University of Nevada, Reno

The Role of shNDPK and E-Cadherin in Growth and Metastasis of an Inflammatory Breast Cancer Model (MARY-X)

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in Biochemistry and Molecular Biology and the Honors Program

by

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May, 2011

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

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entitled

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be accepted in partial fulfillment of the
requirements for the degree of

BACHELOR OF SCIENCE, BIOCHEMISTRY AND MOLECULAR BIOLOGY

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May, 20011

ABSTRACT:

MARY-X, an inflammatory breast cancer (IBC) murine xenograft, exhibits the formation of florid lymphovascular emboli in vivo and spheroid formation in vitro. IBC is highly invasive and metastatic and our model presents the same in mice as it does in human. Therefore, studying the molecular processes involved in this IBC murine model will allow us to uncover the mechanisms promoting cancer growth and metastasis of IBC specifically and cancer in general. In this study we determined the extracellular expression of secreted NDPK in mice implanted with the MARY-X tumor as well as intracellular expression of NDPK in the MARY-X tumor cells. NDPK, which regenerates ATP, acts as an pro-angiogenic factor. NDPK promotes tumor growth by inducing endothelial cell proliferation. E-Cadherin, an adherence protein, is overexpressed in MARY-X spheroids and is responsible for the densely aggregated configuration and the stem cell-like nature of the *in vivo* emboli and the *in vitro* spheroids. We will also investigate specific E-Cadherin expression within MARY-X spheroids in order to identify the role of the unique expression of E-Cadherin in the stem cell-like nature of the MARY-X spheroids. These findings may uncover potential drug targets against IBC allowing development of more effective treatments preventing the growth and metastasis of IBC and other cancers.

ACKNOWLEDGEMENTS

I would like to give special thanks to Dr. Sanford H. Barsky, Dr. I.O. Buxton, Dr. Yin Ye, Dr. Joe Tellez, Nucharee Yokdang, Honglin Tian, Devin Steelman, Maria Durazo, Nick Tschernia, Andrew Tschernia, and Devon Bateman for their added expertise, humor, support, advice and patience. I have had a wonderful experience researching and writing this thesis and it's all due to these fine people. I have learned lots of technical skills, but also I have learned so much about the importance of research. Dr. Ye one said to me that in research, ideas are cheap. It's all about execution, and weighing the payoff against the time and effort you put into it. All these people have shown me that research is about creativity as much as it is about knowledge. It's about telling a story, just as much as it is about advancing science. Again, thank you all.

TABLE OF CONTENTS

TABLE OF FIGURES

INTRODUCTION

Inflammatory breast cancer (IBC) is uniquely characterized by an extraordinarily high degree of invasion into the dermal lymphatic vessels by tight aggregates of tumor cells called lymphovascular emboli. IBC is accompanied by inflammation of the cancer site as well as redness of the skin. We have previously established the first transplantable human inflammatory breast carcinoma xenograft model in mice, termed MARY-X, that presents the same in mice as it does in human. Also, MARY-X exhibits many similatiries to human IBC on a protein expression level and identically mimics human IBC RNA expression.[1] This model has allowed us to study many of the specific and detailed molecular mechanisms that regulate IBC revealing the forces behind this cancer's deadly character. MARY-X exhibits an extremely aggressive invasiveness and presents an extreme view of cancer's mechanism of growth and metastasis.

Metastasis is a crucial factor in survival rates of breast cancer patients [2]. Distant metastasis correlates with poor prognosis. Women who experience a breast cancer recurrence at distant sites in the body have only a 9% chance of living an additional 10 years, as opposed to the 56% survival rate shown for women who experience a local recurrence in only the breast [3]. The metastatic process is still an enigma to cancer researchers the world over. Thus, identifying the molecular processes of metastasis would identify potential drug targets and refocus the way we investigate and treat cancer in the future.

As mentioned, in vivo, MARY-X presents as florid lymphovascular emboli. In vitro, it forms a similar structure of tight cellular aggregates termed spheroids. When first prepared in a crude shake, the emboli are more loosely aggregated, but after a 24-48 hour incubation, these aggregates become very tight and round [1]. The spheroids exhibit a stem cell-like phenotype and

seem embryonal in nature. These spheroids are resistant to radiation and chemotherapy treatments, but the cells become highly apoptotic when disadhered from the main spheroid mass.[4]

