatments [61]. However, the majority of breast cancer related deaths are due to metastasis. This suggests that a better understanding of the
metastatic process and methods to target and treat the condition are warranted. The following discussion introduces observations that challenge current thinking regarding surgical treatment of breast cancer.

Removal of the primary tumor may not decrease the likelihood of remission. According to a continuous growth model (Figure 1-1A), after surgical removal of the tumor, the recurrence rate would be predicted as a sigmoidal trend [137]. This is based on the assumption that tumors continuously grow until they reach a saturating size, in which the circulatory system cannot support the constant growth of the tumor and thus tapers to a sigmoidal curve. In contrast, in a study involving 1173 women, Demicheli et al. have shown that recurrence of breast cancer after surgery has a bimodal distribution, in which the highest chance of recurring disease lies within the first 18 months after locoregional surgery, and the next increase of recurrence at 5 years [137] (Figure 1-1B). Recurrence at 18 months was correlated with larger primary tumor mass at surgery while recurrence at 5 years was associated with smaller tumors at surgery. Importantly, these women were clinically followed but had no post-surgical treatment. The bimodal pattern of recurrence is not dependent on estrogen receptor status [138]. This suggests that recurrence is a progressive and mechanistic process that is triggered after primary tumor removal. One possible explanation is that the primary tumor has an inhibitory effect on the secondary sites where tumor cells lie dormant. When the primary tumor is removed, provided that the surgery itself does not disseminate cells, the inhibitor is removed; thus allowing cells at the secondary sites to grow as metastases [139]. Evidence for this notion
came from the discovery of anti-angiogenic compounds. Several anti-angiogenic factors have been purified from tumors. Some of these factors include angiostatin [140], endostatin [141], and thrombospondin [142].

In a 1998 to 2011 California study, Gomez et al. concluded that there was no change in 10-year mortality among patients with breast-conserving surgery and radiation treatment versus patients who underwent bilateral mastectomy [143]. In addition, according to data analyzed from the Surveillance, Epidemiology, and End Results (SEER) 18 registries research database, radiotherapy and mastectomy as a preventative measure to invasive unilateral breast recurrence did not prevent morality from breast cancer in DCIS patients [144]. This means that even if a patient undergoes mastectomy, it does not decrease the mortality rate. This evidence supports the contention that current surgical therapy ranges from ineffective to exacerbating, and suggests that the cells that will eventually develop as breast cancer metastases leave the breast as early as they transform in the breast. An inevitable conclusion described as an inconvenient truth [145].
Figure 1-1: Hazard rates of cancer recurrence and death from breast cancer patients post surgical treatment. Adapted from Demicheli, R., et al. [137]. (A) Predicted recurrence rate based off a continuous growth model. (B, C) The hazard rate of recurrence (B) and death (C) from data collected on 1,173 breast cancer patients who underwent mastectomy. These were pre- and post-menopausal patients.
There is a great need for more biomarker development for not only earlier detection but also improvement of treatment based on the type of breast cancer [146]. Some breast cancer patients develop a non-invasive slow-growing cancer that may not require surgery. These patients would instead require careful monitoring, and radiation and chemotherapy would result in subjecting these patients to harsh treatments. This can lead to over-treatment of the patient; demonstrating that we need to rethink our standards of detection and care for breast cancer patients.

Our current knowledge of breast cancer metastasis is lacking and we need to gain a deeper understanding to address these concerns and improve treatment. It is important to increase the scientific knowledge by also exploring alternative mechanisms. Many researchers follow preconceived dogmas that fixate on accepted concepts that may not incorporate all observations made in the research/clinical setting. Research described in this dissertation has challenged many of the established thoughts regarding the protein, nucleoside diphosphate kinase, and its function in breast cancer. This protein has been touted as a metastasis suppressor since its discovery in 1988 by Patricia Steeg [147].

In the following sections, nucleoside diphosphate kinase and its role in cancer-mediated purinergic signaling will be provided. A review of purine signaling (also known as purinergic signaling) will be discussed followed by experiments supporting
the hypothesis that extracellular nucleoside diphosphate kinase acting extracellularly as delivered via exosomes contributes to the metastatic process.

1.6. Purinergic mechanisms and nucleotide signaling

ATP, first discovered in the 1929 by German chemist Karl Lohmann, is the universal energy currency in all living cells. Although it was originally thought of solely as a mediator of energy transfer, ATP was first documented in 1972 as a non-adrenergic, non-cholinergic neurotransmitter [148,149]. This discovery had major implications for ATP as both an intra- and extracellular signaling molecule and facilitated later studies in purinergic signaling [149]. Purinergic signaling is involved in both short-term (neurotransmission, modulation, and secretion) and long-term (proliferation, differentiation, apoptosis, and motility) signaling [150]. As formulated in the Nucleotide Axis Hypothesis, extracellular ATP is released from endothelial cells where it acts at extracellularly-directed endothelial purinergic receptors to stimulate the release of other vasoactive factors in blood vessels including prostacyclin and nitric oxide, in addition to ATP itself [151]. These mediators relax terminal arterioles and once metabolized to adenosine, promote venous dilation. This intraluminal axis favors the movement of circulating tumor cells into distant tissues. Circulating ATP and adenosine prevent platelet aggregation further helping to ensure dissemination of tumor cells. Moreover, the tumor microenvironment has been characterized as having high ATP levels [152,153]. Adenosine, ADP, and ATP activate purine receptors, P1 and P2.
P1 receptors are only bound by adenosine. P1 receptors are divided into A1, A2A, A2B, and A3 receptors. The human P1 receptors share about 39-61% sequence homology [154]. These seven transmembrane domain G-protein coupled receptors (GPCR) regulate the activity of adenylate cyclase to modulate intracellular cyclic AMP and Ca\(^{2+}\). A1 and A3 are coupled to \(G_{i/o}\) to downregulate adenylate cyclase and A2A and A2B are coupled to \(G_s\) to stimulate adenylate cyclase. Extracellular adenosine is mostly supplied by the breakdown of ATP, ADP, and AMP by nucleotidases [155,156]. Adenosine has been shown to be elevated in the tumor microenvironment [157,158].

P2 receptors are broadly divided into P2X channels and P2Y receptors. The P2X channels are a family of seven ATP-gated ion channels (P2X1- P2X7), of which P2X5 and P2X7 have been suggested to promote tumor cell death when activated by ATP [159]. The P2Y receptors are GPCRs that can be activated by ATP, ADP, UTP, and UDP. There are currently eight members of the P2Y family in humans; P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 [153,160–162]. Human P2Y receptors are broadly divided into 2 groups, the P2Y1-like receptors coupled to \(G_q\) proteins and the P2Y12-like receptors that are coupled to \(G_i\) proteins [163]. In platelets, activation of P2Y1 receptors recruits \(G_q\) proteins to activate phospholipase C and increase intracellular Ca\(^{2+}\) [164,165]. Activated P2Y12 receptors recruit \(G_i\) proteins, which inhibit the synthesis of cAMP through adenylate cyclase. Both of these G-coupled functions play a significant role in platelet aggregation and vascular function. P2Y receptors can form homo or heterodimers with other P2Y receptors or GPCRs as well.
as mediate cross talk between receptors [166,167]. Since 1972 the scientific literature on purinergic signaling has grown significantly [168].

Many studies have shown an involvement of P1 and P2 receptors in cancer. Adenosine in the tumor microenvironment aids in the suppression of pro-inflammatory signaling and evasion of immune surveillance [158,169-172]. Adenosine binding to A2A receptors on T-cells induces arrest, which includes decreased proliferation and decreased cytokine production [157,169,173,174]. In addition, adenosine binding to A2A on antigen presenting cells decreases cytokine IL-12 and induces IL-10 production to inhibit immune response [169,175,176]. ATP was shown to increase invasion and migration of T47D breast cancer cells by activation of P2X7 receptors through the AKT pathway [177]. Activation of P2Y2 receptors by ATP and UTP increased cell proliferation, invasion, and migration of MDA-MB-231 breast cancer cells [178,179]. In addition, this activation could mediate angiogenesis through increased MMP activity and VEGF production [178]. ATP and UTP also increased ICAM-1 and VCAM-1 expression in endothelial cells, which increased adhesion of the breast cancer cells to endothelial cells.

In this dissertation, the focus is placed on P2Y1 receptors. P2Y1 is an ADP/ATP specific receptor encoded by the P2YR1 gene. P2Y1 was first cloned from chick brain in 1994 by Webb et al. [180]. P2Y1 activation in platelets prevents aggregation, and recently has been a candidate for antiplatelet drug therapy [181]. P2Y receptors play a role in cell proliferation. In Müller glial cells, ATP activation of P2Y receptors transactivated platelet-derived growth factor receptor (PDGFR) and
epidermal growth factor receptor (EGFR) which led to downstream activation of ERK1/2 and PI3K causing increased proliferation [182]. Activation of P2Y1 by ADP leads to activation of the coupled G protein, typically G_q, followed by a signaling cascade. There are a number of potent selective antagonists to P2Y1 receptors including; MRS2179, MRS2279, MRS2500, and MRS2365 (Figure 1-2). All of these MRS compounds share similar structures to ADP/ATP in which they contain the 5-carbon sugar and the adenine nitrogenous base group. 2-(Methylthio)adenosine 5’-triphosphate/diphosphate (MeSATP/ADP) are common P2Y receptor agonists for selective receptor activation.

Other players in purinergic signaling are the pannexin and connexin channels [183]. Pannexin and connexin are hemichannels that promote release of ATP. The connexins are integral multi-pass transmembrane proteins that have four transmembrane domains linked by one intracellular loop and two extracellular loops with N- and C-terminal cytoplasmic domains [184]. Connexin hexamers, called hemichannels form in the secretory pathway and are then trafficked to the cell surface where they can act as stand-alone channels, or dock with hemichannels in appositional membranes to form intact gap junctions (GJ). There are 21 members of the human connexin family [185] annotated as Cxn, where the n denotes the molecular weight (e.g. Cx43 has a molecular weight of 43 kDa) [186]. Connexin mediated release of ATP is a response to decreased extracellular calcium levels [187], CO_2 [188], and membrane depolarization (40-60 mV) [184]. Pannexins are composed of Panx1, Panx2, and Panx3, and are composed of a four transmembrane structure,
two extracellular loops and three intracellular structures, which include the N-terminus, a loop, and the C-terminus. Pannexins exhibit ATP conductance, with activation of Panx1 channels during normal resting potentials [185], high extracellular potassium levels [189,190], elevated intracellular calcium levels [191], and mechanical stretch [190]. A role for pannexin channels in cancer has been shown when ATP released by dying cancer cells activated these channels on immune cells and recruited immune targeting of the cancer cells. Activation of these channels by ATP stimulates the release of more ATP. The release of extracellular ATP may promote autocrine signaling through activation of purinergic receptors to regulate immune function [192]. Activation of CD3/CD28 stimulated Panx-1 to release ATP from γδ T-lymphocytes [193]. This extracellular ATP would activate P2X4 channels in an autocrine fashion in the T-cells to promote activation. On the other hand, increased release of ATP from Panx1 channels has been shown to facilitate internalization of the Panx1 channel in neural cells [194]. Although it is unknown whether this mechanism occurs in other cell types, the possibility of a negative feedback loop of ATP release and internalization of the Panx1 channel demonstrates a possible pathway of deactivation of Panx1 expressed on cells in the immune system.
Figure 1-2: Agonists and antagonists of P2Y1 receptors. (A) Agonists of the P2Y1 receptor. ADP, MeSATP (2-(Methylthio)adenosine 5’-triphosphate), and MeSADP (2-(Methylthio)adenosine 5’-diphosphate). (B) MRS compounds that are selective P2Y1 antagonists. MRS2179 (2’-Deoxy-N6-methyladenosine 3’,5’-bisphosphate tetrasodium salt), MRS2279 ((1R*,2S*)-4-[2-Chloro-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo [3.1.0]hexane-1-methanol dihydrogen phosphate ester diammmonium salt), MRS2500 ((1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicycle [3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt), MRS2365 (((1R,2R,3S,4R,5S)-4-[(6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo [3.1.0]hex-1-yl[methyl] diphosphoric acid mono ester trisodium salt).

Nucleotides are released into the extracellular environment through lytic and non-lytic means (sheer stress, ATP releasing channels, transporters, and vesicular exocytosis) [195]. In addition, there is a rapid turnover of the nucleotides due to ectonucleotidases on numerous cells, such as ectonucleoside triphosphate diphosphohydrolase (also known as ecto-ATPase or CD39) [156,196]. These
Ectoenzymes catalyze rapid degradation, leading to locally elevated nucleotide levels. Deregulation of ectonucleotidases may be associated with cancer. Mice lacking nucleoside triphosphate diphosphohydrolase-1 developed spontaneous liver cancer from the elevated levels of nucleotides [197]. Nucleotides have been shown to have an important role in endothelial function and vascular regulation. Adenosine can dilate blood vessels and increase blood flow. ADP induces platelet aggregation by its interaction with P2Y1, P2Y12, or P2X1 receptors [164,165].

In our research, we observed that elevated localized ATP and ADP levels resulted in activation of endothelial plasma membrane receptor P2Y1. The P2Y1/2 agonists, ATP, 2-methyl-thio-ATP (2-MeSATP), and 2-methyl-thio-ADP (2-MeSADP), induced tubulogenesis in human cardiac endothelial cells, and this pro-angiogenic effect was suppressed by the VEGFR-2 inhibitor, SU1498, as well as the P2Y1 antagonist, MRS2179 [198,199]. This demonstrated that activation of the P2Y1 receptor transactivated the VEGFR-2 to promote angiogenesis. The source of the elevated ATP and ADP was from extracellular nucleoside diphosphate kinase, which was secreted by human breast cancer cells [200]. Thus began a study of nucleoside diphosphate kinase and purinergic regulation of tumor-mediated angiogenesis.

1.7. A story about NDPK

Nucleoside diphosphate kinase, also known as AWD, NDPK, NM23, and NME, is a family of proteins that transfers terminal gamma-phosphate groups to diphosphate nucleosides. They play a role in maintaining triphosphate nucleotide pools. In addition, NDPK has a role in development [201,202], hematopoietic function
[203], and proliferation [204]. In humans, there are ten genes for NDPK but NDPK-A and NDPK-B are the best studied. NDPK-A and NDPK-B proteins share 80% sequence homology. NDPK-A and NDPK-B are 17-19 kDa and can form homo or hetero-multimers. Common multimeric structures of NDPK include dimers, trimers, and hexamers (consisting of three pairs of dimers) (Figure 1-3A). In NDPK-A, cysteine(C)4 and C145 residues interact to form disulfide bonds during oxidative stress [205] that destabilize the hexameric structure into dimers [206]. Mutation of C145 to serine in NDPK-A has been shown to increase DNase and phosphotransfer activities and decrease the dissociation of the hexameric form [207]. The enzymatic function of NDPK-A/B is the transfer of the gamma phosphate group from the nucleoside triphosphate (NTP) to the nucleoside diphosphate (NDP) by a ping-pong mechanism [208]. For example, the step-wise progression of the ping-pong mechanism at the active site of NDPK-A/B would be (Figure 1-3C):

1. A GTP molecule binding and donating the terminal phosphate group, in which the GTP is now hydrolyzed to GDP and will leave the pocket;
2. An ADP molecule will then attach and bind the phosphate group, and ATP is now generated and will leave the active site.
Figure 1-3: 3D model and enzymatic reaction of NDPK. The structures were generated from the Protein Database (www.rscb.org). The reference structure used was 2HVD adapted from Giraud, M., et al. [209]. (A) 3D model of the quaternary structure of NDPK from using the RSCB-Protein Workshop Viewer software. The two illustrations are of the hexameric structure to show the dimerization of a trimer. Subunits are differentiated by color. NDPK can enzymatically function as a monomer, dimer, trimer, and hexamer. (B) Display of the 3D model of hexameric NDPK in secondary structures with ADP substrates. In the close up image, ADP interacts with the residues H118 and S120 in both the wildtype (cyan) and S120G mutant (yellow) NDPK, adapted from Giraud, M., et al. [209]. (C) NDPK transphosphorylation mechanism. NDPK transfers the terminal-gamma phosphate group to an N1TP to and N2DP by a ping-pong mechanism.
Hydrolysis of this phosphate group occurs at the amino acid residue H118 (Figure 1-3B). This phosphotransfer mechanism is dependent on divalent metal ions, in which Mg\textsuperscript{2+} is the most efficient [210]. Although NDPK does not display dramatic affinity differences between nucleotide substrates, the ribose 3'-hydroxyl group plays a role in catalysis and by replacing this group there is a dramatic decrease in kinetics [210,211]. Intermolecular phosphotransfer (auto phosphorylation) between the histidine (H117 in \textit{E. coli} and H118 in humans) and serine residues (S119 in \textit{E. coli} and S122 in humans) in the active site generates a phosphointermediate state to promote the catalytic activity of the enzyme [212,213]. Mutation of H118 to a tyrosine creates a dominate negative catalytically dysfunctional mutant [214]. The dysfunctional H118 mutant has been used to demonstrate the involvement of the transphosphorylation activity in biological processes. Besides transphosphorylation activity, NDPK-A and B exhibit multiple functions, including endonuclease activity [215–217], protein histidine kinase [218–220], c-Myc binding protein [221], and association with membrane lipids [222].

