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Purinergic Mechanisms in Breast Cancer Support Intravasation, Extravasation and Angiogenesis

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Abstract

Several advances have recently expanded models of tumor growth and promoted the concept of tumor homeostasis, the hypothesis that primary tumors exert an anti-proliferative effect on both themselves and subclinical secondary metastases. Recent trials indicate that the characterization of tumor growth as uncontrolled is inconsistent with animal models, clinical models, and epidemiological models. There is a growing body of evidence which lends support to an updated concept of tumor growth: tumor homeostasis. In the case of breast cancer, if not all metastasizing tumors, these advances suggest an inconvenient truth. That is, if breast tumor cells metastasize to distant sites early in the tumorigenesis process, then removal of a breast tumor may hasten the development of its metastases. We explore the heretofore unappreciated notion that nucleotides generated by tumor cells following the secretion of an ADP-kinase can promote metastasis and support angiogenesis. Evidence is presented that blockade of the actions of nucleotides in the setting of newly diagnosed breast cancer may provide a useful adjunct to current anti-angiogenesis treatment.

Keywords

Secreted nucleoside diphosphate kinase-B (sNDPK-B); nucleotide receptors; angiogenesis; VEGFR2; transactivation

1. Introduction

Breast cancer is the most common cancer among women in the United States [1] and while it is the second deadliest cancer with a yearly mortality exceeded only by lung cancer, management of breast cancer in the US ranks among the best in the world [2]. The typical treatment regime under which this has been accomplished involves either mastectomy or lumpectomy plus postoperative (adjuvant) treatment. Nonetheless, more than forty-thousand women will die of the disease this year [1]. Clinical oncologists have known that breast cancer specific mortality is almost exclusively a function of a metastatic process [3]. Of all the types of breast cancer recurrence, the presence of distant metastases is associated with the least favorable outcome. Those whose breast cancer reoccurs at distant sites in the body have only a 9% chance of living an additional 10 years, compared to a 56% survival rate for women with a reoccurrence of breast cancer which is isolated to the breast [4]. Thus the imperative in breast

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cancer research is to understand the metastatic process and the philosophy of breast cancer care must be to utilize this understanding to prevent metastases.

Metastatic growth of tumors has been classically characterized as unlimited, yet several limiting factors do act as a brake on tumor growth. Adequate blood supply dictates that tumor cells must reside within 100 μm of a capillary blood vessel or risk necrosis. The rich vascularity of metastatic tumors has lead to the discovery that proliferating tumor cells have an intrinsic ability to generate the growth of new blood vessels; angiogenesis, that is a hallmark of cancer [5].

Excision of a primary tumor can lead to tumor metastases appearing shortly thereafter [6-8]. Assuming that cells weren't *"dislodged'* during surgery, an intellectually displeasing notion, it is reasonable to take this as evidence to suggest the presence of subclinical distant metastases at the time of loco-regional treatment. Indeed, although fine needle biopsy and large needle core biopsy have been shown to significantly impact the appearance of sentinel node metastases [9], tumor manipulation during excision is not thought to explain distant metastases [10]. Demichili and coauthors examined the hazard rate for recurrence of breast cancer in patients undergoing mastectomy, showing an early peak in recurrence, with the majority of recurrences around 18 months post-mastectomy [11]. This clearly indicates that the surgery is an aggravating event, with the result becoming clinically apparent approximately 18 months thereafter. This data strongly suggests that metastases present at the time of mastectomy have existed in a dormant state and have proliferated only once the antiangiogenic effects of the primary tumor have been removed. Animal studies confirmed this behavior of human tumor cells a decade ago [12-13]. Moreover, there is even compelling, if troubling evidence to indicate that the natural history of untreated breast cancer may rarely involve spontaneous regressions and even resolution rather than progression [14]. The notion of metastases as dormant, extant lesions awaiting events that trigger their conversion to rapidly growing tumors that attract a blood supply is supported by recent genomic studies that implicate nucleotide metabolism among other critical changes in gene transcription [15]. Efforts to maintain dormancy of metastases in patients when first seen by the oncologist might offer a new strategy for adjuvant treatments to prevent their growth.

Removal of a primary tumor and development of metastases is a likely clinical course even among the 10% of low rise (node negative) patients that will eventually develop distant metastases [1]. In consideration of these unique temporal features regarding metastasis development, a model of tumor homeostasis has been described in which the primary tumor exerts an anti-proliferative effect *via* an unknown mechanism upon subclinical micrometastases [14]. Furthermore, evidence indicates that this trend towards dormancy is a function of the primary tumor preventing the initiation to angiogenesis being made [16]. Thus, mechanisms that orchestrate primary tumor growth and development are disparate, although no doubt related, to those that encourage and support the growth of metastases.