Nucleoside diphosphate kinase (NDPK) is a housekeeping protein that regenerates ATP by transferring a phosphate group from GTP to ADP (donor/acceptor). ATP is consumed during a variety of cellular processes. NDPK is produced intracellularly and secreted extracellularly in women under certain conditions such as during the menstral cycle. Secreted NDPK regenerates extracellular ATP, which can acts as a ligand with the purinergic P2Y receptors on the surface of endothelial cells. Activating the P2Y receptor transactivates the VEGFR2 receptor even in the presence of a VEGFR2 receptor inihibitor [5]. The VEGFR2 receptor triggers a protein cascade, which phosphorylates and activates ERK1/2 leading to increased proliferation of endothelial cells, one of the main characteristics of angiogenesis. Furthermore, NDPK has been shown to be secreted at varying levels by human cell lines of several different breast carcinomas while no expression of shNDPK was seen in women without cancer (Yokdang, unpublished results).

Based on findings that secreted human NDPK (shNDPK) has been shown to promote proliferation and migration of human endothelial cells in culture, [5] it is hypothesized that NDPK plays a vital role in angiogenesis which promotes tumor growth and metastasis resulting in more aggressive and life-threatening forms of breast cancer. Since MARY-X is fast-growing and highly angiogenic, invasive and aggressive, we propose that MARY-X should express shNDPK which should be found in the serum of mice implanted with Mary-X tumors. We also propose to show that this level of secretion is high compared to other less aggressive breast carcinomas. It has been proposed that NDPK is a pro-angiogenic factor, implicating it in upstream ERK phosphorylation signaling.[10] We will also look at the total ERK levels

compared to the phosphorylated ERK levels in MARY-X spheroids that have been stimulated with NDPK.

It has been previously shown that E-Cadherin is overexpressed in the MARY-X spheroids not only in the adherent and tight junctions of normal cells, but in a global fashion.[4] We believe that this type of E-cadherin over-expression contributes to the tightness of the spheroids and perhaps disruption of E-cadherin binding leads to the apoptotic signal that seems to occur after disadherence [7]. It has been supposed that this expression of E-Cadherin contributes to the spheroids stem-cell like nature while also aiding in its motility and therefore its metastasis.[4] It has also been supposed that the E-Cadherin overexpression does not come from transcriptional activity, but from altered trafficking within the MARY-X cells. We will be investigating the source of this global expression of E-Cadherin and this altered trafficking.[4] It has been shown that in MARY-X tissue as well as spheroids E-Cadherin exists as different fragments (Ye, unpublished results). All 5 fragments are seen in the MARY-X xenograft, while only NTF1 and full length E-cadherin are seen in the MARY-X spheroids. Full length E-cadherin is cleaved by calpain to yield the NTF1 fragment. In this cleavage, the E-Cadherin is freed from the beta-catenin and the cytoskeletal structure. This allows the protein to move away from the tight junctions, where its expression is usually restricted. This, in turn, is hypothesized to allow the global distribution of E-cadherin that is seen in the MARY-X spheroids.

MATERIALS AND METHODS

Spheroid Preparation

The MARY-X tumor was extracted from the mouse, and minced into a crude "shake" with high glucose DMEM. The shake was incubated at 37ºC and 70-80% humidity (5% CO2) for 24-48 hours. At this time, the shake is run through different size mesh strainers (first 100um and second 40 um) to isolate 40-100 um spheroids. We then use ficoll paque (GE Healthcare) to separate the dead material from the living. The living spheroids are then plated with high glucose DMEM with 10% FBS and 1% PenStrep.

Blood Collection

Small pieces from previously harvested tumors were implanted subcutaneously on the rear backs of NU/NU Nude Mice (Charles River Laboratories) that were 3-8 weeks old. Tumors are grown to a maximum of 2cm by 2cm. At different times and size measurements, the jugular vein was lanced and 100-125 ul of blood was collected. The blood was clotted in a microtainer tube (BD Biosciences, catalog no. 365956) at room temperature for 1–2 hr. Serum was separated by centrifugation at 6,000 × g for 10 min. Serum was used in experiments or stored at −80°C for future use.

Tumors were implanted in 3-6 week old SCID mice in the mammary fat pad. At two week intervals blood was collected as specified above.

NDPK Detection in Serum Samples

ELISAs were performed according to the protocol in Yokdang, et al.[9] Briefly, we performed an indirect ELISA using NDPK-B protein (Abnova, catalog no. H00004831-P01, 0.24ug/ul),

primary antibody mouse anti-H Nm2 AB (Abcam, catalog no. AB 60602) and secondary antibody rabbit anti-mouse IgG, HRP conjugate (Southernbiotech, catalog no. 6175-05). Signal from secondary antibody was detected after addition of Diethanolamine Peroxidase substrate (OPD) with a BIO-RAD Model 680 Microplate reader at 490 nm.

