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PRINCIPAL INVESTIGATION: Joseph M. Backer, Ph.D.

CONTRACTING ORGANIZATION: New York Medical College
Valhalla, New York 10595

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Joseph M. Backer, Ph.D.

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New York Medical College
Valhalla, New York 10595

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Metastatic dissemination of primary breast tumors is controlled by positive and negative regulators. We found that expression of a catalytically inactive nucleoside diphosphate kinase B (NDPK B/T) in MDA-MB-435 human breast carcinoma cells significantly decreases their metastatic dissemination in a nude mouse assay. Inhibition of metastatic dissemination of primary tumors formed by these cells occurs prior to formation of detectable micrometastasis. Our structural studies suggests that wild type NDPK B and highly homologous NDPK A form different oligomers that may occupy specific binding sites We hypothesize that NDPK B/T displaces wild type NDPK B from specific binding sites, acting in effect as a dominant negative mutant. These results suggest that NDPK B acts as a positive regulator of metastatic potential of human tumor cells and that specific inhibition of NDPK B may be a target for potential anti-metastasis therapeutics.

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Introduction

Metastatic dissemination of primary tumors is the major cause of death for patients with breast carcinoma. Thus understanding the molecular mechanisms involved in the metastatic process is crucial for identification of new targets for the development of anti-breast cancer therapeutics. Potential targets include genes and/or gene products involved in positive and negative regulation of the metastatic phenotype of human breast tumor cells.

There are two highly homologous human *nm23* genes which encode nucleoside diphosphate kinases (NDPK) A and B which form homo and heteromers AxBy. It was reported that a low level of *nm23-1* gene expression correlates with the increased metastatic potential of human breast cancer. The *nm23-1* gene was proposed to be a metastasis suppressor gene and function as a negative regulator of metastasis. We hypothesize that both *nm23-1* and *nm23-2* genes control the metastatic potential of human breast tumor cells as negative and positive regulators, respectively. We proposed to test this hypothesis by establishing metastatic potential of MDA-MB-435 human breast carcinoma cells transfected with nm23 constructs encoding wild type and catalytically inactive NDPK A and B. We expected that catalytically inactive NDPKs may act as dominant negative mutants of the wild type NDPKs and, thus, reveal their regulatory functions. Furthermore, we proposed a specific biochemical mechanism that may link NDPK activity to survival of cells under hypoxic conditions in micrometastatic lesions.

If our hypothesis is correct, then we will establish novel cellular and molecular functions that account for the development and progression of breast cancer. Moreover, if this hypothesis is correct then the inhibition of NDPK B may become a feasible goal for the development of anti-metastasis therapeutics.

Nature of the Problem

Several lines of evidence indicate that conversion of tumor cells to a metastatic phenotype may be controlled by a limited number of specific genes (see for review, Backer & Hamby, 1997). These genes are the primary targets for the development of anti-metastatic therapeutic strategies. At least theoretically, it is easier to develop inhibitors of positive regulators, than stimulators of negative regulators. Unfortunately, until now positive regulators of the metastatic potential of human breast tumor cells have not been identified. We hypothesize that the *nm23-2* gene may function as a positive regulator of the metastatic potential of human breast tumor cells. This proposal is designed to test this hypothesis and to test a specific mechanistic model that may explain how the *nm23-2* gene functions.

Background and Preliminary Results from our Studies.

Cell fusion and microcell-mediated chromosome transfer experiments established that the metastatic potential of tumor cells can be suppressed independently from their tumorigenic potential. Furthermore, significant alterations of metastatic potential of tumor cells were achieved by overexpression of cloned genes and by overexpression of ribozymes directed against specific mRNA (see for review, Backer & Hamby, 1997). Although for obvious experimental and ethical reasons experiments with human tumor cells were performed in immunodeficient mice, these findings are consistent with the results obtained with animal tumors.

There are two reports on genetic control of the metastatic potential of human breast carcinoma cells. Phillips, et al., found that introduction of human chromosome 11

into highly metastatic MDA-MB-435 human breast carcinoma cells yielded clones of tumorigenic, but weakly metastatic clones (1996). Leone, et al., reported that transfection of nm23-1 cDNA into human breast carcinoma MDA-MB-435 cells yielded clones of tumorigenic but weakly metastatic cells (1993). These experiments support the hypothesis that the *nm23-1* gene may function as a negative regulator of metastatic potential.

The *nm23-1* gene encodes nucleoside diphosphate kinase A (NDPK A) and a highly homologous gene, *nm23-2*, encodes nucleoside diphosphate kinase B (NDPK B). We have recently found that overexpression of a dominant negative mutant of NDPK B in human melanoma cells suppresses their metastatic potential without affecting their tumorigenicity (Backer, et al., 1997). The suppression was observed with pooled clones of transfected cells eliminating the possibility that this effect was due to a serendipitous integration of a transgene, or a serendipitous mutation in one of the genes that control metastasis. Our results suggest that the wild type *nm23-2* gene may function as a positive regulator of metastatic potential in human breast tumor cells.

Purpose of the Present Work

The overall purpose of the present work is to establish the mechanisms through which *nm23* genes control metastatic dissemination of the human breast tumor cells. Previously it was proposed that the *nm23-1* gene functions as a negative regulator of metastatic phenotype. We proposed that the *nm23-2* gene functions as a positive regulator of metastatic phenotype. To test this hypothesis we determined the metastatic potential of MDA-MB-435 cells transfected with constructs for expression of wild type and catalytically inactive NDPK A and B (products of *nm23-1* and *nm23-2* genes, respectively). Furthermore, we began to test specific mechanism(s) through which *nm23-1* and *nm23-2* genes may regulate the metastatic potential of human breast tumor cells.