Autopsy studies have sought to determine the prevalence of pre-clinical, presumably dormant breast cancers, and generally report a median prevalence of 8.9% of ductal carcinoma *in situ* (DCIS) [17]. When this data is contrasted with a median prevalence of DCIS of 1.3% of the female population, it suggests a large reservoir of clinically occult cancers which would never progress into metastatic disease. This is further supported by data showing that less than half of untreated low-grade DCIS will develop into metastatic disease over a 40 year period of time [18]. This may be due in part to a failure of the these occult tumor cells to induce angiogenesis. Thus, the balance of factors suppressing the growth of distant metastases as well as those that promote it is critical to the fact of metastatic disease in a given patient. Such a balance may be upset in favor of the growth of metastases in patients when primary tumors are removed and mechanisms such as those we discuss here can proceed to support angiogenesis. The notion

that the extracellular actions of ATP contribute to dormancy of primary tumors or might stimulate their growth *in situ* cannot be clarified at preset. There is tantalizing evidence that either possibility might occur and is discussed below. The events that permitted intravasation and extravasation in the passage of tumor cells to distant sites may be proceed early on and unopposed by the primary tumor whose influence is that which suppresses angiogenesis. With the importance of angiogenesis increasingly apparent in clinical oncology, the formation and extracellular actions of nucleotides will be a valuable mechanism to exploit in decreasing the frequency of metastasis and subsequent deaths from cancer. If a role for purines can be firmly established in both intravasation/extravasation as well as angiogenesis and safe and effective antagonists of these mechanisms identified, a dual benefit could be realized with women at high risk for developing disease.

2. Extracellular ATP

Although originally viewed with much skepticism, the extracellular presence of ATP released from nerves as well as non-excitable cells [19] is well established and has been frequently reviewed in recent years [20]. From the first suggestion of the phenomenon by Burnstock in 1976 [21], to the first actual experimental evidence for the fact of ATP release by Westfall et al., in 1978 [22], there is now an extensive understanding of a near ubiquitous role for extracellular purines in most organ systems. Nucleotide release has been extensively studied in endothelial cells of blood vessels that are the object of interest in this review, the phenomenon has been described in epithelial cells by Schwiebert and colleagues who have offered a valuable discussion of the mechanisms of release that are beyond the scope of this discussion [23].

3. Tumor Cell Migration

Accepting that metastases in breast cancer are not the result of surgical manipulation, forces the inconvenient truth that in many cases, cells from a tumor in the breast have already spread to distant parts of the body at the time of diagnosis. Understanding the mechanisms of tumor invasion and migration is a central focus of current investigations. While a thorough review of these mechanisms is beyond the scope of this review, it is now possible to propose an important role for extracellular purines in the process of tumor cell invasion and metastasis.

As pointed out recently by Christofori [24], cells commonly move into and out of tissue spaces in the normal processes involved in embryonic development and immunity. It is reasonable to suspect therefore that tumor cells mimic many of these processes in their passage from primary tumor to metastatic site. Because it is known that breast cancer cells move in the body *via* both blood and lymph, we focus our discussion here to metastasis *via* blood simply because far more is known about purinergic mechanisms in blood vessels than in the lymphatics.

It is well known that leukocytes leave blood vessels to fight infection. The transendothelial migration of white cells out the blood stream and into tissue spaces requires that they move between capillary cells and through basement membrane of both the capillary and tissue. This process, diapedesis or transendothelial migration, is known to involve the interaction of leukocytes with the vessel wall [25]. Leukocytes must first bind to and then move along the capillary wall to the endothelial borders where they traverse the endothelium and the sub-endothelial basement membrane and migrate through the interstitial space. Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 cluster on the endothelial cell and appear to be responsible for leukocyte binding based on an interaction with specific leukocyte molecules (*e.g.*, CD11, CD18, CD49 and CD29). The many steps that follow, including those that subserve transcellular versus paracellular passage of leukocytes [26], involve enzymatic steps and both leukocyte and endothelial cell deformation to permit transit to occur [27].

While little attention has been paid to a role for nucleotides or ATP receptors in this process, it is known that locally generated ATP may enhance leukocyte binding to endothelium [28] and that the expression of adhesion molecules by endothelium is enhanced by extracellular ATP acting at purine nucleotide receptors [29]. Moreover, polymorphonuclear leukocytes are known to elaborate ATP themselves [30], so that along with endothelial cells that are well known to elaborate ATP extracellularly [31], ATP receptors will be activated. Rapid metabolism of ATP locally would generate adenosine consistent with reversing the permeability of the endothelium [32] and dilating venous vessels to regulate flow down-stream [33]. Taken together with the known ability of ATP receptor stimulation to lead to endothelial cell contraction and thus, vascular permeability [34-36], it is reasonable to consider mechanisms that can maintain ATP levels extracellularly as consistent with promoting transendothelial migration.