Statistical Analysis of ELISA

Graphs were prepared using Prism Graphing Software (V4.03; GraphPad Software, San Diego, CA, USA) and statistical analyses were performed using InStat Statistical Software (V3.0; GraphPad Software). A $P \leq 0.05$ was considered significant. All experiments were tested for statistical significance using ANOVA and Kruskal–Wallis multiple comparisons post-test unless otherwise stated.

Detection of ERK 1/2 Phosphorylation

Four samples of MARY-X spheroids placed in high glucose DMEM containing 0% FBS and 1% PenStrep, were stimulated with NDPK/ADP/GTP, only ADP/GTP, 10%FBS, and 0% FBS (as control). The cells were lysed using RIPA buffer. Total ERK protein and phosphorylated protein were labeled on the same blot with different fluorescently tagged antibodies. Primary antibodies used were goat anti ERK 1/2, used at 1:1000 dilution and rabbit anti-Phosphorylated ERK 1/2 at a 1:2000 dilution. The secondary antibodies used were donkey anti-goat conjugated to a tag that fluoresces at 800nm used at a 1:25,000 dilution and donkey anti-rabbit conjugated to a tag that fluoresces at 680 nm at a 1:50,000 dilution. The Western membranes were read at 700 and 800 nm using a LI-COR Odyssey Imager. Analysis was performed using the accompanying Odyssey software.

Immunohistostaining and Confocal Imaging of MARY-X Spheroids.

MARY-X spheroids treated with calpeptin (50 µM) and vehicle control were subjected to double immunofluorescent studies using the following combination of primary and secondary antibodies at their respective dilutions: CDH1 rabbit mAb (OriGene, Rockville, MD, USA), 1: 250 in 1X PBS with 0.1% Triton X-100, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), 1:200 in 1X PBS, E-Cadherin (24E10) rabbit mAb -- Alexa Fluor 488 conjugate (Cell signaling Technology, Boston, MA, USA) diluted 1:50 in 1XPBS.

In order to immobilize the spheroids, glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) were coated with Cell-TEK adhesive (BD, San Jose, CA, USA) overnight. The plates were then rinsed with DI water, and dried. DMEM (Invitrogen, Carlsbad, CA, USA) containing spheroids and 10% FBS (Invitrogen, Carlsbad, CA, USA) was added to dry coated plates. The spheroids which adhered were fixed acetone (Fisher Scientific, Pittsburgh, PA, USA) and methanol (Sigma, St. Louis, MO, USA) at 1:1 ratio, then permeablized with TritonX-100 (Sigma, St. Louis, MO, USA) and blocked with 1.5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove,PA, USA) and 0.1% triton X-100 in PBS. The CDH1 primary antibody solution was added and incubated overnight at room temperature, then washed with PBS 3 times, 10 minutes each. The AlexaFluor 594 secondary antibody solution was added and incubated for 1 hour, then washed 4-5 times, 10 minutes each. The 24E10 primary antibody solution was added, incubated for 4 hour at room temperature, then washed with PBS 3 times, 10 minutes each, and DI water once. The spheroids were then mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA).

Images were taken with the Fluoview 1000 Confocal Microscope (Olympus, Center Valley, PA, USA).

Immunohistostaining and Confocal Imaging of MARY-X Histology Sections.

Frozen MARY-X histology sections were taken and were subjected to double immunofluorescent studies using the following combination of primary and secondary antibodies at their respective dilutions: CDH1 rabbit mAb (OriGene, Rockville, MD, USA), 1: 250 in 1X PBS with 0.1% Triton X-100, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), 1:200 in 1X PBS, E-Cadherin (24E10) rabbit mAb -- Alexa Fluor 488 conjugate (Cell signaling Technology, Boston, MA, USA) diluted 1:50 in 1XPBS.

The tissue sections were fixed with an acetone (Fisher Scientific, Pittsburgh, PA, USA) and methanol (Sigma, St. Louis, MO, USA) solution at 1:1 ratio, then permeablized with TritonX-100 (Sigma, St. Louis, MO, USA) and blocked with 1.5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove,PA, USA) and 0.1% triton X-100 in PBS. The CDH1 primary antibody solution was added and incubated overnight at room temperature, then washed with PBS 3 times, 10 minutes each. The AlexaFluor 594 secondary antibody solution was added and incubated for 1 hour, then washed 4-5 times, 10 minutes each. The 24E10 primary antibody solution was added, incubated for 4 hour at room temperature, then washed with PBS 3 times, 10 minutes each, and DI water once. The tissue sections were then mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken with the Fluoview 1000 Confocal Microscope (Olympus, Center Valley, PA, USA).