The following is a description of our results as related to the Statement of Work of our Proposal.

Technical objective 1: Tumorigenicity and metastatic dissemination studies of NDPK overexpressing MDA-MB-435 cells.

Task 1: Tumorigenicity studies in nude mice. Quantitation of micro- and macrometastasis during tumor progression.

The following β-glucuronidase tagged pooled transfected cells were injected in the mammary fatpad of nude mice (10^6 cells/mouse):

Pooled Cell Lines	Designation
MDA-MB-435 cells transfected with empty vector	V
MDA-MB-435 cells with transgene encoding NDPK A	A
MDA-MB-435 cells with transgene encoding catalytically inactive NDPK A/T	A/T
MDA-MB-435 cells with transgene encoding NDPK B	B
MDA-MB-435 cells transgene encoding catalytically inactive NDPK B/T	B/T

We found that the tumor take was similar for tumors formed by different transfected cell lines (Table 1 and Figure 1). The rate of tumor growth for tumors formed by A and B/T cells was approximately two times lower that of other tumors. However, we did not find a difference in tumor growth rates when cells were injected in the mammary fatpad of SCID mice (data not shown; collaboration with Dr. Z. Bhujwalla, The Johns Hopkins University). These results indicate that *nm23* genes do not strongly

influence growth of primary tumors. It is important to note that in our experiments primary tumors were started with a large inoculum of tumor cells (10^6 cells/mouse). Thus, we could not evaluate the role of *nm23* genes in the early stages of tumor progression from a single transformed cell.

We found that the incidence of macrometastasis in lungs of tumor-bearing animals was significantly lower only in mice carrying tumors formed by B/T cells as compared with V, A, and A/T cells (Table 1 and Figure 2). Although tumors formed by A cells appears to be less metastatic, the difference with control was not statistically significant. Data for B cells are being analyzed. For comparison, Figure 2 also presents data obtained in similar experiments with human melanoma cells transfected with the same nm23 constructs. In this system we found that metastatic potential was suppressed only in B/T cells. Taken together, our findings with human breast carcinoma and melanoma cells indicate a common mechanism of suppression of metastatic potential of tumor cells upon overexpression of catalytically inactive NDPK B/T.

Theoretically, the development of macrometastasis formed by B/T cells may be inhibited before or after formation of micrometastasis. The cell lines used in our experiments have been tagged with bacterial β-glucuronidase and can be visualized in the sections of frozen tissue by staining with X-glu. Examples of the staining are shown in Figure 3. We completely sectioned several lungs from macrometastasis-free animals with primary tumors formed either by V, or by B/T cells. These sections were stained with X-glu and screened visually under the light microscope. We found numerous small clumps of X-glu stained cells in lungs of macrometastasis-free animals with primary tumors formed by V cells. These clumps are mostly confined to thin wall vessels (Figure 3). In contrast, we did not find X-glu stained cells in lungs of macrometastasis-free animals with primary tumors formed by B/T cells. It should be noted that the lack of X-glu stained cells may be attributed to the loss or underexpression of bacterial β-glucuronidase. We are currently evaluating the presence of human cells in lungs of macrometastasis-free animals by PCR, using primers specific for the transfected DNA.

Our data support previous findings that overexpression of wild type NDPK A suppresses metastatic potential of MDA-MB-435 cells (Leone, et al., 1993). However, a larger number of animals is required to obtain statistically significant results. This effect depends on the catalytic activity of NDPK A, since cells overexpressing catalytically inactive NDPK A/T were highly metastatic. Our data indicate that overexpression of catalytically inactive NDPK B/T in metastatic MDA-MB-435 cells suppresses their metastatic potential without significantly affecting their tumorigenicity. This effect cannot be attributed to a fortuitous integration of NDPK B/T cDNA in the host DNA since it is observed with pooled cell lines containing at least 50 different clones. To explain the mechanism of action of NDPK B/T we hypothesize that it acts a dominant negative mutant of the wild type NDPK B. We propose that catalytically active wild type NDPK B associated with as yet unknown binding sites acts as a positive regulator of metastatic potential. Displacement of wild type NDPK B with NDPK B/T eliminates this positive regulation and inhibit metastatic dissemination.

Task 2: Analysis of clonal origin of primary tumors and metastasis.

As described above significant suppression of metastatic potential was observed with pooled B/T cells. However, tumors formed by these cells did not generate micrometastasis as judged by X-glu staining. We decided not to analyze clonal origin of metastatic tumors. However, if PCR analysis reveals a presence of micrometastasis in lungs of animals with B/T tumors we will analyze their clonal origin.

Technical objectives 2: Hypoxic studies

Task 3: Hypoxic studies in vitro.

We assayed the growth of transfected MDA-MB-435 cells under hypoxic conditions and found that oxygen deprivation does not affect the growth of these cells (data not shown). These results suggest that cells derive energy from glycolysis, rather than from mitochondrial oxidative phosphorylation. To test this hypothesis we assayed the effects of several inhibitors of mitochondrial ATP synthesis on cell growth. We tested oligomycin that directly inhibits mitochondrial ATPase, antimycin that inhibits mitochondrial electron transport chain that supplies energy for ATP synthesis, and FCCP that uncouples generation of energy in the electron transport chain from ATP synthesis. These inhibitors individually, or together did not affect cell growth when cells were grown in glucose-containing (4.5 g/L) media.