Movement of breast tumor cells in the blood stream in a fashion that permits their targeting to particular distant sites is as yet not fully explained but is unlikely to be a mere mechanical consequence of where the relatively large tumor cell [37] becomes lodged in a capillary [38]. In fact, such trapping might reasonably result in regional infarcts rather than regulated extravasation were it not for the fact that tumor cells deform significantly in their passage in the blood stream and adhere specifically to endothelium in certain vascular beds [39-40]. In addition to the specific ability of tumor cells to elaborate surface factors in support of specific tumor–endothelial adhesive interactions in the microvasculature [41], is the knowledge that they release factors that ensure their passage to distant sites.

In the case of intravasation and extravasation events, cancer cells may mimic the normal events that leukocytes use to move through the endothelial barrier [42]. In addition to the proteolytic and cell-cell binding events that are critical in transendothelial passage and that are receiving much attention [43], is the release of factors in support of activation of purinergic receptors on endothelial cells. First, hypoxic tumors release purines the their immediate environment [44-45]. In this context, metabolism of such purine is proposed to be associated with tumor progression in breast cancer [46]. Human breast cancer cells in culture secrete a nucleoside diphosphate kinase (sNDPK-B) that supports the activation of ATP receptors (P2Y) on endothelial cells [47]. This kinase, secreted as a phosphoprotein capable of generating ATP from ADP locally [48], would support a cascade of events that would subserve vasodilation [49] and elicit additional vasoactive factor release from endothelial cells including ATP [31, 50]. Although the many roles for NDPK that have been described in biology have recently been reviewed [51], the established role for sNDPK-B in breast cancer was ignored, and those seeking to describe cellular localization of NDPKs have not considered its extracellular role [52]. Nonetheless, the ability of metastasizing cells to harness purinergic mechanisms in blood vessels by extracellular elaboration of NDPK-B is well supported by invitro work [47-48, 53-55]. The presence and subsequent metabolism of ATP in the blood stream will suppress platelet aggregation as both ATP [56-57] released by endothelium and/or regenerated by sNDPK-B at the site of intravasation/extravasation, as well as in the form of adenosine. Adenosine is a venous vasodilator [58] formed form ATP and ADP by endothelial ectonucleotidases [59]. These actions of purines are both useful if the invading cell is to enter past endothelium and passage to distant sites successfully (Fig 1).

4. ATP and Cancer

In addition to a mechanism for generating ATP locally, many tumor cells express extracellularly-directed ATP receptors and there is evidence that ATP can suppress the growth of some tumors when carried in animals [60]. The role for purinergic receptor-mediated cancer growth regulation is, however unclear since ATP receptor activation on colorectal cancer cells in culture stimulates growth [61] while this is inhibitory in epithelial carcinoma cells [62]. In

the breast cancer cell line MCF-7, the P2U receptor agonized by both ATP and UTP stimulates growth. The explanation for the disparate effects of ATP on growth likely lies in the subtypes of purinergic receptors expressed. Human breast cancer cell lines such as MDA-MB-231 do not express ATP receptors and as such, consideration of the role of ATP P2X or P2Y receptors in tumor suppression in this model is mute. We are not aware that purinergic receptor expression in tumors as they develop and metastasize in women has been addressed but it seems reasonable at this juncture in our understanding to include proliferation of tumor cells in the milieu of purinergic mechanisms that influence tumorigenesis (Fig. 1).

5. Angiogenesis

Tumor angiogenesis is the vital process of new blood vessel formation in support of tumor growth, development and metastasis [63-66]. Without access to oxygen and nutrients tumors cannot grow beyond 2-3 mm^3 [67-68]. In normal endothelium, cell turnover occurs slowly despite the abundance of angiogenic factors present in the blood stream [69]. The maintenance of endothelial quiescence is thought to be due to the presence of a delicate balance of endogenous negative (anti-angiogenic) and positive (pro-angiogenic) regulators [70] [71]. Tumor formation is favored when these homeostatic regulators fail to function properly. The moment when this failure occurs, when a tumor begins to over-express pro-angiogenic factors and normal endothelial cells proliferate to form new blood vessels is called the angiogenic switch [65,72-75]. Cancer cells begin promoting angiogenesis early in tumorigenesis under mutagenic/hypoxic conditions that drive expression of pro-angiogenic proteins, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin 8 (IL-8) and transforming growth factor β (TGF-β) [75-77]. Supported by *in vitro* data [47-48,54-55], we have proposed that under these mutagenic/hypoxic conditions breast cancer cells secrete nucleoside diphosphate kinase (sNDPK-B), which generates ATP from ADP locally and acts on the endothelial nucleotide receptor (P2Y receptor) to regulate blood flow and vascular permeability, which in turn enables metastasis (Fig. 2).

6. Antiangiogenesis

Angiogenesis inhibitors which block development of new blood vessels, are one promising avenue of cancer research. Many antiangiogenic compounds have been described, including a number which, curiously, are found to be secreted by primary tumors. Angiostatin is a fragment of murine plasminogen generated by implanted primary human tumors and has proven to be a potent angiogenesis inhibitor. Angiostatin suppresses the formation of metastatic secondary tumors in the mouse model [12] and blocks the action of sNDPK-B in *in vitro* studies [78].