RESULTS

The serum from nu/nu nude mice implanted with different tumor lines (MARY-X, BAC, Reiter, HMS-6X and HMS-X) were tested with ELISA assay and the NDPK concentration found was compared to tumor size and timeline of growth (Figure 1). Various patterns are observed, though no specific correlation seems apparent. Nude mice were found to express a high level of endogenous antibodies which would interact with any anti-mouse secondary antibody used. This is because nude mice have a suppressed immune system, but some immune function is still viable. This causes a high background level to occur in the ELISA assay results. SCID mice, on the other hand, have almost no immune system whatsoever, so the level of endogenous antibodies produced in SCID mice is much lower (Figure 2). The SCID was thought to be a better model for the ELISA because of the lower background level produced.

The serum form SCID mice implanted with MARY-X were tested with ELISA assay and, again, the NDPK concentration was compared to varying time points and tumor sizes (Figure 3). Patterns observed are not clear, however the most notable peak happens at the size 300 mm^3. At this time point the NDPK concentration in the mouse serum is more than twice that of any other given concentration at any time or size. To verify the significance of this, more experimentation is needed.

The level of phosphorylation of ERK in NDPK stimulated MARY-X spheroids was also tested (Figure 4). The level of ERK phosphorylation was much higher in the presence of NDPK, and the GTP/ADP donor/acceptor pair than in the presence of ADP and GTP alone. This is significant, implicating NDPK in the angiogenic pathway of MARY-X.

To measure E-cadherin expression, Spheroids and Histological sections were stained and imaged with a confocal microscope. We saw a decrease in expression of NTF1 and full-length

E-cadherin in spheroids that had been treated with calpeptin, a protein known to prevent calpain from cleaving full length E-cadherin (Figure 5A). We also observed lower expression of NTF1 and full-length E-cadherin in the non-emboli tissue compared to the emboli (Figure 5B).