The ability of transfected MDA-MB-435 cells to grow normally under hypoxic conditions or in the presence of mitochondrial inhibitors led to the initial conclusion that for these cells metastatic dissemination cannot depend on the survival of cells under hypoxic conditions. However, recent experiments indicated that this conclusion could be premature. We found that when cells were placed in low-glucose (0.45 g/L) media they continued to grow. However, addition of mitochondrial inhibitors stopped cell growth, induced contraction of cells and resulted in death within 24 to 48 hours. Thus, these cells utilize mitochondrial oxidative phosphorylation for ATP production when they encounter a low glucose environment.

Since oxygen diffuses from blood into tissue better that nutrients it is reasonable to assume that in tumors or metastases oxygen deprivation follows nutrient deprivation. We are therefore now screening for tissue culture conditions of glucose/serum deprivation under which we can monitor the effects of oxygen deprivation. The objective is to find conditions that will establish whether there is a differential response of MDA-MB-435 cells overexpressing different NDPKs.

Task 4. Construction and analysis of cells overexpressing NDPK A and catalytically inactive NDPK B/T under control of an inducible promoter.

Initially, we planned to transfect cells with plasmids with NDPK under the control of a dexametasone-sensitive MMTV promoter and under control of a metal-sensitive sheep metallotheonine promoter. However, these promoters are fairly "leaky" and experiments with these plasmids imply that a long term exposure of cells and eventually animals to dexamethasone and/or heavy metals would be required. The side effects of these exposures may present problems.

Recently a new system of inducible expression of transfected genes in eucaryotic cells has been commercialized by ClonTech. This system "Tet-On" consists of two plasmids:

1. A regulatory plasmid contains a "reverse" Tet repressor (rTetR) fused to the C-terminal 127 amino acid residues of the VP16 protein of herpes simplex virus. This fusion protein (tTA) is expressed under control of the immedate early promoter of cytomegalovirus (pCMV). In the presence of tetracycline tTA binds to the tet operator sequence (*tetO*) from E. Coli and acts as a transcriptional activator for any promoter downstream of *tetO*. This plasmid is integrated into the chromosomal DNA of target cells and rTerT is constitutively expressed.

2. A response plasmid contains seven copies of *tetO* upstream of the minimal pCMV followed by a multiple cloning site for insertion of the gene of interest. The minimal pCMV lacks a strong enhancer and cannot drive expression of downstream genes. Thus, when the gene of interest is inserted in the vector and is integrated into chromosomal DNA there is no expression. However, when the regulatory plasmid is integrated into chromosomal DNA of the same cell, then addition of tetracycline causes binding of rTetR to *tetO* and transcription is turned on.

We are in the process of constructing MDA-MB-435 cells that will express NDPK A and NDPK B/T in response to exposure to tetracycline.

An alternative approach would to downregulate the expression of NDPK B. We considered the development of MDA-MB-435 cells with knocked-out *nm23-2* gene. Both *nm23* genes are mapped to chromosome 17q21.3 (Backer, et al., 1993). Unfortunately, our FISH experiments established that chromosome 17 is tri- and tetraploid in the majority of MDA-MB-435 cells, rendering knock-out experiments unfeasible (Figure 4).

As an alternative approach we have designed ribozymes directed against NDPK B mRNA (see, Figure 5). This ribozyme will be tested in an *in vitro* transcription/translation system. If it proves to be effective it will be transfected into MDA-MB-435 cells and the ability of ribozymes to inhibit production of NDPK B will be tested. According to our model, cells carrying ribozymes should express a phenotype similar to the cells transfected with NDPK B/T.

Currently, we have two tissue culture assays that discriminate between highly metastatic V cells and weakly metastatic B/T cells. B/T cells grow worse in soft agar and better in matrigel than V cells. Both assays are cumbersome and do not have molecular explanations. We therefore decided to develop immunological reagents that discriminate between V and B/T cells. The traditional approach would be to develop a large panel of monoclonal antibodies from mice immunized with V or B/T cells and to screen these antibodies for selective reactivity with V and B/T. However, this route can generate only a rather limited repertoire of mouse monoclonal antibodies. Recently, combinatorial methods of generating large ($\geq 10^8$) repertoires of human single-chain variable fragment (scFv) antibodies has been developed (see for review, Winter, et al., 1994). These antibodies can be expressed as fusion proteins on the surface of filamentous phage. The resulting phage display library can be screened in a high throughput assay to yield phage clones which selectively bind to a specific antigen. The scFv antibodies carried on the surface of selected phages as fusion proteins can be expressed as soluble scFv antibodies for conventional immunochemical and immunohistochemical assays. Furthermore, DNA sequencing can identify selected scFv antibodies with unique specificities.

Differential panning of V and B/T cells (see, Figure 6 for details) yielded phages that bind only to V cells. The selective reactivity of 170 selected phages against V cells was further tested in an ELISA assay using anti-phage antibody. The distribution of ELISA's O.D. readings for phages from the 15 most reactive clones is shown in Figure 6. The range of reactivities revealed by ELISA testing suggests the existence of different cell-surface antigens that are expressed in V and absent in B/T cells. In preliminary experiments soluble scFv's produced by these 15 clones display strong binding to V cells in ELISA assays (Figure 6B).

Further development of these immunological reagents and identification of unique molecules expressed on the surface of highly metastatic V cells, but not on the surface of the weakly metastatic B/T cells is a subject of a separate grant application. However, for the purpose of this project we will use scFv antibodies to establish whether overexpression of B/T under control of an inducible promoter or an anti-NDPK B ribozyme would lead to the disappearance of the cell surface markers recognized by selected scFv antibodies.