NDPK was first discovered in 1988 as a \textit{non-metastatic} (NME) gene in which increased mRNA transcripts correlated to a lower metastatic potential in various cancer cell lines [147]. There are various intracellular mechanisms that demonstrate NDPK metastatic suppression. NDPK-B has been shown to suppress motility and this is inhibited by interaction with proto-oncogene MDM2 in renal cell carcinoma [223]. NDPK-B generation of GTP has been shown to play a role in G-protein signaling [224], such that NDPK-B depletion decreased Gβ1 expression, which reduced downstream
cardiac contractility [225]. AMP-activated protein kinase (AMPK) phosphorylation of the S120 residue on NDPK regulated its activity, in which loss of AMPK increased NDPK activity [226]. This led to continuous break down of ATP causing energy depletion to promote cell death in HEK cells. This study suggested that AMPK regulation of NDPK activity demonstrates a relationship between cellular metabolism and tumor suppression. However, there is growing literature that shows NDPK exhibits other pro-metastatic effects. For example, NDPK-A has been shown to interact with prune exopolyphosphatase 1 (h-prune) to promote cancer migration, which is associated with increased cancer disease progression [227–229]. NDPK-A interacted with a membrane-bound MUC1 (a mucin protein thought to mediate cancer cell growth) cleavage product to promote the growth of T47D breast cancer cells [230]. Also this interaction between NDPK-A and MUC1 cleavage product promoted the growth and undifferentiated state of pluripotent stem cells [231,232]. NDPK-B has also been known as PUF, a DNA binding protein that binds to the poly-pyrimidine rich regions of the c-Myc promoter, a transcription factor that plays a role in the cell cycle and has been implicated in some cancers [233–235]. NDPK-B binding to Piwi-like RNA-mediated gene silencing 2 (PIWIL2) upregulated c-Myc and mediated tumor cell proliferation [236]. This mechanism may support a role for NDPK as a transcription factor to promote upregulation of the proto-oncogene c-Myc. In addition, the N-Myc oncogene was shown to upregulate the NM23-H1 and NM23-H2 gene expression and protein products in some neuroblastoma tumors and this was correlated to poor prognosis [237]. High expression of NDPK was detected in
renal cell carcinoma [238], neuroblastoma [239], leukemia [240], breast cancer [241], kidney cancer [242], and colorectal cancer [243]. Additionally, there is a long splice variant containing exons from both NM23-H1 (exons 1-4) and NM23-H2 (exons 2-5) genes [244]. Neuroblastoma tumors showed high expression of NM23-H1, NM23-H2, and NM23-LV. Extracellular NDPK was detected from human breast, colon, pancreas, and lung tumors [245]. Our lab has shown that NDPK has a role in endothelial cell proliferation, migration, and tumor-mediated angiogenesis [198,246]. NDPK was detected in the conditioned media of breast cancer cells and also in the serum of breast cancer patients [246,247]. Extracellular application of NDPK-B promoted the growth of MDA-MB-435s cancer cells and CD31+ endothelial cells [200,246]. Inhibition of the NDPK transphosphorylation activity has been shown with polyphenolic compounds, in which ellagic acid (EA) and epigallocatechin gallate (EGCG) have been shown to be the most potent [248,249]. EGCG and EA decreased growth and migration of endothelial and cancer cells in vitro [200,246]. In addition, EGCG and EA inhibited tubulogenesis of CD31+ endothelial cells. NDPK-B activated endothelial VEGFR-2 and ERK1/2, and blocking NDPK with EA or P2Y1 with MRS2179 inhibited this mechanism. The MeS-ATP activation of VEGFR-2 and ERK1/2 was blocked with PP2, a c-Src tyrosine phosphatase inhibitor. This observation suggested that P2Y1 transactivation of VEGFR-2 was mediated by activation of c-Src kinase. These results demonstrated that NDPK released by breast cancer cells stimulated angiogenesis by increasing proliferation, migration, and
tubulogenesis in endothelial cells. However, the mechanism by which NDPK was released into the extracellular environment remained a mystery.

1.8. Introduction of exosomes

Vesicles released from cells include exosomes, microvesicles (ectosomes and microparticles), and apoptotic bodies (Figure 1-4). The word “exosomes” was first used in 1981 [250] however, the definition was narrowed in 1987 to describe 30-100 nm vesicles that originated from multivesicular bodies. There are many names for extracellular vesicles; including microvesicles, exosomes, and ectosomes. These names can be based on a number of features, such as size (micro/nano vesicles/particles), origin (oncosomes and protostomes) and extracellular presence outside on the cells (ectosomes and exosomes).
Figure 1-4: Classification of extracellular vesicles. Adapted from Colombo, M., et al. [251]. Eukaryotic cells can release a variety of extracellular vesicles that originate from different areas of the cells. Extracellular vesicles are released by either direct budding from the plasma membrane (PM) or intracellular vesicles fusing to the PM. Intracellular vesicles may also fuse to lysosomes for protein degradation.
In the endocytic pathway, the early endosome matures into the late endosome where it accumulates intraluminal vesicles (ILV) that contain specifically sorted lipids, cytosol, and protein components (Figure 1-5) [251]. The ILVs form by inward budding of the membrane on the early endosome. The mature endosomes are also known as multivesicular bodies (MVB). Fusion of the MVB with the plasma membrane, releases the ILV into the ECM where they are known as exosomes. Alternatively, ectosomes and microvesicles form by budding of the cell's plasma membrane and can range in sizes of 100-1000nm in diameter. The main mechanism of ILV and MVB development involves endosomal-sorting complexes required for transport (ESCRT)-0, -I, -II, and -III and many other associated proteins. ESCRT-0 detects and organizes the ubiquitinated transmembrane proteins in the membrane of the endosome. ESCRT-I and -II are involved in remodeling of the membrane in the budding process where the membrane deforms into the bud with the sequestered cargo [252]. ESCRT-III proteins participate in vesicle scission. Although this is the best-studied mechanism of exosome biogenesis, there are also ESCRT-independent mechanisms.
Figure 1-5: Mechanisms of biogenesis and release of exosomes. Adapted from Colombo, M., et al. [251]. (A) These are the most common mechanisms of biosynthesis of exosomes. The early endosome is modified by a variety of ESCRT, tetraspanins, and lipids into the mature endosomes or multivesicular bodies (MVB) where fusion with the plasma membrane (PM) releases the exosomes into the extracellular environment. (B) Various mechanisms of release of exosomes. ESCRT, ARF6, and RAB proteins are commonly studied for playing a role in extracellular vesicle release. Specific RAB proteins are associated with secretion of exosomes that contain particular protein cargoes.
Exosomes and microvesicles can target specific tissues and cells based on the surface molecules expressed. They are taken up by the host cells and promote signaling mechanisms, such as translation of mRNA to protein [253]. Exosomes mediated cell-to-cell communication through paracrine and endocrine signaling across distance. Although exosomes contain many different proteins based on their cells of origin, several protein markers are widely expressed on all exosomes. These include: ALIX, tumor susceptibility gene 101 (TSG101), CD61, CD81, CD9, major histocompatibility complex II (MHCII), and RAB proteins (Figure 1-6). ALIX (also known as programmed cell death 6-interacting protein) is a class e vacuolar sorting protein (VSP) that is involved in sorting and concentrating of the cargo in multivesicular bodies [254]. TSG101 is another protein involved in sorting, interacting with ESCRT-I to recruit ubiquitinated proteins [255]. CD61, CD81, and CD9 are tetraspanins found at the membrane of exosomes that interact with integrins and MHC class I and II [256,257]. MHC class II is a receptor that binds to CD4 on antigen presenting immune cells that is involved in the immune response. RAB proteins are part of the superfamily of Ras-related proteins and a class of small GTPases associated with the membrane and participate in intracellular transport of proteins [254,258–260].
Figure 1-6: Diagram of extracellular vesicle composition. Adapted from Colombo, M., et al. [251]. Extracellular vesicles contain lipids, nucleic acids, and proteins. The types and quantities of these components vary by type of extracellular vesicles, composition of the host cells, and conditions in which the extracellular vesicles were collected.
Shedding of microvesicles from the plasma membrane can be triggered by increased intracellular calcium [261], ATP binding to P2X7 receptors on macrophages [262], activation of complement receptor 1 on leukocytes [263], and changing of fresh media supplemented with fetal calf serum on tumor cells [264]. Exosome secretions can be triggered by specific stimuli, such as treatment with calcium ionophores [265], depolarization of neurons [266], and activation by neurotransmitters [267]. It is worth noting that there are a number of unexpected factors that can influence exosome secretion. In order to prevent collection of contaminating serum exosomes, most cell cultures are changed to serum-starved conditions, which can also affect exosome concentrations and cargo. Therefore, it is critical that experimental conditions remain consistent to reduce batch-to-batch variability associated with exosome collection and purification.

The exosome secretion pathway has not been characterized in its entirety. Various proteins have been implicated to be involved in vesicle release (Figure 1-5). RAB GTPases have roles in intracellular trafficking, vesicle budding, and membrane fusion of the MVB to the plasma membrane. One of the first RAB proteins found to be involved in this process was RAB11 [259]. RAB11 regulates trafficking between recycling of endosomes and the Golgi. RAB27A and RAB27B participated in MVB vesicle docking and when Slp4 and Slac2b (two RAB27 effectors) were silenced, exosome secretion was inhibited [260]. RAB22A has been shown to play a role in microvesicle release by breast cancer cells during hypoxia [268]. SNARE complexes are involved in the direct fusion of the MVB to the plasma membrane and shuttling
out the cargo into the ECM [269]. The SNARE complex forms during the interaction of v-SNARE on the vesicle and t-SNARE on the recipient membrane. VAMP participates in the SNARE complex to facilitate vesicle fusion [270]. Vps4 interacts with Snf7 and Vps2 in ESCRT-III and recycles ESCRT-III to promote membrane remodeling and vesicle scission of the MVBs [271]. Based on the different categories of vesicles, there may be multiple pathways to vesicle release. In the future, these mechanisms would be worth exploring with broad implications in disease treatment.

In the last five years, research associated with extracellular vesicles, particularly exosomes, has exploded. It is now known that all cell types secrete extracellular vesicles including cancer cells. Different types of extracellular vesicles have shown enrichment of cell-type specific proteins and lipids [272], which would make them excellent candidates for biomarker development. In addition, all biological fluids contain exosomes; these include saliva, urine, and breast milk [273,274]. This suggests that exosomes can be used as a non-invasive method for biomarker identification. It has been shown that cancer cells release more exosomes and microvesicles than non-tumor cells [275–277]. An increase in exosome release in an in vivo model has demonstrated that there is more uptake of the tumor secreted microvesicles in the non-tumor cell types than in the reverse experiment [278]. It has also been suggested that only 10% of the released exosomes are from the tumor itself with the remaining exosomes likely released by immune cells [279].

Tumor derived exosomes have been shown to promote angiogenesis [253,280,281]. In addition, extracellular vesicles purified from the serum of breast
cancer patients may play a role in the epithelial-mesenchymal transition of mammary epithelial cells [282]. Highly metastatic tumor secreted exosomes have been shown to educate other less malignant cells to become more metastatic and increase vascular permeability to allow for tumor cells to migrate [275,283]. Pancreatic ductal adenocarcinoma exosomes were shown to initiate the formation of the pre-metastatic niche in the liver before the development of liver lesions [284].

There are currently four databases that compile data on mRNA, miRNA, proteins, and lipids identified with extracellular vesicles: ExoCarta (http://www.exocarta.org) [285], Vesiclepedia (http://www.microvesicles.org) [286], EVpedia (http://evpedia.info) [287], and EV-Track (http://evtrack.org) [288].

1.8.1. RNA and DNA cargo

Exosomes have the potential to become an excellent target for biomarker development. As qPCR is a sensitive technique for detection and identification, the mRNA, miRNA, and DNA cargoes can be characterized and quantified using this assay. In addition, RNA profiles differ greatly among the different types of extracellular vesicles. For example, apoptotic bodies contain ribosomal RNA while microvesicles contained little to no RNA [289]. The plasma membrane of the exosome protects the RNA cargo from extracellular RNases and once it is incorporated into the host cells, the RNA can be translated into protein for downstream signaling cascades [253,290]. Exosomal RNA expression can be altered by the application of external stimuli to the parent cell and this can elicit different effects on the recipient cells [291]. It has been shown through the use of multiplexed reporters, that EV uptake and RNA translation
can occur within 1 hour after horizontal transfer to recipient cancer cells [292]. The typical miRNA process that occurs in cells involves inactivation of mRNA translation, in which the miRNA can bind to either the 3’ or 5’ UTR of mRNA. In addition, there is also another miRNA mechanism that involves toll-like receptors (TLRs) as miRNA receptors [293]. Binding of miRNA to human TLR7 activates NF-κB signaling in immune cells, which leads to secretion of IL-6 and TNF-α to promote cancer cell proliferation.

Double stranded DNA (dsDNA) has also been found in exosomes [294]. The total dsDNA collected in exosomes could assemble the entire genome and has been shown to indicate the mutations accumulated by the parent tumor cells. The detection of these mutations has also been identified in plasma exosomes of mice carrying tumors. In another study, large oncosomes contained more DNA than other extracellular vesicles [295]. This suggests that dsDNA in extracellular vesicles may provide an avenue for early cancer detection. Interestingly, mitochondrial DNA (mtDNA) has been detected in EVs [296]. Cancer-associated fibroblast derived EVs could promote cancer stem-like cell escape from metabolic quiescence and resistance to hormone therapy. EVs have demonstrated to be potent signal mediators as well as potential biomarkers for their ability to carry DNA and RNA.

1.8.2. Protein cargo

Exosomes contain a diverse population of proteins, carried either in their membranes or the lumen. This cargo reflects both the tumor cell membrane-expressed proteins and proteomic signature. The exosome cargo and biological
function depend on the parent cell of origin. B-cell and dendritic cell derived microvesicles play an important role in activation of the immune system as well as antitumor effects. Exosomes can modulate the immune system, such as breast cancer exosomes suppressing macrophages through regulation of TLR2 to down regulate immune function [297]. Expression of specific integrins on both vesicles and target cells has been shown to mediate targeting of cancer extracellular vesicles to target specific organs, initiating pre-metastatic niche formation [298]. Exosome protein cargo has been explored as predictive markers of survival in cancer patients. In one study, it was found that patients with stage IV melanoma had better survival when plasma exosomes were protein-poor (<50 µg/ml) [275]. Exosomes from more malignant melanoma cell lines contained more protein than poorly and non-metastatic cell lines [275]. NDPK has been identified in breast cancer exosomes in two different proteomic studies [299,300].

1.8.3. Lipid cargo

EVs display a phospholipid bilayer. Different cells can be composed of different lipid populations, and also the EVs derived from those cells can display different lipids as well [272]. Exosome lipid composition has become a significant field of study for a number of reasons; (1) a thorough understanding of the exosome lipid composition would be a basis for designing liposomes for effective drug delivery without requiring large batch processes to isolate cell derived EVs, (2) lipid organization does provide a background on EV biogenesis, and (3) elucidation of EVs lipid dynamics could provide a mechanism of how pathogens interact with cells [301].
Lipids play a major role in exosome biogenesis. Ceramide, a lipid molecule composed of a sphingosine and a fatty acid generated by hydrolysis of sphingomyelins, can stimulate exosome secretion [302–304]. It has also been shown that neutral sphingomyelinase (nSMase2) regulates exosome biogenesis and that GW4869 inhibits exosome formation [304,305]. For more information on lipidomics in EVs, the following reviews can provide a more thorough background [306,307].

1.8.4. Metabolite cargo

Although lipids are a type of metabolite, this section is a review of the other metabolites that can be detected associated with EVs. The number of metabolomics studies in EVs has been steadily increasing. Many studies have shown that EVs are enriched with metabolites [308–310]. These studies present some unique challenges, such as background metabolites present in the tissue culture media and the different analytical techniques that can be performed [308]. Neural derived EVs showed active metabolic enzymes, particularly L-asparaginase activity, that were able to produce metabolites independent of parent cells [311]. Exosomes have been shown to provide amino acids to the nutrient-deprived cancer cells to promote tumor growth [312].

1.9. Implications in future therapies

Exosomes have the potential to be implemented for biomarker development but also for delivery of anticancer treatment. The unique traits that allow for EVs to target cancer cells make it ideal to implement for locating and treating cancer. EVs can be conditioned to carry different compounds to be delivered to the cancer cells.
Currently, there are no FDA-approved treatments for breast cancer that involve antiangiogenic compounds. Avastin (bevacizumab), designed to block VEGF, was employed, but the breast cancer indication was withdrawn following clinical trials [313]. This could be because researchers have not determined the exact breast cancer subtype or the optimal treatment regimen that would work well. Another explanation could be vascular or vessel co-option, in which the migrating tumor cells alter the existing tumor blood supply to become antiangiogenic drug resistant [314,315]. The tumor cells may grow and infiltrate the capillaries and large pre-existing vessels to create a disorganized vasculature at metastatic sites. Since this process is not neo-angiogenic, antiangiogenic therapies may not inhibit the development of the modified tumor vasculature and thus lead to resistance of angiogenesis inhibitors. Perhaps, there may be other alternative angiogenic pathways that may need to be targeted at the same time. Our research has proposed another mechanism of angiogenesis that occurs in the absence of VEGF.

A better understanding of these mechanisms may provide other pathways to target and treat breast cancer. Particularly, the role of NDPK in angiogenesis and metastasis may be a candidate for biomarker development, as detection in serum would suggest tumor presence [247] and/or metastasis, and targeting the protein and its receptor of action can decrease breast cancer growth [316].