The mechanism by which anti-angiogenic factors work is unclear. Clinical success has been demonstrated with monoclonal antibodies to VEGF (bevacizumab, Avastin®) and epidermal growth factor receptors (EFGR) cetuximab (Erbitux®), gefitinib (Iressa®), and the related Her2 protein (trastuzumab/Herceptin®). Tyrosine kinase inhibitors have also demonstrated a clinical utility in inhibiting VEGF receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor [79]. However, all of these anti-angiogenic therapies tend to exhibit low response rates when used as a monotherapy [80], are astonishingly expensive (annual treatment cost for Herceptin® is ~$80,000 USD and if given with Avastin®, as is often the case in advanced breast cancer, costs are well over $100,000 a year) [81-82] and resistance to these agents tends to build quickly [83]. This is likely due to the existence of multiple pathways available to stimulate angiogenesis, and the up-regulation of alternative pro-angiogenic signals in an evasive manner by the tumor [84]. Clearly clinicians are only able to partially prevent the angiogenic switch from being thrown.

Standard chemotherapy employs a varied arsenal of drugs that attack all dividing cells in the body leading to treatment-related toxicity [85]. Anti-angiogenic therapy, on the other hand

targets only dividing endothelial cells with no measurable toxicity [86]. Genetic instability of tumor cells (high rate of mutation), which leads to the overgrowth of drug-resistant tumor cell populations, is a major problem with standard chemotherapeutic agents [87]. Tumor cells also over-express p-glycoprotein, a transport pump which pumps chemotherapeutics drugs out of the cells, reducing therapeutic effectiveness [87-89] and compounding treatment failure.

Anti-angiogenic drugs, such as Avastin® (Genentech), have proven useful in combination with therapies directly aimed at tumor cells, by normalizing the vasculature in the tumor, thereby allowing better drug access and perfusion [90-92]. Moreover, anti-angiogenic therapy is progressing toward the goal of terminating cancer growth completely by denying the tumor a blood supply. In addition to Avastin®, two more FDA-approved drugs sorafenib (Nexavar®, Onyx) and sunitinib (Sutent® [SU11248], Pfizer) are mixed tyrosine kinase inhibitors that inhibit several protein kinases, such as Raf kinase and VEGFR2 kinase [93].

7. The Nucleotide Axis Hypothesis

Although once viewed as improbable if not even foolish to propose, it is now well established by many laboratories [94], including our own that cells release ATP as a local hormone [31, 95]. Endothelial cells release ATP in response to sheer stress [96] as well as vasoactive mediators such as bradykinin and even ATP itself acting at the P2Y receptor [95]. Red blood cells are a rich source of ATP and release the nucleotide under hypoxic conditions [97]. ATP acting at its endothelial membrane receptor stimulates the release of nitric oxide and prostacyclin [98], both potent vasorelaxants that increase blood flow in local vascular beds. The endothelial cell expresses both P2Y1 as well as P2Y2 receptors [99] and both are stimulated by ATP and can be specifically antagonized by selective compounds [100-101]. We have proposed that nucleotides and adenosine in the blood stream constitute an hormonal axis that subserves the regulation of blood flow in vascular beds not primarily served by nervous regulation [31,49]. ATP acts immediately upon release as an autocrine and paracrine hormone by stimulating endothelial nucleotide receptors (P2Y1/2), and again following its metabolism to ADP (a more potent P2Y1 agonist than ATP) to dilate arterial resistance vessels since ADP is a nucleotide receptor agonist. The presence on endothelium of enzyme activities that degrade ATP to ADP [102] as well as an *ecto*-ADP-kinase that can regenerate ATP from ADP [95] suggests that nucleotide levels in the relatively acellular boundary layer next to endothelium [103] can be maintained to act in time and space in the blood stream at nucleotide receptors. The released purine can finally act again in the venous circulation as adenosine where it dilates the venous circulation to accommodate the increased flow signaled upstream and activates endothelial adenosine receptors [104] and inhibits platelet aggregation (Fig.2).

8. Intravasation/Extravasation of Tumor Cells

The extracellular actions of nucleotides also mediate changes in endothelial cell behavior consistent with events required for intravasation and extravasation of cancer cells to and from the blood stream [105]. Given then that nucleotides appear in the blood stream [106] and act to promote vasodilatation, antagonize platelet aggregation and stimulate endothelial cell migration, it is reasonable to wonder if tumor cells might exploit such mechanisms to metastasize (Fig. 1). In addition, the regulation of extracellular nucleotides is being recognized as important to the proliferation of various cancers [60,107]. The current clinical trials of the nucleotide receptor inhibitor drug suramin [108-109] which is known to inhibit growth factor signaling including that of nucleotide receptors involved in cancer growth and angiogenesis [110-111], emphasizes the role of nucleotides in tumor progression and legitimizes interest in nucleotide generating mechanisms in metastasis.