Technical objective 3: Mitochondrial and peptide inhibitors studies.

Task 6. Effect of NDPK-based peptide inhibitors.

Four peptides corresponding to different regions of NDPK B have been synthesized (see Figure 7). The first of these peptides links amino acids from the α-helix region α1 and β-sheet region β2 of NDPK B via a PGGGP spacer. We expect that the resulting peptide would interfere with the dimerization of NDPK B. The DSKPG and the CG-DSKPG-GGC peptides cover the kpn-loop of NDPK B with the latter peptide designed with N-terminal CG- and C-terminal GGC extensions for cyclization. The *killer prune* (kpn) mutation in the kpn loop in the Drosophila's NDPK is lethal when combined with the *prune* mutation (Biggs, et al., 1988). Lascu et al. demonstrated that this mutation affects the stability of the NDPK, but does not affect catalytic activity of the enzyme (Lascu et al., 1992). It has been suggested that kpn-loop is involved in the interactions with other proteins (Dumas, et al. 1992; Williams et al., 1995, Webb, et al., 1995), and we plan to use peptides 2 and 3 to probe these interactions (see lelow). Two other peptides cover the C-terminus of the NDPK B. According to X-ray structures the C-terminus of NDPK B forms a belt around mature hexamer of NDPK B and may contribute to the stabilization of the mature NDPK (Webb, et al., 1995). On the other hand, the last 10 amino acids residues (142-152) of the C-terminus have 4 differences between NDPK A and NDPK B. It is possible that this part of the molecule participate in the NDPK A and NDPK B specific interactions with other cellular proteins.

So far we found that at 10 μg/ml none of these peptides affected the growth of any cells in DMEM media containing 10% serum. We plan to come back to experiments with peptides, once we find tissue culture conditions that discriminate between MDA-MB-435 cells overexpressing different NDPKs.

Task 7: Development of protocols for isolation and characterization of intermembrane mitochondrial NDPK.

We have developed a protocol for detection of human NDPK A and NDPK B in cellular fractions using an autophosphorylation reaction:

$$[\gamma\,^{32}P\,]\text{-ATP} + \text{E-his118} = \text{E-his118}\sim{}^{32}P + \text{ADP}$$

Phosphohistidine adducts are thermo- and acid labile and therefore phosphoproteins can be detected on SDS-PAGE only if samples are not boiled prior to loading and gels are not acetic acid fixed prior to radioautography. When dialyzed NP-40 extracts of MDA-MB-435 cells are incubated with [γ ^{32}P]-ATP, boiled and separated on SDS-PAGE only 36 kDa and 96 kDa phosphoprotein bands are detected (Figure 8, Lane 1). However, if the sample is not boiled prior to loading on SDS-PAGE there are additional 21 kDa and 17 kDa bands corresponding to phosphorylated NDPK A and NDPK B respectively (Figure 8, Lane 2). Only these two bands are detected when the autophosphorylation reaction is carried out on the proteins immunoprecipitated with a monoclonal antibody that recognize NDPK A and NDPK B (Figure 8, Lane 6).

recognize NDPK A and NDPK B (Figure 8, Lane 6).

NDPK A and NDPK B may exist as homomers and/or heteromers AxBy. To establish the presence of AxBy heteromers we fractionated NP-40 extracts on Q-Sepharose. NDPK A is a slightly acidic protein (pI=6.7) while NDPK B is a basic protein (pI=8.7). We reasoned that only NDPK A and heteromers AxBy containing preferentially NDPK A would bind to the Q-Sepharose anion-exchange resin while NDPK B would not bind to this resin. Indeed, we found that material that did not bind to Q-Sepharose contains preferentially NDPK B (Figure 9, Lane 3 for boiled sample, and Lane 4 for unboiled sample). On the other hand, Q-Sepharose bound material contained both NDPK A and NDPK B (Figure 8, Lane 5) indicating the presence of AxBy heteromers in the cellular extracts.

In order to further characterize the status of NDPK A and NDPK B in MDA-MB-435 cells we fractionated an NP-40 cellular extract on a TSK S3000 HPLC gel filtration column and assayed for the presence of NDPKs in the fractions in the autophosphorylation assay (Figure 9). We found that NDPKs are eluted between 29 kDa and 69 kDa markers. Since NDPKs are 17 kDa proteins our gel-filtration experiments indicate that mature proteins exist as dimers, trimers and tetramers.

It is interesting that the last active fraction, containing mostly NDPK B is eluted close to the 29 kDa marker, suggesting that NDPK B exist as a dimer, or in a form that is distinctively different from AxBy heteromers. In turn, it suggests that NDPK B dimers may have specific docking sites which can be displaced only by dimers of catalytically inactive NDPK B/T but not by other AxBy heteromers.

Our gel-filtration findings are unexpected, since human NDPK has been purified from red blood cells as a hexamer and recombinant human NDPK B has been crystallized as a hexamer (Webb, et al., 1995). We were unable to detect the presence of phosphorylated hexamers even after prolonged exposure of radioautographs (data not shown). It is possible that dimers, trimers and tetramers of NDPK detected in our experiments appear during NP-40 extraction of cells under non-reducing conditions. To test this possibility we plan to treat cells with a cross-linking reagent prior to NP-40 extraction and gel filtration to determine the size of cross-linked NDPKs. At that point the characterization of NDPK in cellular extracts will be completed and we will characterize the intermembrane mitochondrial NDPK from MDA-MB-435 cells.

Conclusions.