The drug combination of MRS2179 and EA may be a possible neoadjuvant therapy for a woman diagnosed with an early stage 0-1 breast cancer prior to her undergoing surgery. It is currently unknown whether or at what concentrations
MRS2179 can be tolerated by humans. Antagonists of the P2Y1 receptor have shown potential as antiplatelet aggregation drugs and may be clinically tested in the future. Alternatively, EA is an antioxidant found in walnuts and the skins of blueberries. Dietary antioxidants are not FDA regulated, but some are commercially available for regular consumption. Early treatment has been linked with increased survival rates and the introduction of an early treatment or preventative regimen in which EA is administered as a neoadjuvant may improve patient prognosis.

1.10. Hypothesis

The hypothesis of this dissertation is that exosomal NDPK promoted tumor mediated angiogenesis and metastasis. In addition, treatment with EA and MRS2179 block angiogenesis and suppress metastasis.

1.11. Summary

Extracellular NDPK has been shown to promote endothelial proliferation, migration, and tumor-mediated angiogenesis. This study investigates how NDPK is released into the ECM by exosomes secreted by breast cancer cells and whether this exosomal NDPK mediates angiogenesis and metastasis through purinergic mechanisms.

Chapter 2 covers the physiological role of NDPK in vivo. Two orthotopic disease models are developed to study the role of NDPK on metastasis. In one model, a triple negative breast cancer cell line is injected into the mammary fat pads of female SCID mice and the other is a murine-derived mammary carcinoma injected
into BALB/c mice. These tumors are monitored and measured to determine size and the presence of metastasis. Treatment with NDPK inhibitor and P2Y1 receptor antagonists are employed to demonstrate the role of NDPK in tumor growth and metastasis.

In chapter 3, we show that enzymatically active NDPK associates with the exosomes with a transphosphorylation activity assay. NDPK concentration of the exosomes is quantified based on activity levels. This functional extracellular NDPK also could stimulate tubulogenesis in endothelial cells. In addition, inhibition of NDPK and the P2Y1 receptor decreases tubule formation.

In chapter 4, we describe studies focused on purification and characterization of exosomes released by triple negative breast cancer (MDA-MB-231) cells and compared them to the non-tumorigenic human mammary epithelial (hTERT-HME1) cells. Also we determine the presence of NDPK associated with the breast cancer secreted exosomes.

Lastly, chapter 5 will discuss the conclusions and the future directions for this study. Methods of generating a dysfunctional NDPK cell model and potential experiments for applying this model are described.

The appendices discuss the optimization process of the transphosphorylation assay. Additional results on the generation and purification of human recombinant NDPK-A and NDPK-B proteins are also included.
Chapter 2: Breast Cancer Orthotopic Mouse Models to Study the \textit{in vivo} Role of Nucleoside Diphosphate Kinase

2.1. Abstract

The leading causes of death in breast cancer patients are complications that arise in association with metastatic disease. Although, anti-cancer therapies have improved, there is still a lack of complete understanding in cancer metastasis. Nucleoside diphosphate kinase (NDPK) secreted by breast cancer cells has been shown to facilitate blood vessel formation and may have a role as an early factor supporting tumor growth and metastasis. In this study, we investigate the influence of NDPK/P2Y1 inhibition on breast cancer tumor and metastasis development in two orthotopic mouse models. MDA-MB-231-Luc2 and 4T1-Luc2 cells were implanted into the mammary fat pads of female SCID and BALB/c mice, respectively. The mice were divided between treatment groups to study the influence of inhibition of NDPK and/or its stimulation of nucleotide receptors. Primary tumor and metastasis development were monitored throughout the study. Treatment with NDPK inhibitor ellagic acid (EA) and/or the purinergic receptor antagonist MRS2179 decreased the growth rate of the primary tumor and metastasis in both models. NDPK was detectable in the serum of the SCID mice carrying the MDA-MB-231-Luc2 tumors. These data demonstrate that NDPK may have implications as a breast cancer biomarker and target for therapy.
2.2. Introduction

While breast cancer is the most common cancer diagnosed in women, most patient related deaths are due to metastatic disease and not the primary tumor [317]. The mechanisms of metastasis are not completely known, but are known to include intravasation, migration, extravasation, and propagation [318]. The breast cancer cell undergoes a phenotypic change called epithelial-mesenchymal transition (EMT) to express proteins that will assist the cell to migrate away from the primary tumor. During the primary tumor development, angio- and vasculogenesis can occur to provide a pathway for the cancer cells to escape and travel to other regions of the body. Vasculogenesis is the process of blood vessel formation de novo, while angiogenesis is the formation of blood vessels from pre-existing vessels. The breast cancer cells will escape the primary tumor through the blood vessels by a process known as intravasation and then migrate to distant areas of the body. Through extravasation, the cells will leave the vessels and settle at a secondary site where they undergo mesenchymal- epithelial transition (MET) to become a stationary cell type. Dissemination of breast cancer cells can occur early during tumorigenesis [53,54,110]. Once the cells have migrated, they can remain latent for a period of time until a trigger activates growth. While this is a general outline of the metastatic process, the details need to be elucidated. Our lab has shown that extracellular NDPK stimulates endothelial cell migration, proliferation, and tumor-mediated angiogenesis [200,246]. Extracellular NDPK may have implications in tumorigenesis and metastasis.
The conventional role of NDPK is to regulate the intracellular nucleotide triphosphate pools. The enzymatic mechanism of the protein catalyzes the transfer of a terminal gamma phosphate group of a nucleoside triphosphate to a nucleotide diphosphate [210,211]. This intracellular regulatory pathway assists in the maintenance of the cell. On the other hand, extracellular NDPK has been shown to promote a tumorigenic environment [145]. NDPK has been detected in the serum of breast cancer patients and not in healthy individuals [316]. The activity of NDPK elevates local extracellular ATP levels and this facilitates the activation of the P2Y1 receptor on adjacent endothelial cells [200]. VEGFR-2 signaling can be stimulated by activated purinergic receptors in the absence of VEGF to promote angiogenesis [198,199].

While there is no perfect animal model for studying cancer, orthotopic mouse models are representative in vivo models of breast cancer growth in the mammary fat pad, a model for the human breast. This is advantageous since the tumors are implanted in the same microenvironment as the origin of the cells to recapitulate the disease state. The 231 and 4T1 mouse models are two established orthotopic mouse models that will be studied to evaluate the impact of inhibiting NDPK and P2Y1. The 231 and 4T1 models replicate metastasis to the same organs as humans. The luciferase tagged cell lines improved the capability to monitor and track the primary tumor and metastases in the mouse model by luminescence imaging. The 4T1 mouse model has been called the syngeneic model due to the implantation of cancer cells derived from the same mouse strain. This allows for the examination of the influence
of the immune system on tumorigenesis. However, the disadvantages of this system are that the mechanisms are based on the mouse background/microenvironment. The 231 mouse model is based on human breast cancer cell implanted into a severely compromised immunodeficient (SCID) mouse. While this model of human breast cancer can be studied, the system is deprived of an immune system, which can be inadequate for comprehensive studies.

To evaluate whether NDPK plays a role in metastasis, we tested both mouse models to examine the effect of inhibition on NDPK and P2Y1 on both primary tumor and lung metastasis growth. The breast cancer cell lines were luciferase-tagged for sensitive and rapid imaging to monitor the growth of the tumor and metastases. These cells were implanted into the mammary fat pads. Treatment with MRS2179, EA, MRS2179+EA (combo), and endostatin was started after palpable tumors developed. If the role of NDPK in angiogenesis translated to the in vivo models, then inhibiting NDPK transphosphorylation activity and the activation of the P2Y1 receptor would impact the ability of the breast cancer cells to form tumors and metastasize in the mice.

2.3. Materials and Methods

2.3.1. Reagents

MRS2179, endostatin, and ellagic acid (EA) compounds were purchased from Sigma Aldrich (MO).
2.3.2. Cell Culture

A luciferase-tagged human breast carcinoma, MDA-MB-231-Luc2 cells (231-Luc2) (Perkin Elmer, Hopkinton, MA) was grown in Eagle's Minimum Essential Medium (Thermo Fischer Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 2% penicillin/streptomycin (P/S, Thermo Fisher Scientific, Waltham, MA). The cells were trypsinized with 0.05% trypsin, and 2.5x10^6 231-Luc2 cells were resuspended in 50:50(v/v) with Matrigel (Corning, MA) and culture media for in vivo injection. The luciferase-tagged murine breast carcinoma, 4T1-Luc2 cells (Perkin Elmer, Hopkinton, MA) were grown in RPMI media (Thermo Fischer Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 2% penicillin/streptomycin (P/S, Thermo Fisher Scientific, Waltham, MA). The 4T1-Luc2 cells were trypsinized with 0.05% trypsin and 1x10^5 cells were resuspended in sterile 1xDPBS for in vivo injection. Both cell lines were grown at 37°C with 5% CO₂.

2.3.3. Mice

All animal procedures were in compliance with IACUC guidelines. For the 231 model, 6-week old female SCID mice were purchased from Charles River Laboratories (Charles River Laboratories, Hollister, CA). For the 4T1 model, 6-week old female BALB/c mice were purchased from Charles River Laboratories (Charles River Laboratories, Hollister, CA).
2.3.4. Orthotropic Human Breast Carcinoma (231) Mouse Model and Purinergic Inhibitor Treatments.

The 50:50 (v/v) MEM/Matrigel-prepared MDA-MB-231-Luc2 cells were subcutaneously injected into the mammary fat pad unilaterally of 4- to 6-week old female SCID mice to establish a primary tumor. The primary tumors were left to grow for 7 days or a primary tumor volume of 100-200 mm³ before the mice were randomly separated into different treatment groups: (1) untreated control, (2) MRS2179 (the ATP-P2Y1 receptor blocker), (3) EA, (4) combination of EA and MRS2179 (combo), and (5) endostatin (angiogenesis inhibitor). Dose levels of EA, MRS2179, and endostatin were tested and selected based on previous studies [316].

There were a total of 30 mice in the study and 6 mice in each treatment group. The 6 mice in the treatment group were divided into 2 subgroups and the subgroups were alternated between imaging sessions. Mice in the MRS2179, combo, and endostatin treatment groups were surgically implanted with mini osmotic pumps (Alzet, Cupertino, CA) to deliver the drugs at a constant rate. The pump (internal volume, 100 μl) continuously delivered drugs at the rate of 0.11 μl/hr for 30 days. MRS2179 was delivered at a rate of 8.5 µg/day/mouse. Every 28 days, these pumps were replaced with new pumps to maintain treatment regimen. The EA and combo treatment groups were orally administered with EA via drinking water at a concentration of 120 μg/ml. Upon the end of the study, the mice were euthanized with CO₂ gas and cervical dislocation as a secondary measure. The organs were harvested for bioluminescent imaging for metastasis.
2.3.5. **Syngeneic Murine Breast Carcinoma (4T1) Mouse Model and Purinergic Inhibitor Treatments.**

Freshly harvested 4T1-Luc2 cells were prepared at a concentration of $1 \times 10^5$ cells/100 μl to be subcutaneously injected into the mammary fat pad unilaterally of thirty 6-week old female BALB/c mice to establish a primary tumor. The primary tumors were left to grow for 7 days before the mice were randomly separated into the different treatment groups, each with 6 mice. Mice in the MRS2179, combo, and endostatin treatment groups were surgically implanted with mini osmotic pumps to deliver the drugs at a constant rate. The mini osmotic pump (internal volume, 100 μl) continuously delivered drugs at the rate of 0.11 μl/hr for 30 days. MRS2179 was delivered at a rate of 8.5 μg/day/mouse. Every 28 days, these pumps were replaced with new pumps to maintain treatment regimen. The EA and combo treatment groups were orally administered with EA via drinking water at a concentration of 120 μg/ml. Upon the end of the study, the mice were euthanized with CO₂ gas and cervical dislocation. The organs were harvested for bioluminescent imaging for metastasis.

2.3.6. **Primary Tumor and Metastases Imaging by Non-invasive in vivo Bioluminescence Imaging**

All animals carrying the luciferase-tagged cells were anesthetized with isoflurane/O₂ (1-5%) to undergo non-invasive whole body imaging twice each week (4T1) or once every 1-2 weeks (231) to visualize the presence of the bioluminescence cells growing as both primary and metastatic tumors by the Lumina II *in vivo* Imaging System (IVIS, Perkin Elmers, Waltham, MA). This procedure involved an injection of
d-luciferin substrate (Caliper Sciences, Hopkinton, MA) subcutaneous at the neck 5-20 minutes before the imaging procedure. Mice were imaged by the IVIS and injections staggered accordingly. The images were analyzed using Living Image software (Perkin Elmer, Waltham, MA).

2.3.7. Mouse Serum Collection

Every two weeks, 100-125 μl of blood was collected from the jugular vein. The blood was allowed to clot in a serum separator tube (Becton Dockinson, USA) at room temperature for 1-2 hrs. Serum was separated by centrifugation at 6,000 × g for 10 minutes. Serum was collected for experiments or stored at −80°C until further processes.

2.3.8. Serum NDPK ELISA

Mouse serum samples were diluted two-fold and 50 μl aliquots were added onto a high-binding 96 well plate. Recombinant human NM23-H2 (H00004831-P01, Abnova, Taiwan) was serially diluted to concentrations of 1000 ng/ml to 0.3 ng/ml and added to the wells. Sample and standards on the plate were incubated overnight at 4°C. Afterwards, the wells were blocked with 5% BSA for 1 hour and followed by several washes with 1xPBS containing 0.05% tween (PBST). The primary antibody of anti-Nm23 (Abcam, Cambridge, MA) was diluted 1:750 and incubated for 2 hours at 37°C. The wells were washed 3 times with PBST. Next, secondary antibody of anti-mouse IgG-HRP was diluted 1:2000, added to the wells, and incubated for 2 hours at 37°C. Again, the wells were washed 3 times with PBST and developed with OPD (Thermo Fisher Scientific, Waltham, MA) according to manufacturer’s
recommendations. The absorbance was measured at 450nm using a microplate reader.

2.3.9. Statistics

Graphs were all generated using Prism Graphing Software (V5; GraphPad Software, San Diego, CA, USA). All statistical analyses were performed with InStat Statistical Software (V3.0; GraphPad Software, Seattle, WA). Statistical significance was calculated using ANOVA, and p<0.05 was considered significant. The data points and error bars were represented as mean ± SEM.

2.4. Results

2.4.1. Murine breast carcinoma in vivo model

The 4T1 mouse model is a powerful tool as a breast cancer metastasis model. The 4T1 breast cancer cells were originally derived from a spontaneous adenocarcinoma that had arisen in a BALB/c murine mammary gland [319]. The cells can be implanted to grow in the mammary fat pads of BALB/c mice [320]. This allows for the murine breast cancer to develop in its natural tumor microenvironment. The implanted 4T1 cells can metastasize to the lungs, liver, and bones [321,322], which recapitulates the most frequent metastatic sites in human breast cancer patients. Along with that, the 4T1 BALB/c mouse model is an immunocompetent system for the study of the impact of immunomodulatory elements [321].

Female BALB/c mice were implanted with 4T1-Luc2 cells, which expressed luciferase to allow for bioluminescence imaging for tracking and monitoring the
breast cancer cells. All mice developed palpable tumors after one-week post injections of the cells into the mammary fat pad. These mice were divided into five treatments groups: control, EA, MRS2179, combo (EA + MRS2179), and endostatin. The primary tumor growth was measured by tumor bioluminescence (Figure 2-1A). As the tumors grew larger, the bioluminescence reported from the IVIS increased to show the growth of the primary tumor. By day 17, EA showed significantly lower tumor growth than the control group. At days 21 and 28, there were significantly decreased growth rates of the primary tumor in all treatment groups compared to the control group. In addition, day 28 reflected significantly lower metastasis in the chest area than the control (Figure 2-1B). This can be clearly observed from the representative images that show a smaller mammary tumor and less metastasis in the chest area in the combo treatment group (Figure 2-1C). These results suggest that combination treatment does decrease primary tumor and metastasis growth at day 28.
Figure 2-1: 4T1 tumor growth in BALB/C mice. The 4T1 tumor growth rate was measured by bioluminescence for 33 days after initial injection with the 4T1-Luc2 cells. Bioluminescence was recorded as photons emitted per sec/cm² from the regions of interest. The bioluminescence correlated with increased size of the tumor. (A) 4T1 primary tumor growth. (Inset) Quantification of primary tumor size at day 28 by bioluminescence. (B) 4T1 tumor growth in the chest area. (Inset) Quantification of 4T1 metastases in the chest area by bioluminescence at day 28. (C) Representative images of the mice on day 28. n=3-5 per group. *data shown with p<0.05. Green*data shown only significant with ellagic acid at p<0.05.
One of the most common areas of metastasis is the lung. The incidence rate for lung metastasis by week 2 or 3 in the 4T1 model was 79% [320]. At the terminal time point, the mice were sacrificed and the lungs were harvested and imaged. The metastasis of the 4T1 cells was detected by bioluminescence. There was a reduction of metastasis in the lungs of the MRS2179, EA, and combo groups compared to endostatin treatment (Figure 2-2). Taken together, these results indicated that by inhibiting NDPK and the P2Y1 receptor, there was less metastasis of 4T1 cells to the lungs.
Figure 2-2: Inhibition of NDPK and P2Y1 receptor decreased 4T1 metastasis to the lungs. At the 33 days, the mice were sacrificed and lungs were harvested and imaged. The bioluminescence was measured from the whole organ. The lung metastatic lesions were decreased in mice treated with MRS2179, EA, and combination compared to endostatin. Representative images of the lungs in each treatment group are shown. n=3-5 per group. *data shown with p< 0.05.
2.4.2. Orthotopic human breast cancer in vivo model

The 231-xenograft mouse model has been a well-established mouse model, which is based on the implantation of a human triple negative breast cancer cell line into an immunocompromised mouse. The MDA-MB-231 cell line was derived from a plural effusion of a patient with breast adenocarcinoma [323]. They are characterized as triple negative, in which these cells do not express estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2). Metastasis progression to the lung has been shown to occur as early as 4 weeks [324,325].