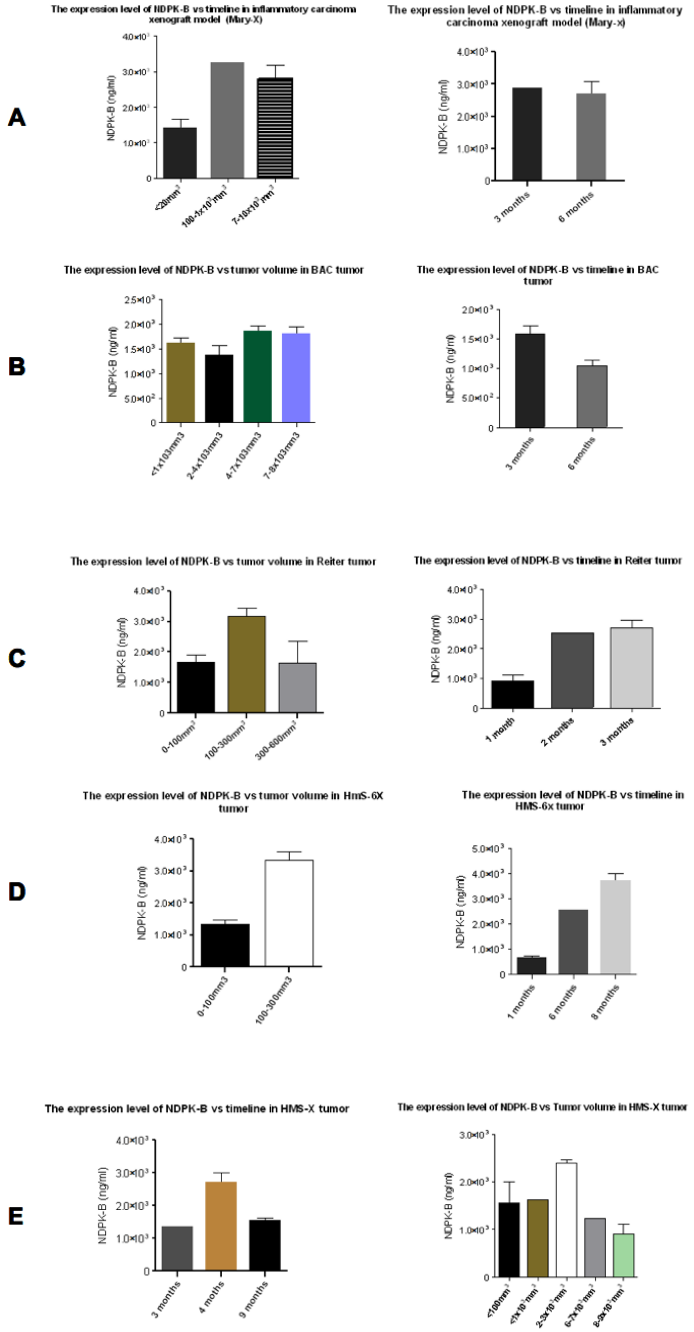

Figure 1. NDPK in Nude Mice. NDPK levels from blood serum of nude mice implanted with respective tumor lines were measured using ELISA assay. (A) NDPK measurement in MARY-X implanted nude mice presented by tumor size as well as time after implantation. Similar results given for (B) BAC, (C) Reiter, (D) HMS-6X, (E) HMS-X.

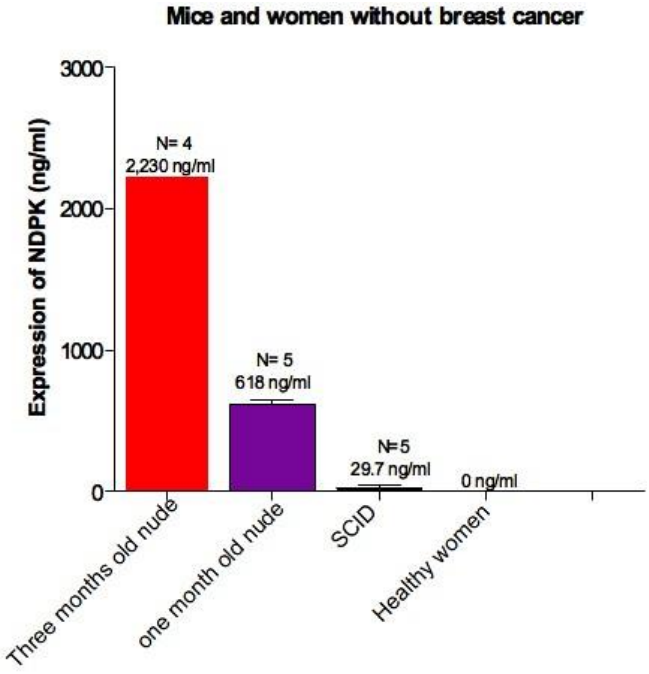

Figure 2. Endogenous Antibody Expression in Nude and SCID mice. High levels of endogenous antibodies were detected in nude mice due to their weakened, but not completely absent, immune systems.