We have identified a cancer cell secreted molecule, nucleotide diphosphate kinase (sNDPK-B) that we propose can pathologically elevate extracellular trinucleotide levels at the expense

of necrotic cells in the developing tumor, purines in the region of intravasation/extravasation from vessels and substrate in the blood stream. Inhibition of this nucleoside kinase and/or blockade of the actions of nucleotides at their receptors may constitute an additional therapeutic target in highly metastatic tumors such as breast cancer.

9. NDPK Inhibitors

Two NDPK-B inhibitors, epigallocatechin gallate (EGCG) and ellagic acid (EA), are both anticancer agents [112] and anti-angiogenic agents [113-114]. EGCG has been shown to inhibit cancer-associated enzymes such as matrix metalloproteinases (MMPs) [115] and the proteasome [116] and our laboratory has shown that EGCG and EA inhibit cancer cell-secreted NDPK-B transphosphorylase activity and inhibit *in vitro* angiogenesis [54]. We have shown that EA is a more potent sNDPK-B inhibitor based on the reduction of extracellular ATP levels [53] that, in part explains why EA is a more potent inhibitor of $CD31^+$ endothelial cell angiogenesis (tubulogenesis) than is EGCG. Investigating the role for sNDPK-B inhibition as a way to manage metastasis in breast cancer and possibly, in other types of solid tumor cancers [48], is worthy of significant interest.

10. A Role for sNDPK-B

Nm23/NDPK enzymatic activity functions primarily as a nucleoside diphosphate kinase regenerating ATP levels for intracellular "*housekeeping*" enzymes by covalently transferring the γ-phosphate from a nucleoside triphosphate (NTP) such as GTP, to a nucleoside diphosphate acceptor (NDP; *e.g.*, ADP) and has also been shown to act as a histidine kinase, a transcription activator and an exonuclease [117]. There are eight isoforms of this plurifunctional protein [118]. NDPK is distributed in the cytosol and plasma membrane, as well as the nucleus [119] where the NDPK-B isoform functions as PuF, a c-*MYC* transcription factor [117]. NM23 was originally described as non-metastatic 23 gene, which was found in mouse carcinoma cells as a homolog of the drosophila *awd* protein (altered wing disk) and was strictly thought to be inversely related to metastasis potential [120-121] although this has been shown to be less straightforward than originally proposed [117,122-124]. Indeed, less attention has been paid to NDPK-B's enzymatic function in cancer and metastasis, and no attention paid other than our own findings to the secreted sNDPK-B isoform even though there is now evidence to support NM23's putative role in promoting metastasis [117,125]. Nm23-h1 (NDPK-A) protein levels have been found in studies of patients with breast carcinoma [121, 126-128] but our hypothesis regarding the secretion and extracellular actions of the sNDPK-B have not as yet been evaluated *in vivo*.

Ecto-NDPK (*on the cell surface*) is known to regulate extracellular nucleotide levels in various cell types [46,129-130]. Hamby *et al.* confirmed that the catalytically inactive H118Y mutant of NDPK-B significantly suppressed the lung metastasis of human melanoma cells *in vivo* [131]. NDPK (A or B) is secreted by, or present in the blood stream from various solid and hematological malignancies [48,132-135]. The disruption of CD39 (*ecto*-apyrase; EC 3.6.1.5) activity, the dominant vascular ectonucleotidases, and its regulation of nucleotide signaling has been observed to inhibit tumor angiogenesis and metastasis [136-137] consistent with, if not directly demonstrative of a role for sNDPK-B in promoting angiogenesis. We discovered that both MDA-MB-435S and MDA-MB-231 metastatic human breast carcinoma cells secrete sNDPK-B into their surrounding environment when cultured *in vitro* [48] while the non-metastatic breast cell line MCF-12 does not (Yokdang, *unpublished observation).*

Recently we have provided evidence for a nucleotide-dependent regulation of angiogenesis by breast cancer secreted sNDPK-B [47,54]. This can be mechanistically related to extracellular nucleotide elevation and subsequent activation of nucleotide receptors to regulate cancer growth [20,60] and tumor angiogenesis [136-137]. Our results show that pathologically-

secreted sNDPK-B and its regulation of extracellular nucleotides utilize P2Y receptors to stimulate angiogenesis [55]. Based on our findings and published reports from other laboratories, we propose that secreted sNDPK-B regulates growth and development of metastases by stimulating angiogenesis and may facilitate intravasation, migration and extravasation early in the metastatic process (Fig. 1,2).