1. Expression of a catalytically inactive nucleoside diphosphate kinase B (NDPK B/T) in MDA-MB-435 human breast carcinoma cells significantly decreases their metastatic dissemination in a nude mouse assay. This result suggests that wild type NDPK B may act as a positive regulator of metastatic potential and that specific inhibition of NDPK B may be considered as a target for potential anti-metastasis therapeutics.
2. Inhibition of metastatic dissemination of primary tumors formed by these cells occurs prior to formation of detectable micrometastastasis.
3. Differential panning of weakly and highly metastatic variants of MDA-MB-435 cells with phage display library of single chain variable fragment antibodies (scFv) revealed presence of unique antigenic determinants on the cell surface of highly metastatic cells.
4. Gel-filtration analysis of cell extracts revealed that NDPK oligomers exist mostly as dimers, trimers and tetramers with unequal distribution of NDPK A and NDPK B between different oligomeric forms.
5. The research supported by this grant generated the following publication and meeting abstracts:

Backer J. M., and Hamby, H. V. (1997) Genetic control of metastasis. *In*: Mackiewicz A, and Seghal PB (Eds.), Molecular Aspects of Cancer and its Therapy, Basel, Birkhauser Publishing LTD, (in press).

Backer, J. M., Mendola, C. E., Price, J., Thomson, J., Sperry, R. G., and Hamby, C. V. (1996) Opposite functions of nucleoside diphosphate kinases A and B in the expression of metastatic phenotype. The 9th International Conference of the International Society of Differentiation, p. 150.

Backer, J. M., Mendola, C. E., Price, J., Thomson, J., Sperry, R. G., Oiseth, S.,and Hamby, C. V. (1996) NME2 (nm23-2) gene may control the expression of metastatic phenotype. 88th Annual Meeting of American Association for Cancer Research, p. 202.

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Backer, J. M., Mendola, C. E., Kovesdi, I., Fairhurst, J. L., O'Hara, B., Eddy, Jr., R. L., Shows, T. B., Mathew, S., Murty, V. V. V. S., and Chaganti, R. S. K. (1993) Chromosomal localization and nucleoside diphosphate kinase activity of human metastasis suppressor genes NM23-1 and NM23-2. Oncogene, 8, 497-502.

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Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D. and Winter, G. (1994) Antibody fragments from `a single pot' phage display library as immunochemical reagent. EMBO J. 13, 692-698.

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Webb, P. A., Perisic, O., Mendola, C. E., Backer, J. M., and Williams, R. L. (1995) The crystall structure of a human nucleoside diphosphate kinase, NM23-H2. J. Mol. Biol., 251, 574-587.

Winter, G., Griffith, A.D., Hawkins, R.E. and Hoogenboom, H.R. (1994) Making antibodies by phage display technology. Annu. Rev. Immunol. 12, 433-435.

Table 1. Tumorigenicity and metastatic potential of MDA-MB-435 cells transfected with β-glucuronidase and variants of nm23 (Collaboration with Dr. J. Price, M. D. Anderson Cancer Center)

Cell Line	Tumor Take[a]	Time to Reach 1 cm[b]	Metastasis[c]
V	15/17	68 days	10/15 (66.6%)
A	5/7	122 days	2/5 (40%)
A/T	8/10	60 days	6/8 (75%)
B (data are being analyzed)			
B/T	7/9	120 days	1/7 (14%)

a. Each cell line was injected into the mammary fatpad of female nude mic, 10^6 cells in 0.1 ml. Tumor take is reported as the number of mice with progressively growing tumor/number of mice injected.

b Determined from growth curves of the tumors.

c Incidence of metastasis/number of mice with tumors.