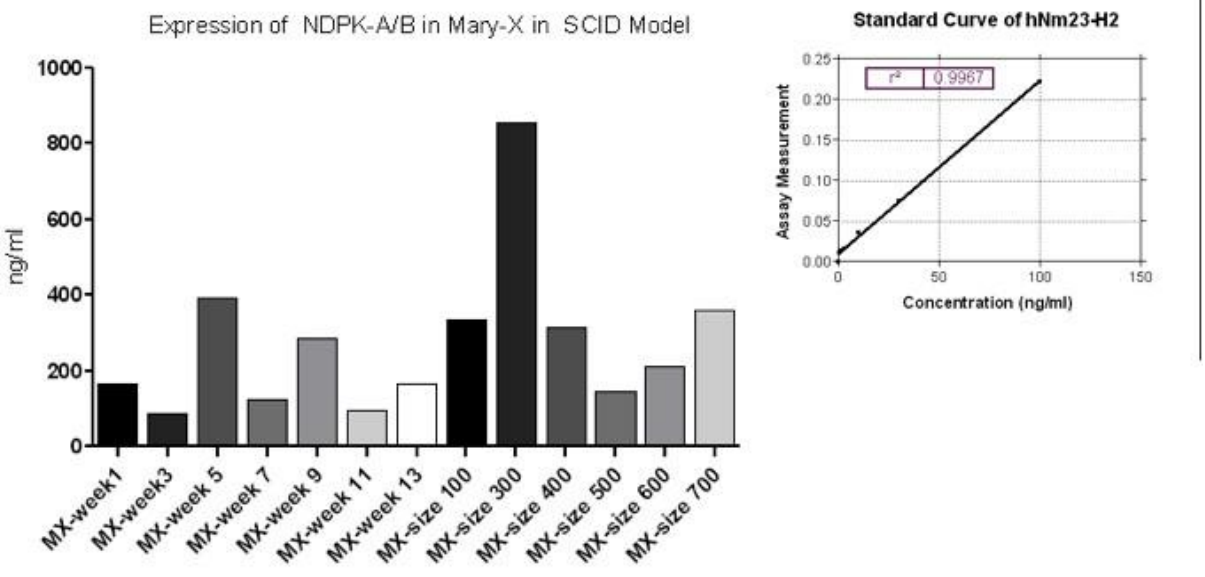

Figure 3. NDPK in MARY-X Implanted SCID Mice. NDPK levels (ng/ml) in SCID mice implanted with MARY-X tumors were measured using ELISA. Samples were chosen for a range in time (in weeks) after transplantation as well as tumor volume (in mm^3).