11. Vascular P2Y Nucleotide Receptors

Purinergic nucleotide (P2) receptors activated by ATP include both ligand-gated ion channels (P2X) and heterotrimeric G protein-coupled (P2Y) receptors [60]. P2Y receptors are recognized as important regulators of carcinogenesis and endothelial cell functions [138-139] and as described above, are integral modulators of platelet aggregation and blood flow regulation. Extracellular ATP activates P2Y1/2 receptors on vascular endothelial cells to release vasoactive mediators such as nitric oxide, prostacyclin, and additional ATP [31,95, 107] which can elicit vasoactive and angiogenic effects and propagate these effects down-stream [140]. There are a myrid of possibilities presented by purinergic receptor expression [99] of both P2X, P2Y and adenosine receptor classes that may describe differences in vascular beds and thus particular targeting of metastasizing cells as well as disparate outcomes of receptor stimulation including apoptosis of tumor cells [141-143]. We focus on P2Y receptors and P2Y1 in particular as inhibitor studies suggest that these receptors are critical, if not exclusive, to understanding the mechanisms that support metastasis and angiogenesis.

In addition to our own work, only a handful of published reports provide evidence of the role of P2Y receptors in angiogenesis [34-35,136-137,144-147]. Therefore, information regarding this subject is limited. What is known is that endothelial P2Y receptors have been shown to stimulate angiogenic properties such as endothelial cell migration [144-145] and vascular permeability [34]. Activated $P2Y_2$ receptors have also been observed to transactivate VEGFR-2, suggesting a direct link between extracellular nucleotide regulation and established growth factor signaling [148]. Recent evidence supports this cooperation and transactivation of a receptor tyrosine kinase (RTK; such as VEGFR2) by P2Y receptors (GPCR): Buvinic *et al*. observed that $P2Y_1R$ utilizes epithelial growth factor receptor (EGFR) signaling to stimulate cell proliferation [149] and we have provided *in vitro* evidence that endothelial $P2Y_1$ receptor activation and subsequent VEGFR-2 signaling explains angiogenic signaling by nucleotides such as ATP [55].

12. Vascular Endothelial Growth Factor Receptor (VEGF-R)

VEGFR-2 (FLK-1) is thought to be the primary regulator of angiogenesis mediating the majority of the angiogenic and permeability-enhancing effects of VEGF [150]. Angiogenesis is generally thought to occur in capillary and post capillary beds [151] initiated by the production of proteases in stimulated endothelial cells, that degrade the surrounding basement membrane [152] and migrate into the tissue, where they proliferate and differentiate to form a new vessel. Endothelial cells produce specific growth factors, such as PDGF and TGF-β, that then attract supporting cells that produce basement membrane and mature the vessel [153-154]. Vessels surrounded by basement membrane and pericytes are regarded as mature.

Embryonic development, ovulation, wound healing and menstruation all require angiogenesis, however pathological angiogenesis occurs in a wide range of diseases, including tumors, chronic inflammatory diseases, and diabetic retinopathy, which results in hyper-permeable vessels with disorganized architecture and blind non-patent endings [155]. Tumor promoted vessels are unstable and immature and lack a complete basement membrane and pericytes [156]. Tumors may also establish circulation independent of angiogenesis, although the role of microvessel mimicry [157] that has been described in inflammatory breast cancer [158] and

may be a feature of about 8% of surgically removed breast cancers [159] is not likely to subserve breast metastatic lesions as it seems to be a feature of non-metastasizing lesions [160].

In mammals, the VEGF pathway involves five secreted ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor) and three primary receptors (VEGF-R1, VEGF-R2, VEGF-R3). The ligands are widely expressed and expression of VEGF-A is induced by hypoxia, which is a common feature of rapidly growing solid tumors and constitutes a key angiogenic signal. VEGF receptors show restricted cell-type expression. VEGF-R1 and VEGF-R2 are prominently expressed by vascular endothelial cells in addition to expression by other selected cell types. VEGF-A interacts with VEGF-R1 and VEGF-R2, whereas placental growth factor interacts strictly with VEGF-R1. The VEGF receptors are tyrosine kinases that autophosphorylate upon dimerization following ligand binding, which in turn activates various down-stream signaling pathways such as the p38 MAPK pathway. VEGF receptors signal diverse effects in endothelial cells including migration, proliferation, cell survival and regulation of gene expression [156].

13. VEGF/ATP in Angiogenesis

In pathological angiogenesis induced by breast cancer cells, the levels of VEGF-R1 and VEGF-R2 mRNA are significantly increased by exposure to the tumor cells and the conditions that accrue such as hypoxia, as compared to the amounts observed in vessels of normal tissues surrounding the tumors [161]. This suggests that the ability of endothelial cells to respond to VEGF may lag behind that of ATP acting at P2Y receptors, whose expression is not delayed [162] and thus the *sNDPK-B:ATP:P2Y* pathway we describe may be effective in initiating angiogenesis and lowering the requirement of the angiogenic process for VEGF-R [55].

VEGF-R2 and P2Y receptors have been shown to localize in caveolar domains in the cell membrane [139,163-166] allowing the potential for substantial pro-angiogenic signaling with small amounts of agonist stimulation if these receptor systems were to interact in a spatially restricted region.