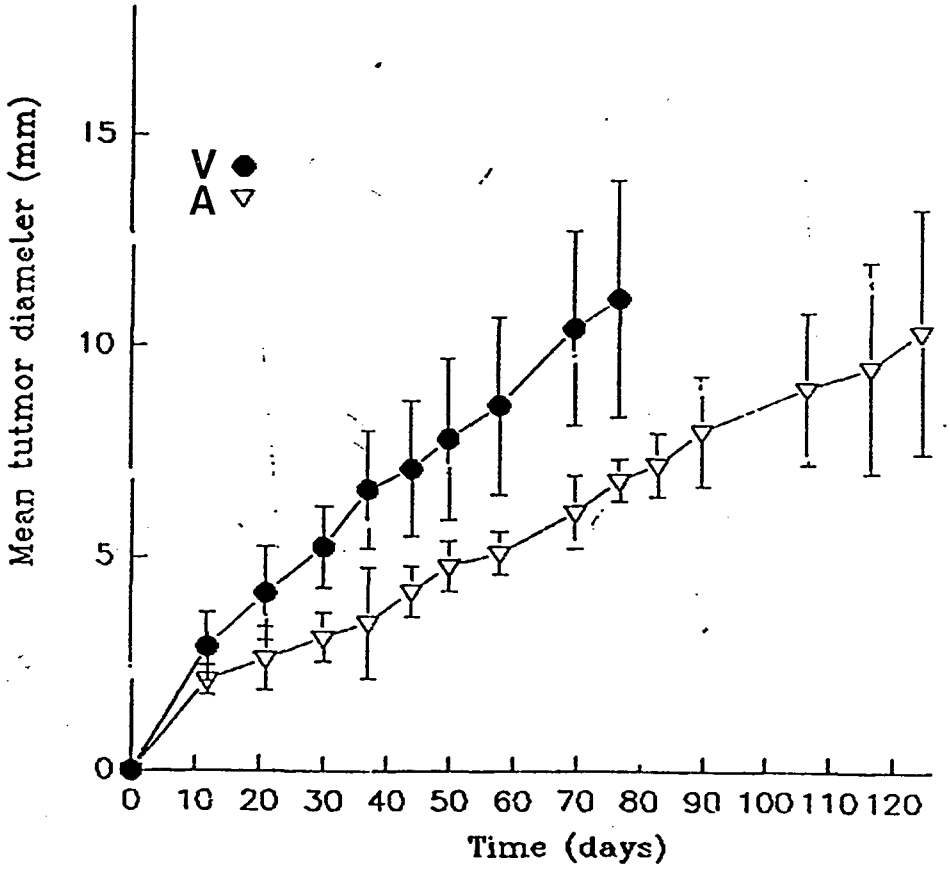


Figure 1. In vivo growth curves of MDA-MB-435 cells transfected with nm23-1 (A) or vector alone (V).

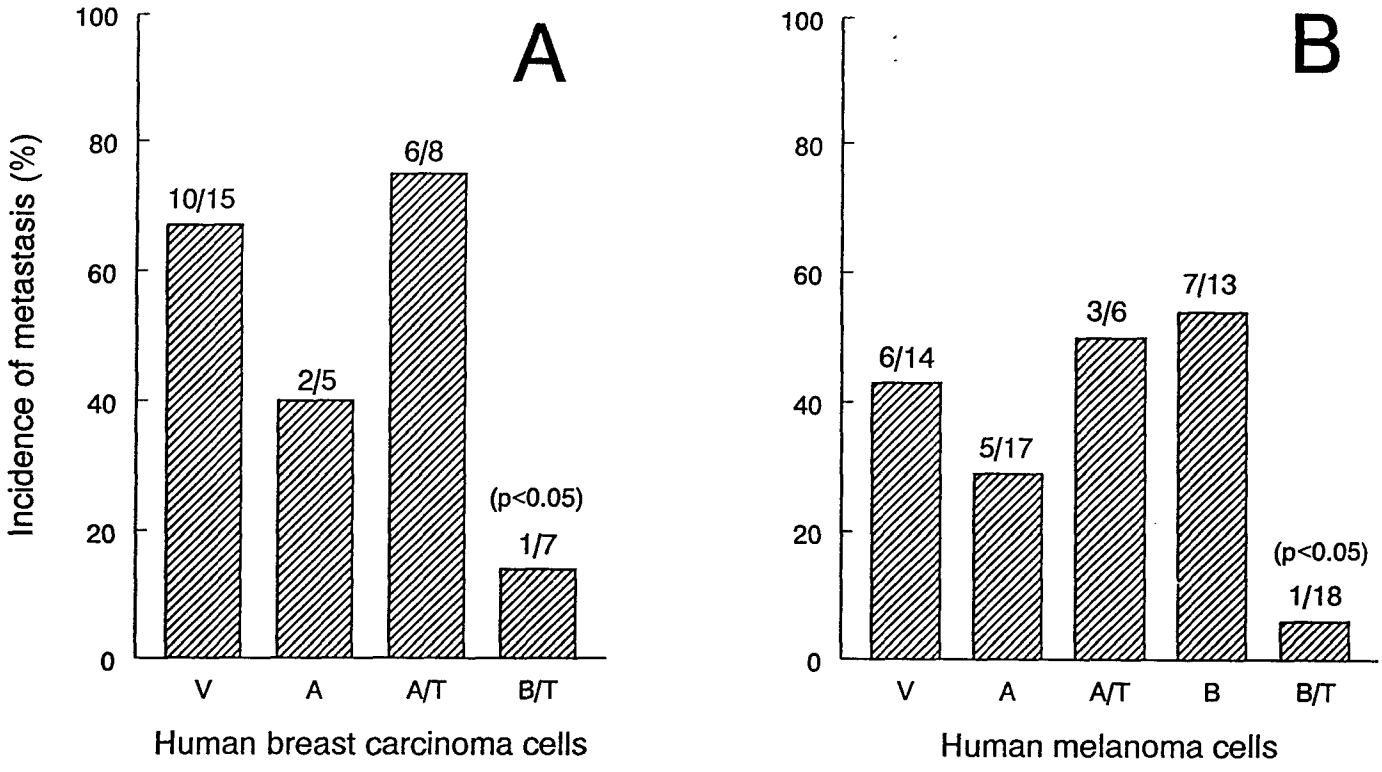


Figure 2. Reduction of the incidence of metastases in nude mice injected with NDPK B/T transfected human MDA-MB-435 breast carcinoma and human IV Cl 1 melanoma cells.

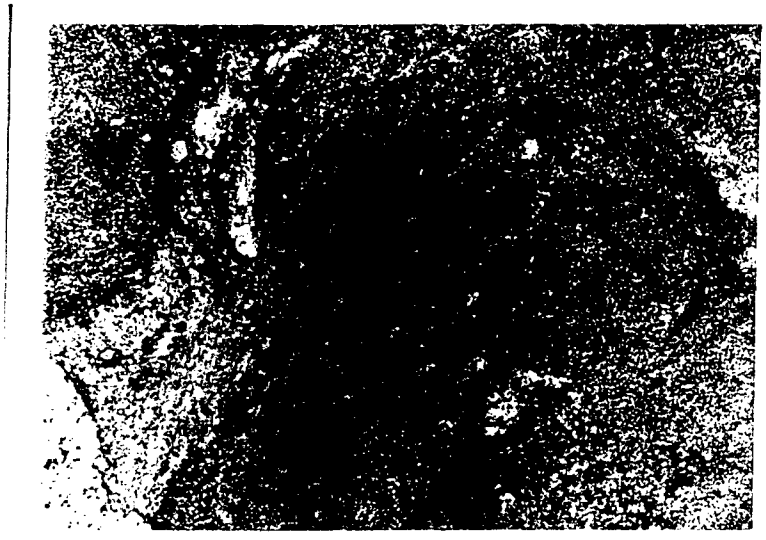


Fig. 3A. A representative section of a primary tumor formed by vector transfected MDA-MB-435 cells containing blue X-glu stained bacterial β-glucuronidase tagged vector transfected MDA-MB-435 human tumor cells adjacent to areas of H&E stained mouse tissue (see "Methods" for details)