The primary tumor size was tracked by caliper measurements and calculated to determine the mean tumor volume (Figure 2-3A). In addition, the growth of the mammary tumor was monitored by bioluminescence (Figure 2-3B). The bioluminescence and mean tumor volumes were plotted and correlated with an R² value of 0.92 (Figure 2-3C). In both Figure 2-3A and 2-3B, MRS, EA, combo, and endostatin treatment groups had dramatically decreased primary tumor growth. Serum NDPK levels were measured by ELISA in healthy and tumor-implanted mice (Figure 2-3D). As tumor growth progressed, the serum NDPK levels also increased. This result supports the hypothesis that as the tumor size increases, there is also an increase in NDPK secretion. Together, these data showed that P2Y1 receptor and/or NDPK inhibition significantly suppressed the primary tumor formation in vivo.
Figure 2-3: Orthotopic growth of 231 cells in SCID mice. Triple negative breast cancer cells were injected into the mammary fat pads of immunocompromised mice. After 2 weeks of tumor growth, the mice were divided into five treatment groups: control, MRS2179, EA, combination, and endostatin. Primary tumor growth was measured by (A) calculated tumor volume and (B) bioluminescence. (C) Correlation of the mean tumor volume and bioluminescence. (D) Serum NDPK levels were measured during tumor growth by an ELISA. Inset image displays representative bioluminescence overlay images of mice bearing tumors.
To determine the extent of metastasis in the 231 model, at the terminal time point, the mice were sacrificed and the lungs, liver, and pancreas were harvested. The metastasis was detected by bioluminescence imaging with the luciferase activity of the 231 cells. Combination and endostatin treatment significantly reduced the metastatic burden when compared to control (Figure 2-4). Also, single drug treatments of MRS2179 and EA showed no significant difference in the metastatic tumor formation compared to control, further supporting that together MRS2179 and EA were more efficacious as a combination treatment.
Figure 2-4: Treatment with combination of MRS2179 and EA decreased 231 metastasis. At the final time point of 16 weeks, mice were euthanized and lungs, liver, and pancreatic tissues were harvested and imaged. The bioluminescence was measured and the averages were plotted. n=4-9 animals per group. *data shown with p< 0.05.

2.5. Discussion

Here we studied the in vivo role of extracellular NDPK and how inhibition of NDPK and the P2Y1 receptor influenced breast cancer growth and metastasis. The
extracellular role of NDPK supports a pro-metastatic activity that has been shown to promote endothelial cell proliferation, migration, and tumor-mediated angiogenesis [200,246]. NDPK was detected in the conditioned media of a wide range of breast cancer cell lines [246] and also in the serum of breast cancer patients [316]. The release of NDPK by breast cancer cells has been shown to stimulate cultured endothelial cells to differentiate into tubule structures that can be inhibited with EA [200]. This tubule formation is triggered by activation of P2Y1 with purine nucleotides generated by NDPK [198,199]. The P2Y1 receptor transactivates the VEGFR-2 to initiate angiogenesis [198].

While this is occurring in the extracellular environment, intracellular NDPK could also regulate angiogenic response in the endothelium. NDPK-B has been shown to be a regulator of VEGFR-2 angiogenesis, in which siRNA knockdown of NDPK-B impaired sprouting angiogenesis in HUVECs [326]. In addition, NDPK-B can interact with caveolins that promote caveolae formation, a sub-cellular compartment where VEGFR-2 resides [327]. Depletion of NDPK-B reduced Cav-1 localization to the plasma membrane and disrupted HUVEC sprouting angiogenesis. The study did not test whether the interaction of Cav-1 and NDPK-B mediated angiogenesis was dependent on NDPK transphosphorylation activity.

Elevated levels of ATP and ADP have been detected in the tumor microenvironment [328]. These purines can activate purinergic receptors on endothelial cells to stimulate angiogenic affects by release of vasoactive factors, such
as nitric oxide, prostacyclin, and more ATP [145,151,329]. These in vitro models demonstrate the relationship of NDPK signaling and angiogenesis.

In order to study the in vivo role of NDPK, we examined two orthotopic models to demonstrate the impact of NDPK on breast cancer metastasis, the 4T1 and 231 mouse models. While both of these model systems develop spontaneous metastasis, each model has advantages and disadvantages.

In the 4T1 mouse model, 4T1 cells were implanted into the mammary fat pads of immunocompetent BALB/c mice. Since the 4T1 cell line was originally derived from a BALB/c mouse, the developing tumors grow in the correct microenvironment with the presence of the immune system. However, the system is completely murine based, in which the treatment response may be different in a human patient. In addition, the cancer progression in the 4T1 model is quite rapid, in contrast to the slow latency exhibited in human breast cancer, making it difficult to study the mechanisms of early cancer development in this model. Due to the rapid progression of the 4T1 cells to metastasize, the studies can be performed in a shorter time frame. However, the 4T1 mammary carcinoma is highly aggressive, which leads to poor survival as could be seen in Figure 2-1A. This is best shown at the last time point, in which all groups were not significant in primary tumor size.

The 231 model is an established model to study human breast cancer, where the 231 cells, a human triple negative breast cancer cell line, were implanted into SCID mice. While the advantage of this model is the ability to study human breast cancer,
it cannot completely recapitulate the human tumor microenvironment. In addition, this system is deprived of an immune system, as the SCID mice are immunocompromised. This model does provide a better understanding of the dynamics of breast cancer latency because of the slow growth of the primary tumor, which enables closer monitoring of the serum NDPK levels. Both cell lines were luciferase-tagged, which allowed for sensitive and rapid imaging to track the growth and metastasis of breast cancer in the mice. In addition, the bioluminescence imaging assisted in the visualization of the metastases in the collected lungs. However, these models mostly showed advanced disease state and were not reflective of the initial mechanisms of spontaneous tumor development.

While both the 4T1 and 231 mouse models exhibited different growth curves, there were similar effects in drug treatment response. Both models yield reproducible tumors that spontaneously metastasize to the lungs. In both models, the primary tumor development was significantly decreased with combo treatment. The 4T1 mouse model had a fast growing primary tumor and showed metastases as early as 2 weeks. Due to the aggressive nature of the 4T1 cells the primary tumor growth rate was non-significant at the terminal time point. The 231 mouse model had slower growing primary tumors and showed distinct differences in the primary tumor growth rates. All treatment groups significantly decreased the growth of the primary tumor growth when compared to untreated mice. The MRS2179, EA, and combination treatment performed as well as endostatin, which was used as a positive control. This showed that inhibition of NDPK as well as the purinergic receptor
influenced the tumor growth rate. The tumor bioluminescence correlated well with the physical caliper measurements, which indicated that measuring with bioluminescence was a sensitive method for tracking breast cancer development.

The lung tissue is a frequent site of metastases in breast cancer. The highly tumorigenic and metastatic nature of the 4T1 model can be adapted for metastasis studies that examine the impact of surgery on recurrence. NDPK and P2Y1 inhibition decreased lung metastases in both the 4T1 and 231 mouse models. It is likely that NDPK and P2Y1R mediated breast cancer cell migration and that inhibiting this mechanism led to the decrease of metastases. In the future, primary tumors would be surgically removed so that the metastatic disease could be studied along with the impact of inhibition on NDPK and its endothelial target. In order to specifically examine the role of NDPK in tumorigenesis, an NDPK knockout breast cancer cell line could also be studied in either of the models. Additionally, serum NDPK levels could be tracked to determine the correlation of NDPK and metastatic disease, using a similar ELISA approach [247,316].

NDPK was first discovered as a tumor suppressor gene [147], and while some studies on the intracellular function of this protein have supported this notion [223,330,331], the role of extracellular NDPK is quite different. NDPK has been shown to interact with a variety of proteins and have multiple functions [332]. These functions include transphosphorylation activity [249], endonuclease activity [215–217], protein histidine kinase [218–220], c-Myc binding protein [221], and association with membrane lipids [222]. NDPK-B can regulate angiogenesis by
VEGFR-2 in which depletion of NDPK has shown decreased vessel formation, reduced VEGF-induced sprouting, and disrupted VEGFR-2 localization [326,327]. This research in parallel with ours shows a complete story about the intracellular and extracellular roles of NDPK to stimulate angiogenesis.

Our work has shown that NDPK plays a role in tumor-mediated angiogenesis [200] and in this study we found a reduction in metastases when NDPK was inhibited in the in vivo models. Inhibition of NDPK could impair blood vessel formation and limit nutrients to the primary tumor to decrease primary tumor growth rate. This would also limit the number of cells that could travel out of the local tumor microenvironment. In addition, the continuous secretion of NDPK by the cancer cells would cloak the cells with purine nucleotides to assist in immune evasion for the cells to travel to distant areas of the body [333]. Our proposed mechanism is that breast cancer-secreted NDPK elevates ATP concentrations in the extracellular matrix to stimulate angiogenesis and permit the escape of cancer cells through the blood stream [145,333]. This pathway was blocked by inhibition of NDPK and the ATP receptor P2Y1. This decreased both primary tumor growth and lung metastases. This study of NDPK has implications for biomarker development and targeted therapy of breast cancer. For the future of breast cancer therapies, it is important that researchers develop epistemic biomarkers that will assist in the identification of disease status and treatment.
Chapter 3: Novel inhibitors of NDPK

3.1. Abstract

Breast cancer is the most prevalent cancer diagnosed in women. Although modern medicine has significantly advanced treatment of breast cancer, there is no cure and new drug treatments are still in need. However, the cost of a new drug to be developed is much greater than for an existing drug to be repurposed. Utilizing the Prestwick Chemical Library, we screened against a nucleoside diphosphate kinase transphosphorylation activity assay to identify novel inhibitors of this enzyme. Eight inhibitors were identified as hits and were further evaluated to determine their IC$_{50}$. Out of the eight, 6,6'-[(3,3-Dimethoxy 1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis [4-amino-5-hydroxy-1,3-napthalenedisulphonic acid] (Chicago Sky Blue 6B (CSB)) was identified as a promising candidate with an IC$_{50}$ of 0.19 µM. In addition, both Chicago Sky Blue 6B and ellagic acid were also tested against an apoptosis assay using a triple negative breast cancer cell line, MDA-MB-231.

3.2. Introduction

Breast cancer is the most diagnosed cancer in women and the second leading cause in cancer related deaths only behind lung cancer [7]. Because the majority of cancer related deaths are due to metastasis, it is imperative to gain a better understanding of the mechanisms that regulate the metastatic process. There is a continuous growing need to develop better drugs to treat breast cancer, as well as
those that can specifically treat subtypes that are notoriously malignant and difficult to manage.

The whole operation of drug development is costly and labor intensive. The repositioning of previously FDA-approved drugs would help decrease the cost and time [334]. Many of these drugs have passed the preclinical and Phase I safety trials in healthy volunteers, which allow the drug to proceed into the next phase where they can be used in patients [335]. The timescale of repositioning a drug to FDA approval is around 6 years compared to the 12-16 years for the standard drug development. Many cancer screening approaches utilize the chemical libraries to reposition existing drugs with new purposes [336,337]. In 2014, around 30% of the FDA-approved drugs and vaccines were repositioned drugs [334].

One of the many characteristics of cancer is an elevation of local ADP/ATP levels within the tumor microenvironment [145,152]. Our lab has proposed that NDPK secreted into the extracellular matrix by breast cancer cells can elevate the local ADP/ATP levels to promote tumor-mediated angiogenesis and metastasis [200,246,338]. Serum from breast cancer patients had elevated levels of NDPK while it was not detected in healthy patients [316]. This increase in ATP activates purinergic receptors on nearby endothelial cells and promotes angiogenesis [198,199]. Various other ectonucleotidases can break down ATP to ADP and adenosine, which cause vasodilation and suppression of the immune system [156,158,329]. Thus, the tumor-secreted NDPK may play a central role in promoting the tumor microenvironment.
We used the NDPK transphosphorylation activity assay to screen the Prestwick Chemical Library to identify novel inhibitors of NDPK. Positive candidates inhibited NDPK activity at the same level or better than 3 µM ellagic acid (EA), a known inhibitor of NDPK [248,249]. Among the positive hits, we selected Chicago Sky Blue 6B (CSB), which showed an IC$_{50}$ of 0.19 µM compared to 0.06 µM for EA, to further study. HPLC analysis confirmed that CSB inhibited NDPK. Then we tested the physiologic affect of CSB with an apoptosis assay on triple negative breast cancer MDA-MB-231 cells. We demonstrated that CSB and EA could induce apoptotic cell death in breast cancer cells.

3.3. Materials and methods

3.3.1. Reagents and materials

Prestwick Chemical Library was kindly donated to our lab by Dr. Dean Burkin (Prestwick Chemical, San Diego, CA). The drugs from this library that resulted in positive hits were purchased from MilliporeSigma (Billerica, MA).

3.3.2. Generation and purification of recombinant NDPK-B protein

Total RNA was purified from human triple negative breast cancer (MDA-MB-231) cells with the MagMAX-96 total RNA Isolation Kit according to manufacturers’ directions. cDNA was generated using Superscript II (Thermo Fisher Scientific, Waltham, MA). NME2 cDNA was generated by PCR using primers AAAGAATTCATGGCCAACCTGGAG and AGTCTCGAGTTATTCATAGACCCAG for NME2. The resulting PCR product was inserted into the pTXB1 vector (New England Biolabs,
Ipswich, MA). The pTXB1-NME2 was transformed into competent Rosetta *E. coli* cells. Cells were induced with 0.5 mM IPTG at an OD of 0.4-0.8 to generate human recombinant NDPK-A and NDPK-B. After induction, the cells were pelleted at 4000 rpm for 10 minutes. The cell pellet was resuspended in RAK buffer (120 mM NaCl, 5 mM KCl, 0.587 mM KH$_2$PO$_4$, 0.589 mM Na$_2$HPO$_4$, 2.5 mM MgCl$_2$, 20 mM α-D glucose, 2.5 mM CaCl$_2$, 25 mM Tris base, and 5 mM NaHCO$_3$ at pH 7.4) and pulse sonicated 3 times for 3 bursts at 4 output control, 40% duty cycle, and 5 second cycle time. The lysate was then centrifuged at 15,000xg for 10 minutes and the supernatant was collected. The liquid was then subjected to ADP agarose (MilliporeSigma, Billerica, MA) column purification. NDPK-B protein was eluted with 10 mM ADP in RAK buffer. The collected recombinant protein was dialyzed in PBS in a snakeskin pouch and concentrated by Amicon Ultra-15 Centrifugal Filter Units (MilliporeSigma, Billerica, MA).

3.3.3. Transphosphorylation activity assay

Purified recombinant NDPK proteins were serially diluted into standards of 1000 ng/ml to 0.3 ng/ml in RAK buffer without CaCl$_2$. For the generation of the NDPK standard curve, standards were incubated with substrates of 10 µM ADP and 30 µM UTP and 10 µl of sterile water for 5 minutes. For the dose response curves, 10 µl of 10 ng/ml NDPK standard was incubated with same substrates and 10 µl of drug was added. The reactions were quenched with 0.1 M HCl in RAK buffer without CaCl$_2$ and incubated for 5 minutes to deactivate the enzyme. The pH was then neutralized back to 7.4 with 0.1 M NaOH in RAK buffer without CaCl$_2$. Next, equal volumes of luciferin-
luciferase ATP detection buffer (containing 0.3 mM luciferin, 6 μg/ml luciferase (0.2 mg/ml luciferase pH 7.6 constituted in 1 M glycine, 10 mM EDTA, and 100 mM MgSO₄·7H₂O), 98 mM glycine, 2 mM Tris-base pH 7.6, 10 mM MgSO₄, 1.1 mM EDTA, 2% BSA, and 2% sodium azide) were added and detected by the Chameleon plate reader with MikroWin 2000 software (Hidex, Turku, Finland).

3.3.4. Generation of anti-NDPK-B magnetic beads

Protein A magnetic beads (Spherotech, Inc., Lake Forest, IL) were crosslinked with CPTC-NME2-3 antibodies (Hybridoma Bank, Iowa City, Iowa) using bis(sulfosuccinimidyl)suberate (BS₃, Thermo Fisher Scientific, Waltham, MA) according to manufacturer’s recommendations. Briefly, at a concentration of 5 μg per 50 μl beads, antibody was diluted in PBS with tween-20 to the magnetic beads. After incubation, the antibody-bead complex was resuspended in 5mM BS₃ with conjugation buffer (20 mM sodium phosphate, 0.15 M NaCl at pH 7-9). Crosslinking occurred for one hour at room temperature, followed by neutralization with quenching buffer (1 M Tris HCl at a pH of 7.5). The crosslinked beads were washed with PBS with tween-20 and stored at 4°C.