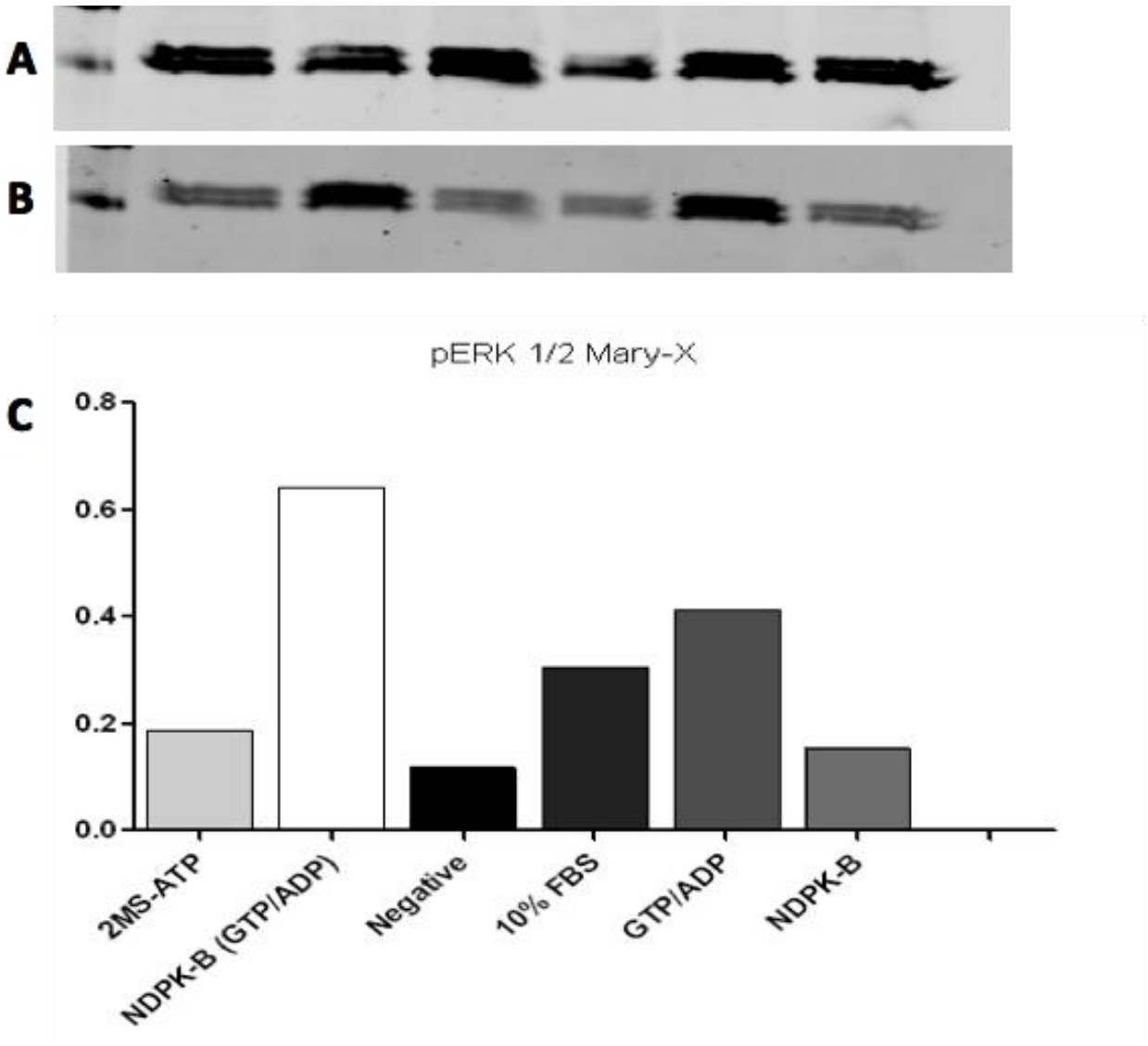

Figure 4.ERK 1/2 and Phosphorylated ERK 1/2 in MARY-X Spheroids. MARY-X Spheroids were stimulated with 2MS-ATP, NDPK-B (in the presence of ADP and GTP), GTP and ADP only, and NDPK-B only. A negative control sample was included of MARY-X spheroids in 0% FBS with no stimulation (Negative). (A) Seen here are the Western Blot results from the labeling of total ERK 1/2. The order of the samples is the same as pictured in part C. (B) Seen here are the Western Blot results from the labeling of Phosphorylated ERK 1/2. The order of the samples is the same as pictured in part C. (C) This is a graph comprised of the ratio of Phosphorylated ERK 1/2 to total ERK 1/2 in the above Western blots.

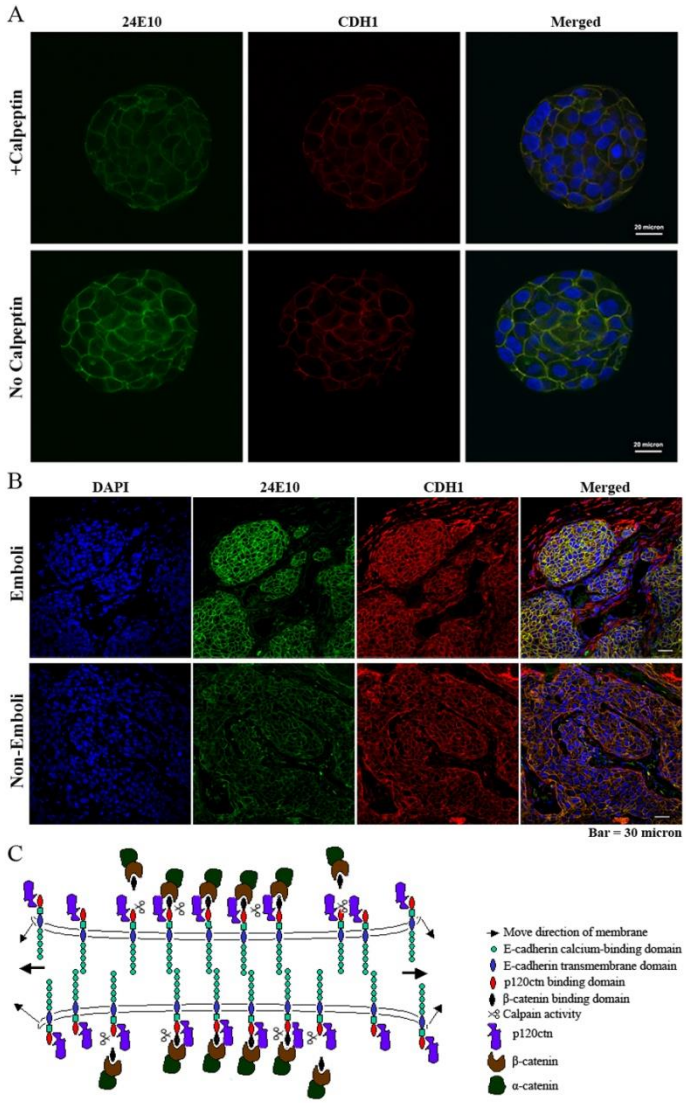

Figure 5. E-Cadherin Expression in MARY-X Spheroids and Lymphovascular Emboli. (A) Labeling of the NTF1 E-Cadherin fragment (green), the nuclei with DAPI (blue) and the CDH1 surface cadherin protein (red) in the presence and absence of Calpeptin are seen. (B) Similarly labeled histology sections of MARY-X tissue are seen here. The difference between emboli and non-emboli tissue are specified. (C) The cleavage of E-Cadherin by calpain releases it from it's cytoskeletal tethers and allows it to move over the cell membrane, causing the global expression of E-cadherin in tumor emboli and MARY-X spheroids.