Conclusions

Our model proposes that sNDPK-B secretion by breast cancer cells both adjacent to (as primary tumors or micrometastases) and within capillaries (as intravasated tumor cells) elevates local ATP concentrations to permit entry of cells into the blood stream and to maintain their ability to move to distant sites once in the blood stream. Once at a distant site, breast tumor cells again benefit from local ATP production to extravasate and form a metastatic tumor. In the absence of suppressive factors such as those released from the primary tumor, distant tumor cells release sNDPK-B to induce endothelial cell tubulogenesis by generating ATP locally leading to activation of the endothelial P2Y receptor and transphosphorylation of VEGFR2 in the absence of VEGF (Fig. 1,2). Once the metastatic cells gain a blood supply and begin to proliferate, VEGF as well as ATP signal further angiogenesis as the tumor grows.

It is clear that elevated ATP levels from sNDPK-B and subsequent P2YR activation by ATP and ADP produce angiogenic signals *in vitro*. While the mechanisms by which P2Y receptors mediate vasodilatation and anti-platelet aggregation (advantageous to the transit of cancer cells to secondary sites) have been well worked out and are accepted, a role for these mechanisms in tumor angiogenesis remains to be defined *in vivo*. Demonstration of a role for P2Y signaling as orchestrated by the circulating cancer cell and subsequent micrometastases offers an exciting opportunity to add a one or more novel therapeutic targets to our adjuvant armamentarium.

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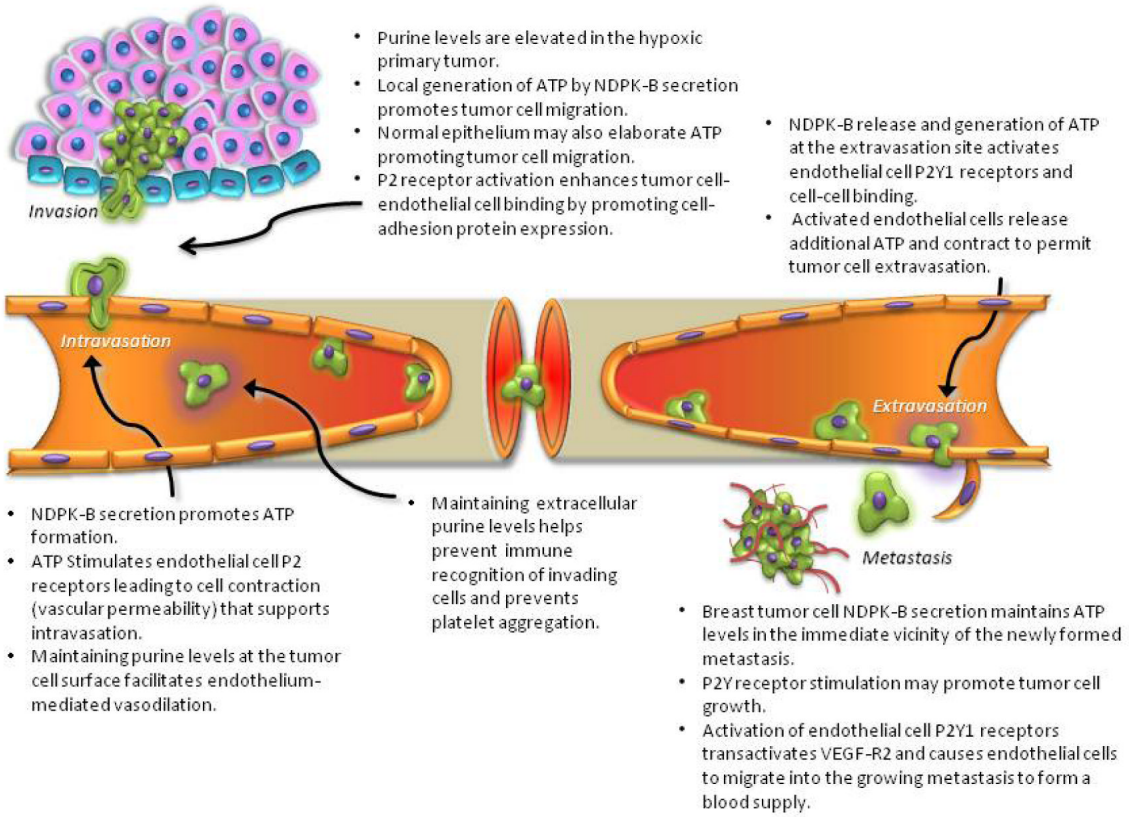
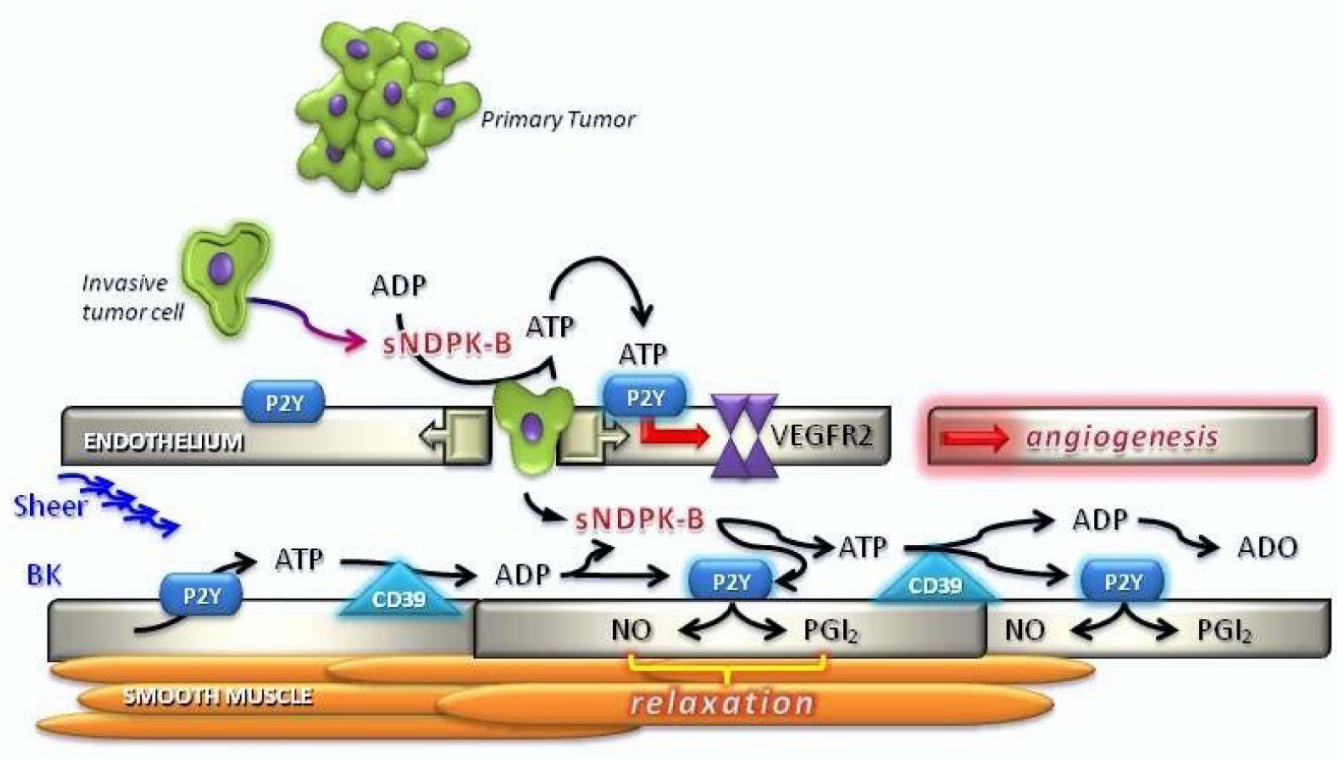