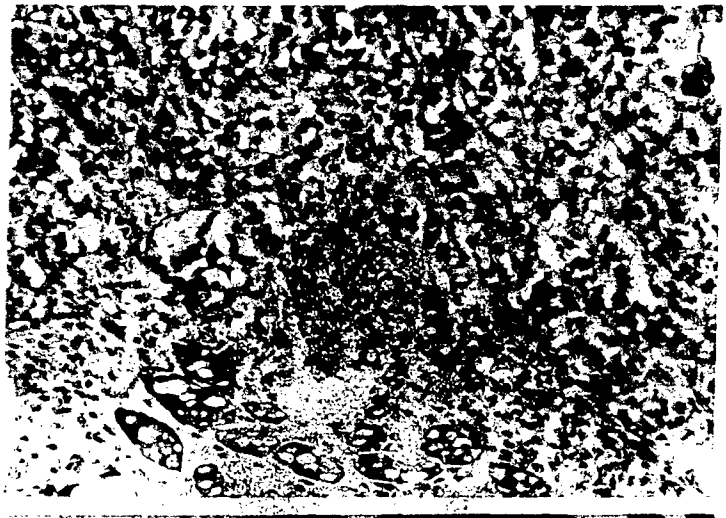


Fig. 3B A representative section of a macrometastatic lesion formed in mouse lung by cells from prime tumor shown on Fig. 3A.

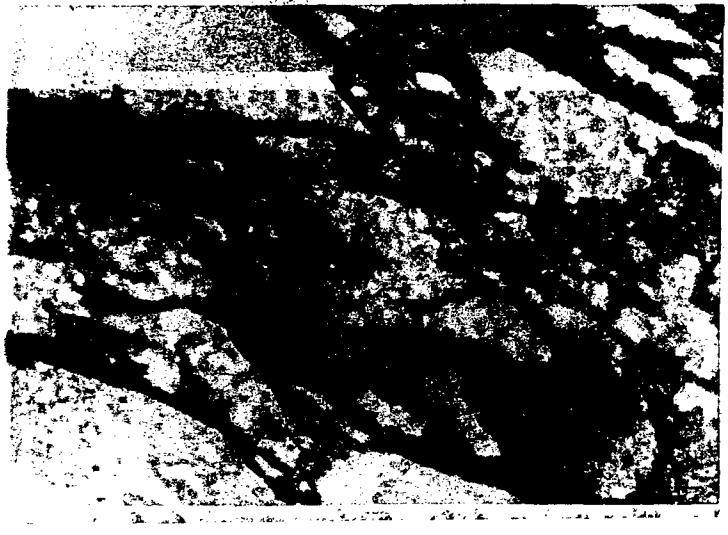
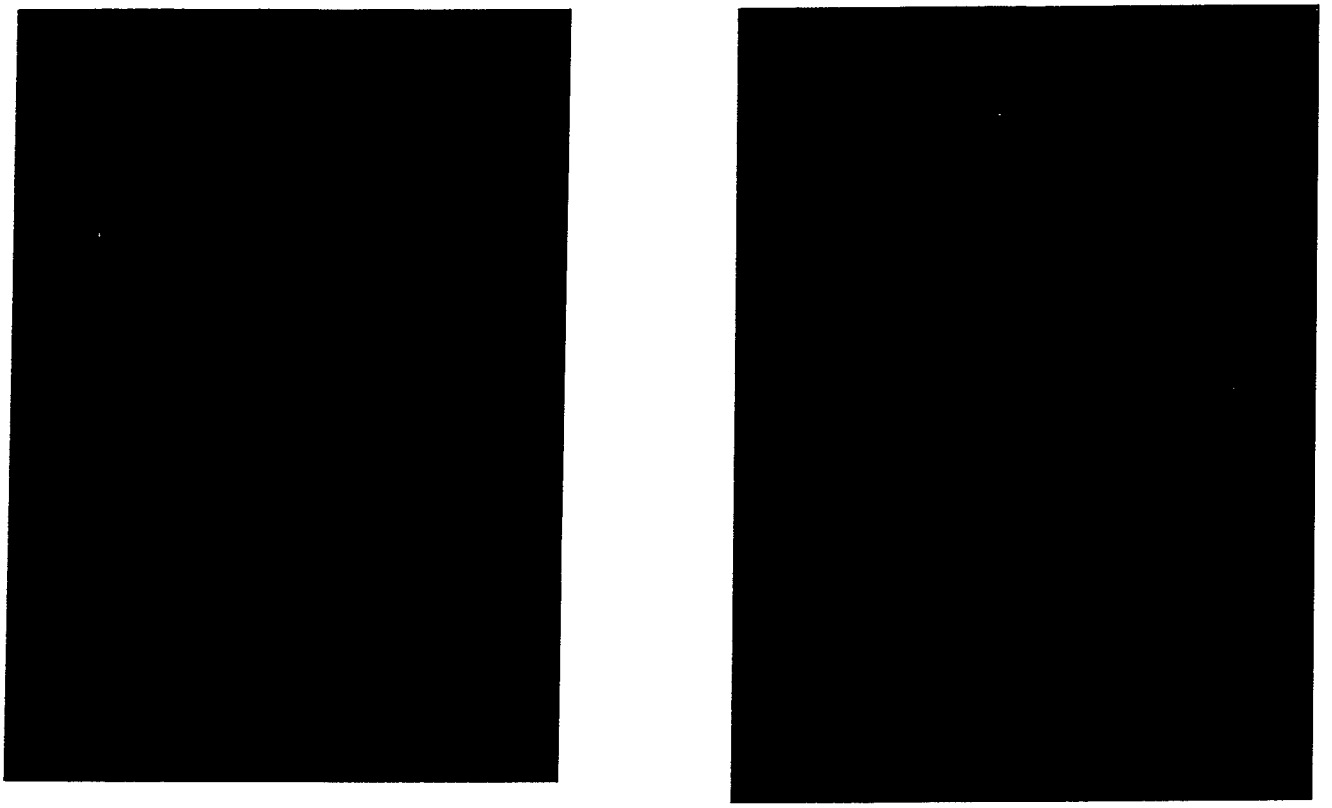