3.3.5. High-performance liquid chromatography (HPLC) analysis of nucleotide products

The above transphosphorylation activity assay was used and after the NDPK reaction was quenched and neutralized, the wells were depleted of NDPK by the anti-NDPK-B magnetic beads. The wells (now containing nucleotide products) were transferred to HPLC vials. The samples were run on an Agilent 1100 HPLC with an
autosampler. Separation was performed with a Polaris C18-A reverse phase column (Agilent Technologies, Santa Clara, CA). The mobile phase consisted of buffer A (10 mM tetra butyl ammonium hydroxide (TBAH) and 10 mM KH$_2$PO$_4$ in deionized water) and buffer B (methanol and 10 mM TBAH). All buffers were sterile filtered with a 0.22 µm filter. The flow rate was held constant at 1.5 ml/min. The nucleotides were eluted from the column with the following gradient: buffer A at 95% for 20 mins, next changed to buffer A at 50% for 7 mins, maintained for 0.1 minutes, then decreased to 5% for 3 minutes, and held constant for 0.1 minutes. At 30.20 minutes, buffer A was changed to 95% and held constant for 3 minutes to elute off any remaining sample. The analyses of the nucleotides were recorded with the detector at 254 nm. Data was analyzed on Chromeleon software (ThermoFisher, Waltham, MA).

3.3.6. Apoptosis assay

MDA-MB-231 cells were grown in DMEM supplemented with 10% fetal bovine serum and 2% penicillin-streptomycin. The cells were passed to 96 wells at a concentration of 5,000 cells/well. After two days of incubation, the media was changed to DMEM supplemented with 2% FBS containing the chemical compounds for analysis. Treatment was incubated overnight. For the apoptosis assay, the cells were trypsinized and centrifuged to pellet. The cells were washed once with sterile PBS. FITC-labeled annexin V and propidium iodide were used to label the cells and then they were run on the Accuri C6 flow cytometer. The data were analyzed on FCS express v6 with compensation calculated from single stained annexin V and propidium iodide only wells.
3.4. Results

3.4.1. NDPK transphosphorylation activity assay

The transphosphorylation activity assay was adapted from Buxton, et al. [245,248,249]. This coupled assay utilizes the NDPK phosphotransfer function to generate ATP from ADP and UTP; the resulting ATP product was measured with a luciferase reaction (Figure 3-1A). The recorded signal could be correlated back to the standard concentration of recombinant NDPK protein, as indicated by the NDPK standard curve (Figure 3-1B). Since both NDPK-A and NDPK-B isoforms show no difference in the activity levels, NDPK-B was arbitrarily selected. An ATP standard curve was generated to show that 1 µM ATP was used as a positive control for the assay (Figure 3-1C). Previously, based on modeling efforts of designing a drug that would fit into the active site of NDPK, EA was determined to be a potent inhibitor of NDPK [249]. EA was used as a negative control and also as a target to measure the level of inhibition of the drugs in the Prestwick Chemical Library (Figure 3-1D).
Figure 3-1: Optimization of the NDPK-B transphosphorylation assay. Human recombinant NDPK-B protein was purified from E. coli and used as a standard for the transphosphorylation reaction at a concentration of 10 ng/ml. The substrates used in the reaction were ADP (10 µM) and UTP (30 µM). After 5 minutes of incubation, the reaction was acid quenched with 0.1 M HCl and pH was neutralized with 0.1 M NaOH. Next, the generated ATP product was measured in a luciferin and luciferase reaction. (A) Schematic of the two-step enzymatic reaction for the NDPK transphosphorylation activity assay. (B) Standard curve of NDPK-B activity. (C) ATP was used as a positive control and (D) ellagic acid, a known inhibitor of NDPK, was used a negative control for the activity assay. n=3.
3.4.2. Screening for novel inhibitors of NDPK

Using the NDPK transphosphorylation activity assay, the Prestwick Chemical Library was screened to identify novel NDPK inhibitors. The screening criteria were determined by reduced activity levels with potency better than or equal to that of 3 µM EA. At the concentration of 3 µM EA, NDPK inhibition displayed the lowest level of activity at the minimum concentration of EA (Figure 3-1D). From the 1,280 compounds screened in the chemical library, nine targets were identified as potential inhibitors of NDPK-B (Figure 3-2 and Figure 3-3). From these nine compounds, individual inhibitory kinetic curves were tested to determine the half maximal inhibitory concentration (IC$_{50}$) values of these compounds (Table 3-1 and Figure 3-4). However, the dose response curve of indoprofen proved it to be a false positive. Based on the dose response curves generated from the NDPK-B transphosphorylation activity assay, CSB had the lowest IC$_{50}$ value at 0.19 µM. The next lowest compounds were acacetin, myricetin, and niclosamide at 0.80, 0.86, and 0.76 µM respectively.
Figure 3-2: Prestwick chemical library screening using NDPK-B transphosphorylation assay. For the drug screening, decreases in luminescence signal (reported as relative light units) were indicative of inhibition of NDPK-B. EA at 3 µM was used as the criteria for determining potential drug candidates. Eight potential compounds were identified with luminescence values equal to or lower than EA. These were indicated as the bars with values below the dotted line. The dotted line represents the relative light units generated by the inhibition of EA on the NDPK-B transphosphorylation reaction. n=3.
Table 3-1: Positive compounds that were identified from the chemical library screening. From these eight compounds, the drug kinetic curves were individually tested and the IC\textsubscript{50} values were calculated.

<table>
<thead>
<tr>
<th>Drug name</th>
<th>IUPAC name</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niclosamide</td>
<td>5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide</td>
<td>0.76</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>(1R,2S,10S,11S,13R,14S,15S,17S)-1-fluoro-13,14,17-tri hydroxy-14-(2-hydroxyacetyl)-2,15-dimethyltetra cyclo [8.7.0.0(^2),7.0(^{11}),15] heptadeca-3,6-dien-5-one</td>
<td>31.18</td>
</tr>
<tr>
<td>Chicago Sky Blue 6B</td>
<td>tetrasodium;(6E)-4-amino-6-[[4-[(Z)]-2-(8-amino-1-oxo-5,7-disulfonatonaphthalen-2-ylidene)hydrazinyl]-3-methoxy phenyl]-2-methoxyphenyl[hydrazinylidene]-5-oxonaphthalene-1,3-disulfonate</td>
<td>0.19</td>
</tr>
<tr>
<td>Myricetin</td>
<td>3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one</td>
<td>0.86</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol</td>
<td>2.51</td>
</tr>
<tr>
<td>Acacetin</td>
<td>5,7-dihydroxy-2-(4-methoxyphenyl)chromen-4-one</td>
<td>0.80</td>
</tr>
<tr>
<td>Oxybenzone</td>
<td>2-benzoyl-5-methoxyphenol</td>
<td>44.78</td>
</tr>
</tbody>
</table>
| Chrys
i
n      | 5,7-dihydroxy-2-phenylchromen-4-one                                         | 3.94                 |
| Ellagic Acid       | 6,7,13,14-tetrahydroxy-2,9-dioxatetracyclo [6.6.2.0\(^4\),16.0\(^{11}\),15] heptadeca-1(15),4,6,8(16),11,13-hexaene-3,10-dione | 0.06                 |
Figure 3-3: Chemical structures of the positive compounds identified from the screening.
Figure 3-4: Dose response curves of potential NDPK-B inhibitors. Drug inhibition curves were tested at concentrations of 1 mM-3.3 nM for (A) Chicago Sky Blue 6B, (B) niclosamide, (C) acacetin, (D) oxybenzone, (E) myricetin, (F) chrysin, (G) triamcinolone, (H) indoprofen, and (I) resveratrol. n=3.

3.4.3. Validation of drug inhibition of NDPK using HPLC

To validate the drug inhibition of NDPK, an HPLC method was developed to directly measure the ATP products. For the experiment, CSB was incubated with NDPK and substrates for five minutes and the reaction was quenched with acid and the pH was neutralized with base. The recombinant NDPK was isolated with antibodies conjugated to magnetic beads. The solution, now containing nucleotide
products and drug, was resolved by HPLC and separated on a C18 reverse phase column. A standard curve of ATP was run to identify the ATP peak on the chromatogram (Figure 3-5A). The dose response curve of CSB generated a higher IC_{50} value (0.82 µM) (Figure 3-5B). From these results, the HPLC method could be used as a secondary measure to determine the dose response inhibition.

![ATP Standard Curve](image1)

![Chicago Sky Blue Inhibition Curve](image2)

**Figure 3-5**: HPLC validation of Chicago Sky Blue 6B inhibition. As a secondary method, HPLC was used to confirm the results from the activity assay. Following the transphosphorylation activity, the substrates were directly measured on HPLC. The HPLC data were collected using Chromeleon 7 software. (A) An ATP standard curve was generated to validate the method and identify the ATP peak. (B) The CSB dose response curve was produced. The calculated IC_{50} value was 0.82 µM. n=3.
3.4.4. Drug induction of apoptosis in 231 cells

We performed an apoptosis assay to determine whether CSB could induce apoptosis in MDA-MB-231 cells at a similar concentration as EA. Annexin-V and propidium iodide labeling were used to assess the potential of using CSB to induce apoptosis in breast cancer cells. Treatment with CSB induced apoptosis in more cells than EA (Figure 3-6A). However, EA displayed significantly more cell death than CSB (Figure 3-6B). Future analysis will involve examining apoptosis in a time course to address this.
Figure 3-6: Analysis of apoptosis in triple negative breast cancer cells with CSB. Triple negative breast cancer cells, MDA-MB-231, were treated overnight with 10 μM EA, 10 μM CSB, or 1% DMSO. These cells were labeled with PI and FITC-annexin V to differentiate between live, dead, and early apoptotic cells on the Accuri C6 Flow Cytometer. The graphs are the quantification of (A) apoptotic cells and (B) dead cells. n=3. *p<0.05.
3.5. Discussion

NDPK has been implicated in cancer tumorigenesis, metastasis, and angiogenesis. A variety of cancer cell lines display extracellular NDPK activity [245]. The conditioned media collected from breast cancer cells stimulated endothelial cell proliferation, migration, and differentiation into tubules [200, 246]. This is mediated by the transphosphorylation activity, in which NDPK acts as a phosphoprotein to transfer a phosphate group to an ADP to generate ATP. This local elevation of purine nucleotides acts on purinergic P2Y1 receptors expressed on adjacent endothelial cells to promote angiogenesis [198, 199]. Inhibition of NDPK by EA inhibited Erk1/2 phosphorylation and VEGFR-2 activation to suppress angiogenesis [246]. Also EA treatment decreased primary tumor growth and lung metastasis in an orthotopic in vivo model of breast cancer [316]. Therefore, inhibition of NDPK has significant implications in breast cancer development and metastasis.

Some of the first identified inhibitors of NDPK were derivatives for c-AMP [339]. Ebselen has been identified as a non-competitive inhibitor of NDPK [340]. In addition, quercetin and acacetin, flavonoid compounds, have been demonstrated as inhibitors of rabbit muscle NDPK [341]. Both quercetin and acacetin were positive hits on our drug screen, which aligned the drugs confirmed in the screening approach with results on NDPK inhibitors from literature. Many of the positive hits in the assay, acacetin, chrysin, myricetin, quercetin, and resveratrol were identified as antioxidants and flavonoids. Resveratrol was also confirmed from previous studies
[248,249]. CSB was a unique candidate in that it had the lowest IC$_{50}$ from the screen with a large complex structure.

CSB does not inhibit only NDPK; there are other proteins that CSB acts to inhibit. Originally, CSB was demonstrated as an inhibitor of glutamate uptake in synaptic vesicles [342]. In addition, CSB has been shown to inhibit Rad51, a protein known to be involved in tumor progression, by blocking the protein from performing homologous recombination [343]. The multiple mechanisms in cancer that are inhibited by CSB show that it may be a potential drug in anti-cancer therapy. As part of the Prestwick Chemical Library, CSB may require less testing, as it is already an FDA approved drug.

To demonstrate that the CSB had a physiologic effect on breast cancer cells, an apoptosis assay was performed. While EA has been shown to decrease growth of breast cancer and endothelial cells [200,246,338], EA can promote apoptosis of breast cancer cells [344]. EA induced apoptotic sensitivity of breast cancer cells to radiation [345]. While CSB induced more apoptosis in the breast cancer cells, EA resulted in significantly more cancer death. Another study in the future would involve examining this mechanism over a time course. The apoptosis assay demonstrated that CSB could potentially be used to inhibit NDPK in a breast cancer model and also induce apoptosis.

Overall, the results from this screening will provide more information about structural commonalities between the inhibitors of NDPK. This may lead to in silico
development of related NDPK inhibitors based on known structures of the drugs to
design a specific and potent compound. On the other hand, repositioning of old drugs
decreases the time required for initial drug development and thus these drugs may
directly go into preclinical and clinical trials. Nevertheless, inhibition of NDPK to
decrease local concentrations of ATP/ADP could be used as an anticancer therapy to
decrease vascular functions that promote pro-tumorigenic effects [333].
Chapter 4: Triple Negative Breast Cancer Cells Secrete Exosomes that Contain Nucleoside Diphosphate Kinase

4.1. Abstract

Extracellular nucleoside diphosphate kinase (NDPK) has been shown to play a role in promoting breast cancer metastasis, but the mechanism by which it is elaborated outside of the cell remained to be elucidated. Here we provide evidence that NDPK can escape into the extracellular matrix through breast cancer secreted exosomes. We utilized transmission electron microscopy, super-resolution microscopy, and flow cytometry to characterize the exosomes purified from a triple negative breast cancer (231) and human mammary epithelial (HME1) cell lines. Transphosphorylation activity assay and western blot were used to demonstrate that NDPK associated with the 231 exosomes. Finally, a tubulogenesis assay was used to demonstrate the physiological effect of NDPK associated with 231 exosomes in human umbilical vein endothelial cells (HUVECs). We found that NDPK from breast cancer cell-released exosomes stimulated tubulogenesis. Also inhibition of exosomal NDPK and the purinergic P2Y1 receptor with the combination treatment of drug inhibitors, ellagic acid (EA) and MRS2179, respectively, decreased endothelial cell tubulogenesis. Taken together, our results reveal that breast cancer cells release exosomes that contain NDPK and that this exosomal NDPK had implications in propagating angiogenesis.


4.2. Introduction

Breast cancer is the most diagnosed cancer and the second leading cause of cancer-related deaths of women in the developed world. Around 30% of all newly diagnosed cancer cases in women are of the breast [7]. Breast cancer related deaths amount to about 14.4% of all estimated cancer deaths in women. Despite the implementations of early detection and aggressive treatment, breast cancer patients may still succumb to the cancer. The majority of cancer related deaths are due to metastasis [8]. This alone highlights the importance of understanding and developing new methods for targeting metastasis. Although there have been considerable advances in cancer medicine, the mechanisms of metastasis are not completely known.

While recent studies have shown that breast cancer cells can disseminate even before the presence of a detectable primary tumor [53,54]; dissemination is an early event during tumorigenesis [346,347]. Once the primary tumor has established a blood supply source, the cancer cells intravasate the vascular system and travel to distance sites of the body [348]. These cells may extravasate from blood vessels, inhabit a host tissue site, and can remain dormant for extended periods of time before they develop into metastases. Disseminated breast cancer cells have a proclivity to form metastases in the brain, bones, lungs, or liver [58]. These tissues may have certain properties that increase the chances of the breast cancer cells to survive outside of the host tissue. Although before the cancer cell may settle, it has been suggested that the secondary tissue area may need to be primed to allow for the
arrival of the cancer cells. This site has been called the pre-metastatic niche [122,124].

Cancer cells in the primary tumor may prime the pre-metastatic niche by the secretion of extracellular vesicles (EVs) and other cell released factors that target specific tissues to prepare for the arrival of the disseminated tumor cells [284]. Exosomes are small vesicles (30-100 nm in diameter) that are released by all cell types to promote a variety of signaling mechanisms [258]. They are derived from inward budding of multivesicular bodies, which fuse to the plasma membrane and dispel the exosomes into the extracellular matrix. These exosomes are mediators of the cancer cells to promote changes in the host tissue, such as remodeling of the ECM, stimulating angiogenesis [98,349], and modulation of the immune system [350,351]. This is achieved through signal transduction mediated by the transfer of the exosome cargo, which contains proteins, lipids, RNA, and DNA. Cancer cells have been shown to elaborate more exosomes than their non-tumorigenic counter parts [352].

Nucleoside diphosphate kinase (also known as NDPK and NM23) was first discovered as a metastasis suppressor [147]. There are ten known isoforms of this protein. Cellular localization of NDPK is ubiquitous, and was detected in the cytosol, plasma membrane, nucleus, and mitochondria [353,354]. NDPK-A and NDPK-B are the two isoforms that have been known to be detectable in the extracellular matrix [246]. Decreased intracellular NDPK transcript levels in breast cancer have been correlated to higher potential of metastasis [147], but extracellular functions of this protein have shown the opposite effect [145,338]. NDPK has been detected in the
conditioned media collected from breast cancer cells, but not from their normal cell type equivalents [245,246]. NDPK has been shown to promote proliferation, migration, and tubulogenesis in endothelial cells [198,200,246]. The intracellular functions of NDPK are histidine kinase [220], exonuclease [217], and transcription regulator [215,235]. Extracellular NDPK found in conditioned media has been shown to generate ATP in the presence of GTP and ADP substrates [245]. The locally elevated ATP/ADP levels activate purinergic (P2Y1) receptors, which transactivate VEGFR-2 on endothelial cells to stimulate angiogenesis [198,199]. Because VEGFR-2 can be activated by purinergic signaling in the absence of VEGF to stimulate angiogenesis, the conventional idea of blocking VEGF will not completely block angiogenesis from occurring. This extracellular role of NDPK suggests an alternative mechanism that supports tumor mediated metastasis and angiogenesis.