Figure 1. Purinergic Mechanisms Supporting Breast Tumor Metastasis

The role of purines is described in the context of the events associated with metastasis. Evidence is described in the text.



Fgure 2. Secreted NDPK Mediates P2YR/VEGFR-2 Activation in Tumor Angiogenesis

We propose that breast cancer-secreted sNDPK-B is a significant angiogenesis promoter. sNDPK-B is secreted as a phosphoprotein [48] and elevates ATP levels in both arterioles and capillaries by re-phosphorylating ADP. Continued rounds of ATP production (enhanced normally from endothelium by sheer and agonists such as bradykinin (BK), are generated by sNDPK-B at the expense of nucleotides such as GTP released from necrotic cells in the metastatic environment. Extracellular ATP and ADP activates $P2Y_1$ receptors on vascular ECs, which transactivates VEGFR2 to induce angiogenesis [55]. Additional events consistent with metastasis are supported by sNDPK-B; ATP inhibits platelet aggregation [167] maintaining blood flow despite the presence of foreign tumor cells; activation of P2Y receptors by ATP and ADP results in the release of nitric oxide (NO) and prostacyclin (PGI_2) from endothelial cells leading to vasodilatation in arterioles. Breakdown of ADP to adenosine (ADO) inhibits platelet aggregation [168] and dilates venules [169] consistent with increasing the dissemination of tumor cells to distant sites. Dynamic regulation of ATP/ADP levels by sNDPK-B activates VEGFR2 even in the absence of VEGF [55]. At distant sites, P2Y receptor activation may be utilized by the metastatic cells to initiate angiogenesis early in metastasis when VEGF levels are low or absent [170] and will sum with VEGF levels to maximize angiogenic potential [55]. Thus we propose that dual inhibition of $P2Y_1R$ and VEGFR2 signaling may provide an effective mode of combination anti-angiogenic adjuvant therapy. $P2Y_1R$-VEGFR2 signaling may be important in describing and understanding the VEGF signaling required for endothelial homeostasis in both tumor as well as normal vasculature.