DISCUSSION

Exhibited in Figure 1, there are varying levels of NDPK in a variety of cell lines (MARY-X, BAC, HMS-6X, Reiter, and HMS-X). MARY-X, as previously stated, is a fast growing, highly invasive tumor, with a high level of angiogenesis. BAC is also fast growing with high angiogenesis. Reiter and HMS-6X are slower growing with minimal angiogenesis and HMS-X is more moderate in growth and angiogenesis. Interestingly, the different tumor cell lines seem to have very different expression patterns of NDPK. It makes sense that the slower growing tumors should have significantly different NDPK stimulation than the fast growing tumors. Here we see that there is not only a difference in secretion level, but also a difference in when secretion occurs. Whether the NDPK secretion is an early or late event and how it affects aggressiveness of the tumor has yet to be seen and will require further investigation. We detected NDPK in nude mice while finding that these mice produce a relatively high background of endogenous antibodies. This background level was subtracted out of Figure 1. So, to improve the reliability of our results we repeated the experiment using SCID mice, which were found to produce a background level close to zero (Figure 2).

ELISA results from the blood serum of MARY-X implanted SCID mice, show an interesting pattern of NDPK secretion suggesting that NDPK is secreted in high levels about 3 weeks after transplantation, or when the tumor is about 200-300 mm^3 (Figure 3). It makes sense that NDPK secretion, or angiogenic growth signaling, would occur relatively early in a fast growing tumor, since it has been shown that NDPK is secreted before VEGF signaling begins. This may add to the important role of NDPK in early tumorogenesis as a replacement for VEGF signaling. It may be even more vital to the tumor since it is such an early growth stimulator. The result given in Figure 3 is a little more detailed than the previous experiments with the nudes

(Figure 1). However it only has one sample per experimental field. More experimentation is needed to confirm this result as well as clarify the secretion pattern. We plan to continue this line of experimentation with SCID mice and broaden this study to the other tumor lines that were previously discussed in Figure 1. Pinpointing the highest expression points of MARY-X and other tumor lines may continue to elucidate the importance of NDPK in growth and metastasis of all cancers and lead to preventative treatments that rival today's standards. For example, Ellagic Acid is known to be an inhibitor of NDPK and has also been shown to decrease the rate of metastasis in SCID mice injected with MBA-MD-231 carcinoma cells (Yokdang, unpublished results). If this same, or similar treatments, could be implemented with MARY-X, we could potentially improve IBC's bleak prognosis.

Figure 4 demonstrates the relative level of Phosphorylated ERK to Total ERK in the MARY-X Spheroids under NDPK stimulation. The level of phosphorylated ERK is much higher with NDPK stimulation than with ADP/GTP presence alone. This supports the idea that NDPK is an angiogenic factor that feeds into the pathway leading to endothelial growth stimulation. Again, this identifies NDPK as a target for treatment of tumor growth by attacking angiogenesis.

Another potential target for the treatment of IBC is E-cadherin. Figure 5A shows the decrease of NTF1 and full length E-cadherin in spheroids treated with calpeptin. Calpeptin stops calpain from cleaving E-cadherin to the NTF1 fragment. The specific place where calpain cleaves E-cadherin, leaves the NTF1 fragment within the cell membrane, while also freeing the protein from beta-catenin and the cytoskeletal structure which limits its expression to specific junctions. This cleavage may have a dispersing effect, which allows E-cadherin to migrate throughout the cell membrane around the cell (Figure 5C). We think this is what causes the global expression of E-cadherin on MARY-X spheroids.

Another notable find was that lower expression in nonembolic tissue was seen in comparison to emboli in MARY-X tissue sections. More fragment types are known to be expressed in the xenograft tissue than in the spheroids (NTF2, NTF3, and NTF4). When these are cleaved, they cannot stay in the cell membrane and are lost. This may account for the lower expression in the non-embolic tissue seen in Figure 5B. This lower expression of full length and NTF1 fragment of E-cadherin means a lower frequency of homotypic binding of E-cadherin of adjacent cells, which means less adhesion. In spheroid disadhesion experiments, using trypsin and calcium depletion, we saw disadherence of the spheroids due to cleavage of the E-cadherin interactions. When the cells are disadhered, a high level of apoptosis occurs. The lower E-cadherin interaction in non-embolic tissue may also explain the necrotic interior of MARY-X; the deeper into the tissue, the more necrotic. All of this is, of course, supposing that spheroids and emboli have the same expression of E-cadherin fragments. To confirm this result, we will analyze the expression of the five E-cadherin types (full-length, NTF1, NTF2, NTF3 and NTF4) in emboli-only samples which we can separate by laser capture microdissection.

Studying the pathways of MARY-X and the important molecular players that make MARY-X such a dangerous disease, such as E-cadherin and NDPK, will not only help us to treat IBC and breast cancers, but will help cancer research as a whole. By better defining such universal concepts in cancer growth and progression, like metastasis and angiogenesis we are making innovative treatment options possible.

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