In this study, exosomes were purified, characterized, and used in angiogenesis studies. Super resolution microscopy, flow cytometry, and western blot analyses demonstrated the presence of NDPK associated with breast cancer-released exosomes. A transphosphorylation activity assay showed the NDPK levels in the exosomes. Tubule formation by human umbilical vein endothelial cells was used to investigate exosomal NDPK-mediated angiogenesis. We demonstrated that breast cancer exosomes contained elevated levels of NDPK protein and induced an angiogenic response mediated by P2YR/VEGF receptor signaling.
4.3. Materials and Methods

4.3.1. Reagents

MRS2179, MRS2279, MRS2500, and ellagic acid (EA) compounds were purchased from Tocris Bioscience (Bio-Technne, Avonmouth, Bristol, United Kingdom). MeSADP was purchased from Sigma-Aldrich (MilliporeSigma, Billerica, MA).

4.3.2. Cell Culture

MDA-MB-231 cells were grown in DMEM (Thermo Fischer Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 2% penicillin/streptomycin (P/S, Thermo Fisher Scientific, Waltham, MA). The non-tumorigenic mammary epithelial cell line used as a control was human telomerized human mammary epithelial cells (hTERT-HME1) (ATCC, Manassas, VA) and was grown in HuMEC complete media (Thermo Fisher Scientific, Waltham, MA) supplemented with bovine pituitary extract, HuMEC supplement kit, and 2% P/S. Human umbilical vein endothelial cells (HUVEC, Lonza, Allendale, NJ) were cultured in complete EGM-2 (Lonza, Allendale, NJ). These HUVEC cells were pooled isolated cells from ten patients. MDA-MB-231-CD63-GFP and hTERT-HME1-CD63-GFP cell lines were generated by transduction of the cells with packaged lentivirus containing pCT-CD63-GFP (System Biosciences, Palo Alto, CA) with 6 μg/ml polybrene. The GFP had a spectrum of excitation at 482 nm and emission at 502 nm. GFP-positive cells were sorted by a FACs flow cytometer.
4.3.3. Exosome Purification

Exosomes were purified with ExoQuick-TC (System Biosciences, Palo Alto, CA) according to manufacturers’ instructions. Cells were grown to >90% confluence, washed twice with sterile PBS for one minute, and washed with media containing 1x insulin, transferrin, and selenium (1xITS, Thermo Fisher Scientific, Waltham, MA) for 2-6 hours before the cells were left in media containing 1xITS for 18 hours. The collected conditioned media was centrifuged at 3000xg for 15 minutes at room temperature to separate out the dead cells and debris. ExoQuick-TC was added to the supernatant and incubated overnight at 4°C. The precipitated exosomes were isolated by centrifugation at 1500xg for 30 minutes.

4.3.4. Transmission Electron Microscopy (TEM) Sample Preparation

Transmission electron microscopy grids were prepared according to Thery et al. Briefly, exosomes were diluted in 4% paraformaldehyde (PFA) to a final concentration of 2% PFA. The samples were adsorbed onto Formvar-carbon coated nickel grids, washed with 50 mM glycine in PBS, and blocked with 0.5% bovine serum albumin (BSA). For immunolabeling, the grids were incubated with antibodies (Biolegend, San Diego, CA) for tetraspannnins associated with exosomes (CD9, CD81, and CD63), then secondary antibodies conjugated with 10nm gold particles (Abcam, Cambridge, MA) were used for labeling, and 1% glutaraldehyde was added to stabilize the reaction. Uranyl oxalate was used to stain the exosomes and 2% methyl cellulose-4% uranyl acetate was used to embed the grids. Grids were analyzed by the UNR imaging core on the Philips CM10 transmission electron microscope with a GATAN
BioScan 792 digital scanner. Images were analyzed on Digital Micrograph 2.1.0 image analysis software.

4.3.5. Examination of Exosomes with the Super-Resolution Microscope

Exosome samples were prepared as immunofluorescence slides. Briefly, glass slides were coated with fibronectin and isolated exosomes were added to adhere to the slides. The slides were then fixed with 4% PFA and blocked with 5% BSA. The slides were then incubated with 1:100 diluted primary antibodies overnight at 4°C. Next day, the slides were washed five times with PBS and incubated with 1:100 secondary antibodies for one hour at room temperature. Again, the slides were washed five times with PBS. Slides were mounted onto depression slips with 100 mM cysteamine (MEA), 50% glucose, 1 M Tris base, and glucose oxidase and catalase solution (GLOX). The slide was then sealed with 1:1 Twinsil (Picodent, Wipperfürth, Germany). The slides were imaged on the Leica GSD super resolution microscope (Leica Microsystems Inc., Buffalo Grove, IL).

4.3.6. Analysis of Exosomes by Flow Cytometry

The purified exosomes were resuspended in 0.1 M sodium phosphate buffer (pH7.4) and diluted in sheath fluid. Brilliant violet-labeled annexin V (Biolegend, San Diego, CA) and annexin binding buffer were added to the samples. For antibody labeling, we used FITC labeled NDPK-A (US Biologicals, Salem, MA), PE-CD63 (Biolegend, San Diego, CA), and their respective fluorophore-isotype controls (Biolegend, San Diego, CA), and incubated in the dark at room temperature for 20 minutes. Samples were submitted to the UNR Flow Cytometry Core B facility to run
on the BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA). Configurations can be found on the UNR website (https://med.unr.edu/physio/cobre-smooth-muscle/core-b/instrument-descriptions-for-publications). Data were analyzed on the FCS Express 6 Flow Cytometry Software.

4.3.7 Western Analysis

Isolated exosome samples were lysed with RIPA buffer containing HALT protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) using the Bullet Blender twice at setting nine for five minutes with a minute vortex in between. The protein lysate was centrifuged at 10,000xg for ten minutes. The protein concentration was measured with an EZQ assay. For each sample, 20 µg of protein were run on a 4-20% mini-PROTEAN TGX gel (BioRad, Hercules, CA) for 45 minutes at 200 V. Next, the gel was transferred onto a Trans-blot Turbo nitrocellulose membrane and immunoblotted with CPTC-NME1-5 (Hybridoma Bank, Iowa City, Iowa) for NDPK-A and NDPK-B and GAPDH (D16H11) XP Rabbit mAb (Cell Signaling Technology, Danver, MA).

4.3.8 Recombinant NDPK-A and NDPK-B Generation and Purification

Total RNA was purified from human triple negative breast cancer (MDA-MB-231) cells with the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturers’ directions. cDNA was generated using Superscript II (Thermo Fisher Scientific, Waltham, MA). NME1 and NME2 cDNA were generated by PCR using custom primers 5’AAAGAATTCTAGGCCAACTGTGA3’ and 5’AGTCTCGAGTCATCATAGATCCAG3’ for NME1 and
5’AAAGAATTCATGGCCAACCTGGAG3’ and 5’AGTCTCGAGTTATTCATAGACCCAG3’ for NME2 (Integrated DNA Technologies, Coralville, IA). The resulting PCR product was inserted into the pTXB1 vector (New England Biolabs, Ipswich, MA). The pTXB1-NME1 and pTXB1-NME2 was transformed into Rosetta competent cells (MilliporeSigma, Billerica, MA). Cells were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at an OD of 0.4-0.8 to generate human recombinant NDPK-A and NDPK-B. After induction, the cells were pelleted at 3000xg for 10 minutes. The cell pellet was resuspended in room air Krebs (RAK) buffer (120 mM NaCl, 5 mM KCl, 0.587 mM KH₂PO₄, 0.589 mM Na₂HPO₄, 2.5 mM MgCl₂, 20 mM α-D glucose, 2.5 mM CaCl₂, 25 mM Tris, and 5 mM NaHCO₃ at pH 7.4) and pulse sonicated 3 times for 3 bursts at 4 output control, 40% duty cycle, and 5 second cycle time. The lysate was then centrifuged at 15,000xg for 10 minutes and the supernatant collected. The liquid was then fractioned by size exclusion chromatography with Bio-Gel P30 beads (BioRad, Hercules, CA). Fractions exhibiting transphosphorylation activity were pooled and subjected to ADP agarose (MilliporeSigma, Billerica, MA) column purification. NDPK-A and NDPK-B protein were eluted with 10 mM ADP in RAK buffer. The collected recombinant protein was dialyzed in PBS in a snakeskin pouch and concentrated by Amicon Ultra-15 Centrifugal Filter Units (MilliporeSigma, Billerica, MA).

4.3.9. NDPK Transphosphorylation Activity Assay

Exosome lysates were prepared in 10% triton X-100 in RAK buffer without CaCl₂. Purified recombinant NDPK-A and NDPK-B were serially diluted into
standards of 1000 ng/ml to 0.3 ng/ml in 10% triton X-100 RAK buffer without CaCl$_2$. The standards were incubated with substrates of 10 $\mu$M ADP and 30 $\mu$M UTP for 6 minutes and quenched with 0.1 M HCl in RAK buffer without CaCl$_2$. The pH was then neutralized back to 7.4 with 0.1 M NaOH in RAK buffer without CaCl$_2$. Next, equal volumes of luciferin-luciferase ATP detection buffer (containing 0.3 mM luciferin, 6 $\mu$g/ml luciferase (0.2 mg/ml luciferase pH 7.6 constituted in 1 M glycine, 10 mM EDTA, and 100 mM MgSO$_4$$\cdot$7H$_2$O), 98 mM glycine, 2 mM Tris-base pH 7.6, 10 mM MgSO$_4$, 1.1 mM EDTA, 2% BSA, and 2% sodium azide) were added and detected by the Chameleon plate reader (Hidex, Turku, Finland) with MikroWin 2000 software.

4.3.10. Tubulogenesis Assay

HUVECs were used between passages 3-6 for the tubulogenesis assay. Geltrex (Thermo Fisher Scientific, Waltham, MA) was coated onto µ-Slide Angiogenesis slides (Ibidi, Madison, WI) and allowed to solidify at room temperature. HUVECs were seeded at 7,500 cells per well in the respective concentrations of reagents. The plate was incubated at 37°C for 6 hours and fixed with 4% PFA. Images were taken with the Keyence BZ-X microscope (Keyence, Itasca, IL).

4.4. Results


NDPK has been detected in the conditioned media of human breast, colon, pancreas, and lung cancer cell lines [245]. In addition, NDPK has been detected in the
serum of breast cancer patients and not in healthy patients [246]. However, these studies did not reveal how NDPK was released into the extracellular environment. NDPK does not contain a secretory signal peptide, and therefore may be secreted by an unconventional mechanism. NDPK has been shown to be associated with lipid rafts on the plasma membrane [222], and around intracellular vesicles [355]. We hypothesized that NDPK might be associated with exosomes, the nano-sized extracellular vesicles that mediate cell-to-cell communication. To test this hypothesis, exosomes were purified and characterized to determine the presence or absence of NDPK.

Exosomes were purified from conditioned media collected from a triple negative human breast cancer cell line, MDA-MB-231 (231) cells, and a non-tumorigenic human mammary epithelial cell line, hTERT-HME1 (HME1) cells. Exosomes were isolated by precipitation with ExoQuick-TC, adsorbed onto nickel grids, stained with uranyl oxalate, and examined by TEM. According to TEM, exosomes were about 30-100 nm in diameter (Figure 4-1A). The average size of a mammalian cell is 25 µm. The exosome morphology displayed a phospholipid bilayer (Figure 4-1B). Next, the 231 and HME1 exosomes were labeled for tetraspanin proteins that were commonly associated with exosomes (Figure 4-1C and 4-1D, respectively). Some of the exosomes were positively labeled for tetraspanins, which indicated that the exosome populations were heterogeneous. These results indicated that the 231 and HME1 purified exosomes were of the correct size and morphology.
**Figure 4-1:** TEM images of purified exosomes. Exosomes were purified from 231 and HME1 cells. (A) TEM image of exosomes isolated from 231 cells. Scale 100 nm. (B) Close up image of a 231 exosome. Scale 20 nm. (C and D) 231 and HME1 exosomes labeled for tetraspanins, respectively. Scale 100 nm.

Purified 231 and HME1 exosomes were further analyzed by flow cytometry to characterize surface markers. To determine that we had purified exosomes that originated from invaginations of the early endosomes and not from apoptotic bodies, we incubated the HME1 exosomes with annexin V. Annexin V binds to phosphatidylserines, which are found on the inner membrane leaflet, and is typically used for the detection of apoptosis in cells. Cells from both 231 and HME1 cell lines were used as a positive control to verify annexin V labeling in binding buffer (data not
shown). We did not observe any annexin V labeling, which indicated that the vesicles were not apoptotic bodies (Figure 4-2).

**Figure 4-2:** Annexin V does not bind to exosomes. HME1 exosomes were incubated with or without brilliant violet 421 annexin V in annexin V binding buffer and analyzed by flow cytometry. Histograms were generated from HME1 exosomes with (black) and without (red) annexin V.
Next, exosomes from both cell lines were labeled for CD63, a tetraspanin protein associated with exosomes. 231 and HME1 exosomes positively labeled for CD63 as indicated by the shift in the exosome populations on the scatter plots when compared to no label and isotype control (Figures 4-3A and 4-3B, respectively). The respective histograms showed a shift in the fluorescence intensity in the 231 and HME1 labeled exosomes, which indicated the presence of CD63 (Figures 4-3C and 4-3D, respectively). Next, the 231 and HME1 exosomes were labeled for NDPK (Figures 4-3E and 4-3F, respectively). 231 exosomes displayed a greater shift on the histogram between isotype control and NDPK labeling when compared to the HME1 exosomes (Figures 4-3G and 4-3H, respectively). These results indicated exosomes could be detected by flow cytometry, and that exosomes from 231 cells expressed more surface NDPK than that of HME1 cells.
Figure 4-3: The identification of CD63 and NDPK on 231 and HME1 exosomes by flow cytometry. Purified exosomes had either no label (black), isotype antibody (red), or CD63/NDPK antibody (blue). (A and B) Scatter plots of 231 and HME1 exosomes, respectively, analyzed for the presence of CD63. (C and D) Histograms of 231 and HME1 exosomes, respectively, generated from the NDPK scatters plots. (E and F) Scatter plots of 231 and HME1 exosomes, respectively, analyzed for the presence of NDPK. (G and H) Histograms of 231 and HME1 exosomes, respectively, rendered from the NDPK scatters plots.
To further show the presence of NDPK on 231 exosomes, we utilized super resolution microscopy to image the purified structures. The exosomes were isolated from the 231 cells that were transduced to express CD63 fused to GFP (Figure 4-4A). The purified exosomes were GFP positive and these were labeled for NDPK. There was co-localization of NDPK-B and CD63-GFP (Figure 4-4B). By Western blot, we analyzed the protein levels of NDPK with an antibody that detected both NDPK-A and NDPK-B isoforms (Figure 4-5A). There was a significantly higher expression of NDPK in the 231 exosomes than the HME1 exosomes (Figure 4-5B). These results indicate that there was more NDPK in the 231 exosomes samples than the HME1 exosomes samples.

**Figure 4-4:** Colocalization of NDPK with CD63-GFP on a 231 exosomes examined by super-resolution microscopy. (A) 231 cells were transduced with lentivirus containing a construct for CD63 fused to GFP. (B) Immunofluorescence of NDPK protein (red) on a purified 231 exosome that expressed CD63-GFP (green) fused protein.
Figure 4-5: NDPK expression in HME1 and 231 exosomes. (A) Representative western blot of NDPK (top) and GAPDH (bottom) protein levels from HME1 and 231 exosomes. (B) Quantification of western results for NDPK normalized to GAPDH. The 231 cell lysate (231 CL) was used as a positive control for NDPK and GAPDH detection. The graph shows mean values and standard deviations. n=3. *p<0.05.
4.4.2. Higher Levels of Exosomal NDPK Transphosphorylation Activity in 231 Exosomes.

Breast cancer cells release NDPK that can act as a trans-phosphorylase in the extracellular environment, by which the enzyme can transfer a terminal γ-phosphate from a nucleoside triphosphate to a nucleoside diphosphate [245,246]. This is performed via a high-energy phosphohistidine intermediate at residue H118. Conditioned media collected from 11 different breast cancer cell lines displayed different levels of NDPK transphosphorylation activity [246]. We utilized the same transphosphorylation activity assay to measure the NDPK activity of exosomes. The assay was a two-step enzymatic reaction. Briefly, NDPK was incubated with substrates of ADP and UTP to generate ATP. Subsequently, the ATP product was used in a reaction with luciferin and luciferase to produce a luminescent signal. The detected luminescence could then be calculated back to the concentration of NDPK using a standard curve. Purified human recombinant NDPK was used as a standard in the enzymatic assay and there was no difference in NDPK activity with the addition of DMSO (Figure 4-6). When normalized to protein concentration, there was significantly more transphosphorylation activity with the 231 exosomes compared to HME1 exosomes indicating there was higher NDPK activity in 231 exosomes (Figure 4-7A). DMSO was used to dissolve EA, a known inhibitor of NDPK [248,249]. Addition of EA inhibited this activity (Figure 4-7B), which is consistent with previous results of EA inhibition of the purified NDPK protein [249]. The results demonstrated that
the NDPK associated with the exosomes was functional and the activity was sensitive to EA inhibition, indicating specificity.

**Figure 4-6:** NDPK-B standard curve and controls for the transphosphorylation activity assay. (A) Purified human recombinant NDPK-B was diluted and used as standards in the transphosphorylation activity assay for the calculation of the amount of exosomal NDPK. (B) Addition of DMSO (DMSO control) to the NDPK-B reaction showed no effect on NDPK-B activity. Substrates alone (ADP + UTP) showed little background activity. n=3.
Figure 4-7: Transphosphorylation activity of exosomal NDPK. (A) NDPK activity normalized to total protein in 231 and HME1 exosomes. (B) NDPK activity was inhibited by ellagic acid. The graphs show mean values and standard deviations. n=3. *p<0.05; **p<0.01; ***p<0.001.

Also, whole and lysed 231 exosomes were tested with substrates of ADP and UTP (Figure 4-8) to determine if the NDPK was located outside or inside of the exosome. Whole exosomes displayed transphosphorylase activity. However, lysed exosomes had an increased level of activity, indicating that NDPK was likely to be more prevalent on the inside of the exosome. When ADP substrate was used alone, there was decreased activity compared to the samples with the addition of UTP, indicating that the ATP generating activity was due to NDPK and not adenylate kinase. Adenylate kinase is an intracellular phosphotransferase enzyme that catalyzes the reaction of two molecules of ADP to generate AMP and ATP.
Figure 4-8: NDPK transphosphorylation activity of lysed or intact exosomes with ADP or ADP/UTP substrates. Exosome samples were either whole or lysed with 10% triton X-100 and tested with either ADP or ADP/UTP substrates to determine where majority of the transphosphorylation activity was located on the outside or the inside of the exosomes. The protein equivalent of 1.5 µg of exosomes was used in each treatment condition.

4.4.3. Exosomal NDPK Meditates Tubulogenesis.

Previously, our lab had shown that conditioned media from MDA-MB-435 cells stimulated tubulogenesis [200]. Upon further investigation, it was determined that NDPK generated ATP/ADP that activated the P2Y1 receptors on the endothelial cells
to induce tubulogenesis. Addition of MRS2179 decreased tubulogenesis, which demonstrated that the mechanism was promoted through the P2Y1 receptor. Cancer secreted exosomes have been shown to stimulate angiogenesis through a number of different exosomal proteins, although the role of exosomal NDPK has not been explored.

A tubulogenesis assay was performed using HUVECs on growth factor-reduced Geltrex. HUVECs were incubated with 100 µM MeSADP, an agonist of the P2Y1 receptor, as a positive control to stimulate tubule formation. NDPK with the addition of its substrates of ADP and GTP stimulated tubulogenesis at a similar level as 10 ng/ml VEGF, 100 µM MeSADP, and EGM-2 controls (Figure 4-9A). Treatment with a combination of EA (10 µM) and MRS compounds (either 10 µM MRS2179, 100 nM MRS2279, or 10 nM MRS2500) significantly decreased tubulogenesis induced by NDPK and substrates. Next, we used exosomes purified from the MDA-MB-231-CD63-GFP cells and incubated them with HUVECs. We showed that GFP exosomes were incorporated with the endothelial cells as seen by the fluorescent tubule structures (Figure 4-9B). Combination treatment with ellagic acid and MRS2179 abolished exosome induced tubule formation. These results demonstrated that 231 exosomes induced tubulogenesis in HUVECs and this effect was abrogated by the combination treatment of EA and MRS2179.
Figure 4-9: Exosomal NDPK stimulates tubulogenesis. (A) Representative images of HUVEC tubulogenesis with different treatments. One (1) µg of human recombinant NDPK with 100 µM ADP + 300 µM GTP, 10 ng/ml VEGF, and 100 µM MeSADP stimulated tubulogenesis. Upon addition of combination (combo) treatment with 10 µM EA (or alone) and MRS compounds (10 µM MRS2179, 100 nM MRS2279, or 10 nM MRS2500) tubulogenesis was inhibited. (B) Fluorescence and brightfield images of HUVECs treated with DMSO control, 231 exosomes, or combination treatment (10 µM EA and 10 µM MRS2179) with 231 exosomes. The representative images were taken from three replicates.
4.5. Discussion

Here we studied the role of extracellular NDPK and its association with exosomes to influence tumor-mediated angiogenesis. NDPK was originally described as a non-metastatic 23 (NM23) gene in mammalian cancer systems [147]. Transcript levels were inversely proportional to metastatic potential [147,356], but other studies have shown inconsistencies with the view that NDPK is associated with tumor suppression [204,238,239,243], particularly, that extracellular NDPK has tumor promoting actions.

Besides transcript levels, another focus of NDPK has been on its enzymatic activity. The major function of intracellular NDPK is to maintain triphosphate nucleotide pools, and the NTP products are used for signal transduction. In addition, intracellular NDPK may function as an exonuclease [217], transcription activator [215,235], and histidine kinase [220]. While intracellular NDPK participates in a myriad of cellular functions, extracellular NDPK has been less studied. Extracellular NDPK was detected in the conditioned media of breast cancer cells [200,245,246] and in the serum of breast cancer patients [338]. We proposed that NDPK elevates ATP and ADP levels in the bloodstream [145,357]. These circulating purine nucleotides were reported to influence platelet aggregation [358,359] and modulate local blood flow [360,361]. Although extracellular NDPK has been shown to promote endothelial cell proliferation and migration [246], the mechanism by which NDPK appears in the tumor microenvironment remained unknown. We examined breast cancer cell-
secreted exosomes as carriers of extracellular NDPK to mediate angiogenesis via purine signaling.

We were able to confirm the presence of 231 and HME1 exosomes with TEM, flow cytometry, and super-resolution. The exosomes visualized by TEM were 30-100 nm in diameter, showed a distinct lipid bilayer, and were tetraspanin positive. According to flow cytometry analysis, both the 231 and HME1 exosomes were CD63 positive. However, when we labeled for NDPK, we found that there was a low signal to noise ratio for NDPK labeling of the 231 exosomes. With the use of super resolution microscopy, this technique increased sensitivity by the detection of single fluorescent molecules to improve spatial resolution [362]. GFP fluorescent exosomes were labeled for NDPK and we were able to directly visualize NDPK on the 231 exosomes. Our findings were consistent with previous studies that have identified NDPK in breast cancer exosome samples by mass spectroscopy [299,300].

The identification of NDPK as a 231 exosomal cargo raises the question whether this protein is functional. To test the activity of NDPK, a transphosphorylase activity assay was developed. This assay has been implemented to examine inhibitors of NDPK [249] and detect functional NDPK in the conditioned media of various cancer and breast cancer cell lines [245,246]. Consistent with the results from conditioned media [246], 231 exosomes displayed higher levels of transphosphorylation activity than the non-tumorigenic HME1 exosomes. While the transphosphorylation activity assay generated ATP from ADP and UTP, there was also the possibility that a phosphate group could be transferred from an ADP to another ADP. This activity
could be from adenylate kinase, which is a phosphotransferase enzyme that generates ATP and AMP from the transfer of a phosphate group between two ADP molecules. However, most of the ATP was generated using the substrates of ADP and UTP, thus demonstrating the activity was mediated by NDPK. Interestingly, the majority of the NDPK activity was from the lysed exosome samples, which indicated that NDPK was also located inside the exosome. This may suggest that NDPK has implications in exosome biogenesis or release. There have been studies demonstrating that NDPK influences membrane dynamics [363,364]. In addition, NDPK-B-deficient mice exhibited higher levels of intracellular vesicles and Cav-1 in brain derived microvascular endothelial cells [327]. NDPK-B was shown to have a role in caveolae formation, in which depletion of NDPK-B in HUVECs and mouse embryonic fibroblasts impaired caveolae formation [327,365]. NDPK-A has been shown to interact with dynamin at adherens junctions to facilitate endocytosis [366]. The relevance we submit for determining exosomal NDPK activity is that the production of ATP is an agonist of the P2Y1 receptor on endothelial cells to stimulate angiogenic responses, which may help promote metastasis of breast cancers.

Previously, it was shown that exogenous application of NDPK stimulated cardiovascular endothelial cell tubulogenesis in vitro [200]. This mechanism occurred in the absence of VEGF because the ATP generated by NDPK activated the P2Y1R to transactivate the VEGFR2 to promote angiogenesis. Another angiogenic mechanism was demonstrated by Feng, et al., in which intracellular NDPK-B was required for VEGF-induced angiogenesis to promote correct recruitment of VEGFR-2
and VE-cadherin to the plasma membrane [326]. Cancer-derived exosomes can travel to distant sites of the body to target recipient cells to promote a receptive environment for the future arrival of the circulating tumor cell [275,284,298]. Additionally, the cancer-derived exosomes can change the nearby tumor microenvironment to favor growth of the primary tumor by stimulating angiogenesis [367,368]. In this study, 231 exosomes stimulated HUVECs to form tubule structures. When the 231 exosome-treated cells were compared to the control cells, the tubules appeared to be highly branched and muddled. This might be influenced by breast cancer exosomes to stimulate vascular leakiness and permeability. The combination MRS2179/EA treatment inhibited tubulogenesis, which demonstrated that the 231 exosomes mediated angiogenesis through the P2YR/VEGF receptor-signaling pathway. This is most likely due to NDPK associated with the exosomes. It is also worth noting, that Geltrex, which is similar to Matrigel, does naturally have a positive effect of endothelial differentiation, as the control wells also have tubule formation. The combination-treated wells show abolishment of tubulogenesis, which indicated that the treatment could even effect basal level angiogenesis. Although the endothelial cells formed fluorescent tubule structures, it could not be determined whether the exosomes were absorbed into the cells or simply attached to the cell surface. Future experiments to investigate this question would include a trypsin or acid treatment of HUVECs treated with exosomes, but these methods may interfere with functional studies [369]. Our data are consistent with previous findings that inhibition of both NDPK and P2Y1 decrease NDPK-mediated tubulogenesis [199,200].
It is worth noting that although we are studying exosomes, based on this precipitation isolation method, the exosomes showed a heterogeneous population. In the future, the ability to study exosomes as a pure population would be desirable. Follow up experiments for this study would include generating a breast cancer cell line with NDPK-A and NDPK-B knocked out to study its influence in vivo, as well as the tracking of exosomal NDPK to metastatic sites in vivo, particularly those targeting to the lungs. These studies would examine if early exposure of breast cancer exosomes may facilitate breast cancer cells to metastasize to the lungs more quickly.

Cancer derived exosomes mediate cell-to-cell communications that are involved in diverse cancer signaling pathways, such as tumor growth, metastasis, and angiogenesis. These exosomes play a role in cultivating the host tissue microenvironment to become susceptible to invasive circulating tumor cells, setting up the so-called pre-metastatic niche. Circulating tumor exosomes would be useful for tumor biomarker development from liquid biopsies. Our findings demonstrate the presence of exosomal NDPK associated with breast cancer cells, thus providing a means for the NDPK to leave the tumor cells and appear in the blood stream. Methods for examining exosomal NDPK may hold value for predicting the likelihood of the progression of breast cancer and providing a target of treatment through the inhibition of both the enzyme and the P2Y1 receptor.
Chapter 5: Conclusions and Future Directions

5.1. Conclusions

An estimated 268,670 new cases of breast cancer will be diagnosed in 2018 and 40,920 women will die of breast cancer this year [7]. That number represents 14.3% of all cancer related deaths in women for 2018. While breast cancer therapies have advanced, we still have an incomplete understanding of metastasis. Dissemination of tumor cells have been known to be an early event during tumorigenesis [110], although recent studies have shown that CTC may disseminate even before the primary tumor forms [53,54]. The CTCs can travel to distant sites in the body and remain dormant for extended periods of time until a trigger signals to grow as metastatic disease. Surgical removal of the primary tumor has shown to progress relapse [137,370,371]. Ongoing discussions have debated the mechanisms of recurrence, whether the primary tumor promoted quiescence of the disseminated tumor cells by secretion of inhibitory factors [140,142,372] or the activation of the wound healing process during surgical removal of the tumor activated CTCs to proliferate [373]. Elucidation of the pathways that control metastasis and resurgence of hibernating tumor cells would have significant impact on cancer therapy.

Here we show that NDPK has an extracellular role in cancer-mediated angiogenesis to support tumorigenesis and metastasis, which is quite different from its intracellular role. NDPK detected in conditioned media collected from breast cancer cells stimulated endothelial cell proliferation, migration, and differentiation into tubules [200,374,375]. Extracellular NDPK elevates the purine nucleotide levels
in the local tumor microenvironment. Activated purinergic receptors on endothelial cells stimulate the VEGFR-2 to promote angiogenesis. This pathway is functional in the absence of VEGF, demonstrating that in the presence of VEGF inhibitors, such as angiostatin, endostatin, and Bevacizumab, angiogenesis can still occur. This angiogenic mechanism was inhibited with EA, an NDPK inhibitor, and MRS2179, an antagonist of the P2Y1 receptor [198,200].

In chapter 2, the role of NDPK in an in vivo model was studied. Two mouse models were examined to show that treatment with a combination of drugs to inhibit both NDPK and purine receptor, P2Y1, not only limited the growth of the primary tumor, but also decreased metastases in the lung. This could infer that inhibition of both the transphosphorylation activity of extracellular NDPK and the P2Y1 ATP receptor on adjacent endothelial cells suppressed the formation of angiogenesis. Thus this would limit the delivery of nutrients to the primary tumor and restrict invasive tumor cells from escaping the primary tissue site.

Chapter 3 described the identification of novel compounds that can inhibit NDPK. The NDPK transphosphorylation activity assay was repurposed for screening the Prestwick Chemical Drug Library to identity potent NDPK inhibitors. EA, a known inhibitor of NDPK [248,249], was used as a positive control and criteria for selecting positive drugs. The positively identified compounds were further tested to generate kinetic curves. In addition, the drugs were also tested in an apoptosis assay in a triple negative breast cancer cell line. This study could provide details for future prospects into 3D modeling of other NDPK inhibitors.
The molecular mechanisms regarding how NDPK is released into the ECM are poorly described. NDPK does not contain a secretory signal and therefore would not follow the classical secretion pathway. Our lab has proposed that breast cancer secreted exosomal NDPK has an extracellular role to stimulate angiogenesis and promote metastasis [357]. Breast cancer-preased NDPK maintains extracellular purines that activate endothelial purinergic receptors [200]. These purinergic receptors stimulate VEGFR-2 to promote angiogenesis in the absence of VEGF [198,199]. However, the mechanism of how NDPK was released into the ECM remained a mystery.

Chapter 4 demonstrated that breast cancer cells secreted exosomes or EVs that contain NDPK. Breast cancer and non-tumorigenic breast epithelial cell line exosomes were collected and characterized to determine their protein markers and morphology. NDPK was identified to be associated with breast cancer exosomes. These exosomes were also tested for transphosphorylation activity and mediated tubulogenesis. Breast cancer exosomes are vehicles that could shuttle NDPK through the circulatory system and also target specific tissues to prime metastatic sites. Future studies measuring exosomal NDPK in the serum of breast cancer patients could likely be used as an indicator of disease status. Additional studies focused on breast cancer exosome targeting of metastatic sites would provide details of what mechanisms regulate this process.

While our lab focused on the extracellular roles of NDPK as a promoter of cancer mediated angiogenesis and metastasis [357], NDPK was originally discovered
to inversely correlate with metastatic potential [147]. By concentrating on correlation aspects, we lose focus on the pro-tumorigenic effects of NDPK, such as purinergic-mediated activation of VEGFR-2 to stimulate angiogenesis. The study expands the function of NDPK and provides the basis for future research into the development of NDPK as a potential biomarker for breast cancer. Breast cancer cells secrete exosomes that contain functional active NDPK to elevate local ATP levels in the tumor microenvironment. The extracellular ATP active P2Y1 receptors present on adjacent endothelial cells to promote angiogenesis by transactivation of the VEGFR-2 (Figure 5-1). This angiogenic response supports the growth of the primary tumor and provides an exit for the dissemination of invasive tumor cells to metastasize to other sites.
Figure 5-1: Model depicting the mechanism of exosomal NDPK mediating angiogenesis. Extracellular NDPK generates ATP that activates P2Y1 receptors on endothelial cells. The P2Y1 transactivates VEGFR-2 through Src to promote angiogenesis. This mechanism occurs in the absence of VEGF.

5.2. Significance

There is a significant need for the development of biomarkers for cancer identification, target for new therapies, and determinants of disease status. Based on the research from our lab, NDPK could be developed as a biomarker for breast cancer, and potentially colorectal and pancreatic. NDPK can be detected in the serum of mice carrying tumors and breast cancer patients [338,376]. Also, NDPK is secreted from
many different subtypes of breast cancer [375], indicating the potential as a general breast cancer biomarker. Other options would be for the detection of disease relapse. Treatment with EA and MRS2179 inhibits NDPK and the corresponding purinergic receptor, respectively, and would decrease tumorigenesis and metastasis [338]. EA and MRS2179 may prove to be an effective adjuvant therapy that would be given during breast cancer treatment. EA is a known antioxidant and could be taken as a supplement even as early as breast cancer diagnosis. While MRS2179 has not been clinically tested in humans, MRS2179 has been shown to be an antiplatelet therapy in collected human blood [377,378]. In addition, Clopidogrel, an antiplatelet drug that inhibits the P2Y12 receptor, has been used in humans to reduce the risk of stroke and heart disease [358]. By extension, this would suggest that inhibition of the P2Y1 receptor could be targetable for therapy.

Utilizing the targeting function of breast cancer exosomes, exosomes could be loaded with EA or other NDPK inhibitors to be delivered systemically. There is considerably potential for the implementation of exosomes to deliver cytotoxins to specific cancer cells for therapy, this method could be direct and limit the amount of toxic reagents in a patient’s body. The exosomes would provide a targeting mechanism as well as increase bioavailability of the drug in circulation. Also the immune evasive exosomes would also protect the drugs from the immune system and degradation until it reached the target cells/tissues. Another role for exosomes would be for detection and imaging of metastasis as the cancer exosomes can specifically travel to potential metastatic sites.
Our studies have shown that exosomal NDPK could be detected in the serum of breast cancer patients and targeted for treatment with a drug combination cocktail of EA and MRS2179 to decrease primary tumor growth and metastasis in an in vivo model. This demonstrated that NDPK has implications as a biomarker for breast cancer.

5.3. Future Directions

Future studies of exosomal NDPK would be to examine if breast cancer exosomes could specifically target the lungs and also affect lung endothelial cell function, such as vascular permeability. Mice would be injected with labeled breast cancer secreted exosomes and the lungs would be harvested, sectioned, and imaged. The serum levels of exosomal NDPK could be measured and monitored in mice that have undergone surgical removal of primary tumors to examine the expression of NDPK during recurrence. In addition, the exosomes could be engineered or loaded with drugs, particularly inhibitors of NDPK, such as Chicago Sky Blue 6B.

Another future study would involve knocking out the NME1 and/or NME2 genes with the CRISPR-Cas9 system in order to study the impacts of NDPK-A and NDPK-B on tumorigenesis and metastasis in the in vivo model. It is likely that double knockout of NME1 and NME2 will not cause cell lethality due to the ability to generate NME1⁻/⁻NME2⁻/⁻ double knockout mice [201,379]. On the other hand, these mice exhibit a hematological phenotype whereby they express severe anemia, impaired erythroid cell development, and die early following birth. The CRISPR-Cas9 technique would allow for targeting of one or both the NME1/NME2 genes. The
editing can occur by the introduction of double strand DNA cuts at the targeted sequence to induce non-homologous end joining (NHEJ) to add or delete base pairs to promote a frameshift mutation to cause a premature stop in gene translation. Also if a template was transfected in, then the system could perform homologous driven repair (HDR) and edit the gene based on the template. HDR would be feasible to generate the H118Y dysfunctional mutant. The H118Y mutant can be generated from a point mutation [214]. However, the efficiency of the HDR is lower than that of the NHEJ. Although, newer methods have been developed to improve HDR efficiency, such as HITI [380,381] or addition of vanillin, an NHEJ inhibitor [382]. One concern of gene editing in the MDA-MB-231 cells, NME1 and NME2 are located on chromosome 17, which are trisomic [383]. This means that in order to create a complete knockout of NDPK-A/B, all three copies would need to be edited.

Although more research is needed, gaining a better understanding of the mechanisms of cancer cell development will lead researchers closer to improving therapies. Exosome research can improve cancer research in terms of cancer biomarker development and anti-cancer targeting diagnostics and therapy. This dissertation expands the understanding of extracellular NDPK in breast cancer dynamics.
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Appendix A: Optimization of the NDPK-A/B

Transphosphorylation Assay using Human Recombinant NDPK-A and NDPK-B from *E. coli*

A.1. Abstract

Here we describe the generation and purification of human recombinant NDPK-A and NDPK-B from *E. coli*. This recombinant protein was used for optimization of the NDPK transphosphorylation activity assay. The results demonstrate that enzymatically active NDPK-A and NDPK-B protein can be purified without tags and be used for transphosphorylation activity assays. This purification process of recombinant NDPK allow for the generation of larger quantities of NDPK to be used in future procedures to determine the role of this protein in breast cancer metastasis.

A.2. Introduction

Extracellular nucleoside diphosphate kinase (eNDPK) has been shown to play a role in tumor mediated angiogenesis, metastasis, and endothelial cell proliferation. Extracellular NDPK has been detected in conditioned media from pancreatic, colon, and breast cancer cells. In addition, extracellular NDPK was detected in many of the breast cancer subtypes meaning that eNDPK is not just in certain types of breast cancer.
A.3. Materials and Methods

A.3.1. Reagents

Nucleotides were purchased from Sigma (MilliporeSigma, Billerica, MA).

A.3.2. Generation of human recombinant NDPK-A and NDPK-B

Total RNA was purified from human triple negative breast cancer (MDA-MB-231) cells with the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturers’ instructions. cDNA was generated using Superscript II (Thermo Fisher Scientific, Waltham, MA). NME1 and NME2 cDNA were generated by PCR using custom primers containing EcoRI and XhoI restriction cut sites 5’AAAGAATTCATGGCCAACTGTGA3’ and 5’AGTCTCGAGTCATTCATAGATCCAG3’ for NME1 and 5’AAAGAATTCATGGCCAACCTGGAG3’ and 5’AGTCTCGAGTTATTCATAGACCCAG3’ for NME2 (Integrated DNA Technologies, Coralville, IA). The resulting PCR product was inserted into the pTXB1 vector (New England Biolabs, Ipswich, MA). The pTXB1-NME1 and pTXB1-NME2 was transformed into Rosetta competent cells (MilliporeSigma, Billerica, MA). Rosetta E. coli cells were grown in TB broth and selected with 50 µg/ml carbenacillin.

A.3.3. Purification of NDPK-A and NDPK-B

Cells were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD of 0.4-0.8 to generate human recombinant NDPK-A and NDPK-B. After induction, the cells were pelleted at 3000 x g for 10 minutes. The cell pellet was
resuspended in room air Krebs (RAK) buffer (120 mM NaCl, 5 mM KCl, 0.587 mM KH$_2$PO$_4$, 0.589 mM Na$_2$HPO$_4$, 2.5 mM MgCl$_2$, 20 mM α-D glucose, 2.5 mM CaCl$_2$, 25 mM Tris-base, and 5 mM NaHCO$_3$ at pH 7.4) and pulse sonicated 3 times for 3 bursts at 4 output control, 40% duty cycle, and 5 second cycle time. The lysate was then centrifuged at 15,000 x $g$ for 10 minutes and the supernatant was collected. The liquid was then fractioned by size exclusion chromatography with Bio-Gel P30 beads (BioRad, Hercules, CA). Fractions exhibiting transphosphorylation activity were pooled and subjected to ADP agarose (MilliporeSigma, Billerica, MA) column purification. NDPK-A and NDPK-B protein was eluted with 10 mM ADP in RAK buffer. The collected recombinant protein was dialyzed in 1xPBS in a snakeskin pouch and concentrated by Amicon Ultra-15 Centrifugal Filter Units (MilliporeSigma, Billerica, MA). Protein concentration was measured with BCA assay.

A.3.4. Coomassie Gel

Protein samples were prepared with 4x sample buffer (SB) and β-mercaptoethanol for a final concentration of 1xSB and 5% β-mercaptoethanol. The samples were run on a 4-20% criterion gel (BioRad, Hercules, CA). The gel was fixed in 10% acetic acid and 40% methanol and stained with 0.1% Coomassie R250, 10% acetic acid, and 40% methanol. The gel was scanned using the BioRad imager.

A.3.5. NDPK Transphosphorylation Activity Assay

Purified human recombinant NDPK-A and NDPK-B protein was serially diluted into standards of 1000 ng/ml to 1 ng/ml in RAK buffer without CaCl$_2$. The standards were incubated with substrates of 10 µM ADP and 30 µM UTP for 6 minutes and
quenched with 0.1 M HCl in RAK buffer without CaCl$_2$. The pH was then neutralized back to 7.4 with 0.1 M NaOH in RAK buffer without CaCl$_2$. Next, equal volumes of luciferin-luciferase ATP detection buffer (containing 0.3 mM luciferin, 6 µg/ml luciferase (0.2 mg/ml luciferase pH7.6 constituted in 1 M glycine, 10 mM EDTA, and 100 mM MgSO$_4$-7H$_2$O), 98 mM glycine, 2 mM Tris-base pH 7.6, 10 mM MgSO$_4$, 1.1 mM EDTA, 2% BSA, and 2% sodium azide) were added and detected by the Chameleon plate reader with MikroWin 2000 software.

**A.4. Results**

Although the pTXB1 plasmid allows for the generation of a cleavable integrin tag for easier protein purification, we inserted the genes, NME1 and NME2, with its endogenous stop codons. Total mRNA was purified from human triple negative breast cancer cells, MDA-MB-231. The mRNA was converted into cDNA and PCR primers designed to amplify the NME1 and NME2 coding sequence with EcoRI and XhoI cut sites to be inserted into pTXB1 (Figure A-1A). The NME1 and NME2 PCR products were 477 and 459, respectively (Figure A-1B). These fragment were inserted into the vector pTXB1, and transformed into DH5α cells. Colonies were grown and plasmids were purified and screened for the detection of NME1 or NME2 inserted into pTXB1 (Figure A-1C and A-1D).
Figure A-1: Construction of pTXB1-NME1/NME2 plasmid. (A) Vector map of pTXB1 with NME1/NME2 insert. (B) NME1 and NME2 transcripts were generated with EcoRI and Xhol cut sites by RT-PCR from total RNA isolated from MDA-MB-231 cells. After the PCR products were inserted into the pTXB1 vector, the plasmid was transformed into DH5α E. coli cells. (C-D) Colonies were screened for the (C) NME1 or (D) NME2 inserts in the plasmid.

The human recombinant NDPK-A and NDPK-B proteins were intended for activity assays. With this in mind, we decided to use affinity purification for the purification of enzymatically active protein. The overall schematic for purification of
NDPK-A and NDPK-B was by size exclusion chromatography (SEC) and then followed by ADP affinity purification (Figure A-2A). Rosetta E. coli cells, which contain the three rare codons for optimal protein translation, were induced with isopropyl B-D-1-thiogalactopyranoside (IPTG) for protein generation. After two hours of induction, the cells were pelleted, resuspended in room air Kreb's buffer, and lysated under sonication to break the bacterial membrane and shear any DNA. The protein lysate was centrifuged and the supernatant was collected. IPTG induced production of recombinant NDPK-B and NDPK-A were identified by coomassie gel and Western blot (Figure A-2B). IPTG induction produced a prominent NDPK band around 17-19 kDa. The NDPK-A antibody detected both NDPK-A and NDPK-B proteins, but the NDPK-B antibody was specific to NDPK-B. The protein lysate was fractioned with SEC (Figure A-2C). Crude protein concentrations were measured at 280 nm and the fractions were tested for transphosphorylation activity. The fractions with the highest protein concentration had the highest level of activity. These fractions were combined and subjected to ADP affinity purification. SEC fractions were run through an ADP agarose bead column and NDPK would bind to the beads. NDPK was eluted with 10 mM ADP and the column was washed with 15 mM ATP to elute off any residue protein. The samples generated from the ADP affinity purification was ran on a coomassie gel (Figure A-2D) and tested for activity (Figure A-2E). The purified NDPK protein was detected in the samples from the ADP elution and concentrated protein lanes. In addition, the ADP eluted sample generated the highest activity when compared to SEC fractions and flow through. The ATP eluted sample also exhibited
high activity levels but this is due to the presence of excess ATP in the sample, as the coomassie gel did not show any bands. Functional recombinant NDPK protein was purified from *E. coli* cells and could be used in transphosphorylation activity assays.

**Figure A-2**: Purification of NDPK-A and NDPK-B recombinant protein. (A) Schematic of the purification process for NDPK from *E. coli*. (B) NDPK was generated by IPTG induction and Western blot analysis was used to identify the protein of interest. (C) The cell lysates were separated into fractions by size exclusion chromatography.
Crude protein concentrations were measured at 280 nm (orange curve) and enzymatic activity (black curve) was measured from selected fractions to be compared to non-induced (NI) cell lysates, 1:10 diluted, and 1:100 diluted IPTG induced crude protein lysates. Next, fractions that displayed the highest activity were pooled and purified on ADP affinity columns. (D) A coomassie gel was ran to determine the purity of NDPK-A/B. Lanes were; 1- NI cell lysate, 2- IPTG induced, 3- pooled SEC fractions, 4- flow through, 5- ladder, 6- washes, 7- ADP eluted, 8- ATP eluted, 9- dialyzed and concentration NDPK. (E) Enzymatic activity was measured from the ADP column samples.

The NDPK transphosphorylation activity assay has been previously described. This two-step enzymatic reaction utilizes GTP and ADP substrates to generate ATP as a substrate for a luminescence reaction (Figure A-3). Briefly, NDPK protein hydrolyzes a gamma-terminal phosphate group from a nucleotide triphosphate and transfers it to an ADP to generate ATP. ATP is utilized as a substrate in a luciferin and luciferase reaction to produce a luminescent signal. The use of GTP and ADP as substrates for the assay has stemmed from the detection and implications of GTP and ADP in the extracellular matrix. Elevated ADP and ATP levels were detected in the tumor microenvironment.
NDPK Transphosphorylation Assay:

(1) NDPK reaction

\[ \text{UTP + ADP} \rightarrow \text{UDP + ATP} \]

(2) Luciferase reaction

**Luciferase and Mg}^{2+}**

\[ \text{ATP + Luciferin + O}_2 \rightarrow \text{Oxyluciferin + Pyrophosphate + AMP + CO}_2 + \text{Light (560nm)} \]

*Figure A-3:* Schematic of the NDPK transphosphorylation activity assay. This assay was a two part enzymatic reaction. The first reaction consisted of NDPK activity with substrates to generate ATP. The second reaction is the detection of ATP by luciferin and luciferase.

A variety of nucleotides were tested to determine the lowest potential background for the assay (Figure A-4). The phosphate acceptor for the assay has to be ADP, but luminescent signal was affected by the type of salt (Figure A-4A and A-4B). Potassium salt ADP had the lowest luminescent signal. For the phosphate donor, UTP in Tris salt generated the lowest background signal (Figure A-4C and A-4D). ADP and UTP were selected as substrates for the NDPK transphosphorylation activity assay.
Figure A-4: NDPK substrates for the transphosphorylation activity assay. In order to lower the background of the assay, various nucleotide substrates were added to luciferase and luciferin to determine background signal. (A-B) ADP was used as the phosphate acceptor for the transfer of the gamma phosphate group. ADP and GTP sample were at 10 μM and 30 μM to demonstrate typical background levels during assay conditions. (C-D) Various NTPs were also examined for background signal. (B,D) Close up graphs of the nucleotide backgrounds. ATP was used as a positive control for the luminescence reaction. n=3.
For optimization of the NDPK transphosphorylation activity assay, recombinant NDPK was used to produce standards of 1000 ng/ml to 0.3 ng/ml. The standard curve showed a robust curve with an $r^2=0.97$ (Figure A-5A). An ATP standard curve was generated to demonstrate the peak signal of 100 μM ATP if all of the 100 μM ADP substrate was converted to ATP (Figure A-5B). Next, NDPK activity was tested between 1 to 20 minutes of incubation with ADP and UTP substrates (Figure A-5C). The optimal reaction time of an enzyme should be in the linear range of signal. The linear range of the reaction was observed during 1 to 10 minutes of incubation (Figure A-5D).
Figure A-5: Optimization of NDPK Transphosphorylation Activity Assay. (A) Recombinant NDPK-A and NDPK-B standard curves were made from 1000 ng/ml to 0.3 ng/ml. (B) ATP was used as a positive control. (C) NDPK activity time course was measured from 1 to 20 minutes using the 30 ng/ml standard. (D) The reaction times between 1 to 10 minutes were in the linear range. n=3.

A.5. Discussion

Since our lab has studied the role of NDPK in triple negative breast cancer cells (MDA-MB-231), human NME1 and NME2 transcripts were generated from the MDA-MB-231 cells. DNA sequencing was performed to determine that the NME1 and NME2 cDNA were not mutants. These cDNA were insert into pTXB1 vectors and transformed into E. coli specialized for protein generation. IPTG concentration and
time of induction were optimized for peak protein generation in the Rosetta *E. coli* cells. Then the cells were pelleted and resuspended in RAK buffer. RAK buffer contained all the necessary salts and sugar levels for physiological conditions. In addition, RAK buffer contained magnesium, which is a cofactor for NDPK. No detergents were used in the protein lysate in order to minimize the effect of detergents on protein activity. Due to the size difference between bacterial (142 amino acids) and human (152 amino acids) NDPK protein, SEC fractionation was used to separate the proteins based on size. Fractions containing high protein concentrations and activity were pooled and further purified with ADP affinity purification. Functional NDPK protein would bind to the ADP agarose and eluted with 10 mM ADP, which is at saturating conditions. The protein would then be dialyzed in 1xPBS and concentrated to be stored for future applications.

Untagged recombinant NDPK may potentially be used for inoculation of BALB/c mice for the generation of a NDPK antibody. Any tag on a protein has the potential to become an epitope for an antibody. In addition, the protein has been dialyzed into 1xPBS.

The optimization of the NDPK transphosphorylation activity assay is important for the potential use of an assay for the identification and measurement of the amount of NDPK in a sample. Future implications for the use of this assay include, discovery of novel NDPK inhibitors, detection of NDPK in conditioned media of various treatments, and the measurement of NDPK from serum samples. One caveat of the assay is that the NDPK protein must be active and functional. If the enzyme is
inactive, the activity assay will not generate any luminescent signal. There is limited information regarding long-term storage of protein samples and the preservation of the enzymatic function in the sample.
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