Fig. 3C. Blue stained human tumor cells are present as thrombi occluded in the capillaries of a section of macrometastasis-free lung recovered from an animal bearing primary tumor formed by bacterial β-glucuronidase tagged vector transfected MDA-MB-435 cells.

Figure 3. Detection of human breast carcinoma cells in mouse lung frozen sections. Lungs are harvested and stored in liquid nitrogen. Frozen lungs are cut on AO Histostat Microtome (Scientific Instrument) into 6 μm sections which are placed on poly-L-lysine coated slides (Baxter). Slides with frozen sections are incubated at 37^0C overnight in an oxidation catalyst buffer containing 0.1 M Na_2HPO_4, pH=7.0, 0.5 mM potassium ferrocyanide 0.5 mM potassium ferricyanide 10 mM EDTA and 1 mg/ml of 5-bromo-4-chloro-3-indol-1-glucuronide (X-glu). Slides are then rinsed 5 times with tap water and examined microscopically at a magnification of 200 for the presence of X-glu stained blue cells. **Hematoxylin and Eosin counter staining of mouse lung frozen sections.** Slides are fixed in 95 % alcohol 0.1 % acetic acid for 30 sec and then rinsed 5 times in deionized water. Rinsed slides are placed in Harris Conventional Hematoxylin Solution (Sigma) for 1 min and then rinsed 5 times with deionized water. Rinsed slides are then dipped 3 times in Scott's Tap Water Substitute, (Sigma) diluted 1:10 with deionized water and rinsed 5 times in deionized water. Slides are then dipped in 95 % alcohol for 30 sec and placed in Eosin Y, Alcoholic, (Sigma) for 1 min. Slides are then dipped sequentially in two bath with 95 % alcohol, 3 times in each bath, in three bath with 100% alcohol, 3 times in each bath, and in three bath with xylene, 5 times in each. Processed slides are mounted. Slides are then dipped 3 times in 100% alcohol 3 sequential times. Slides are then dipped 5 times in xylene 3 sequential times. Slides are then mounted and viewed.



A B

Figure 4. Chromosome 17 (visualized as red dots) is diploid in most human melanoma Line IV Cl 1 cells and aneuploid in most human breast carcinoma MDA-MD-435 cells.

The ploidy of chromosome 17 was determined in human Line IV Cl 1 melanoma cells (A) and human MDA-MD-435 breast carcinoma cells (B) by fluorescence in situ hybridization. Interphase nuclei were prepared from cell lines by conventional methods and aged 1-2 weeks to maintain morphology during denaturation. Slide treatment included washes in 2xSSC, 37°C, for 30', dehydration in 70-80-95% ethanol at room temperature, denaturation in 70% formamide/ 0.65xSSC, 72°C for 2 min, followed by dehydration in a 70-80-90-100% ethanol series at -20°C. The centromeric region of chromosome 17 was prepared as a probe as described (Weier et al., Human Genet., 87, 489-494, 1991). Briefly, a 17-alpha-satellite was selectively amplified from human monochromosomal hybrid DNA isolated from cell line GM 10498 by 2 rounds of PCR with 23-mer primers and during the second round of PCR the alpha-satellite was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, IN). The PCR product was precipitated and redissolved in hybridization solution (50% formamide, 2xSSC, 10% dextran sulfate). The probe was denatured at 78°C for 10 min and applied in 10 µl of hybridization mixture under a 22x24 mm coverslip. Slides were placed in a moist chamber for 16 h at 37°C and washed in decreasing concentrations of SSC at 45°C. The probe was detected by incubation with rhodamine conjugated anti-digoxigenin antibody (Boehringer Mannheim) at a final concentration of 1 µg/ml in 50 µl of blocking solution. Slides were washed 3 times in 4xSSC/0.1% Tween-20 at 45°C for 5 min and mounted in antifade solution (Vector Laboratories, CA) containing DAPI. Slides were viewed under a Zeiss Axiophot microscope equipped with filters to detect DAPI-stained nuclei and rhodamine-stained chromosomes separately as well as with a dual band pass filter set to detect signals simultaneously. Images were collected and merged using a cooled CCD camera and IP Lab Spectrum software.

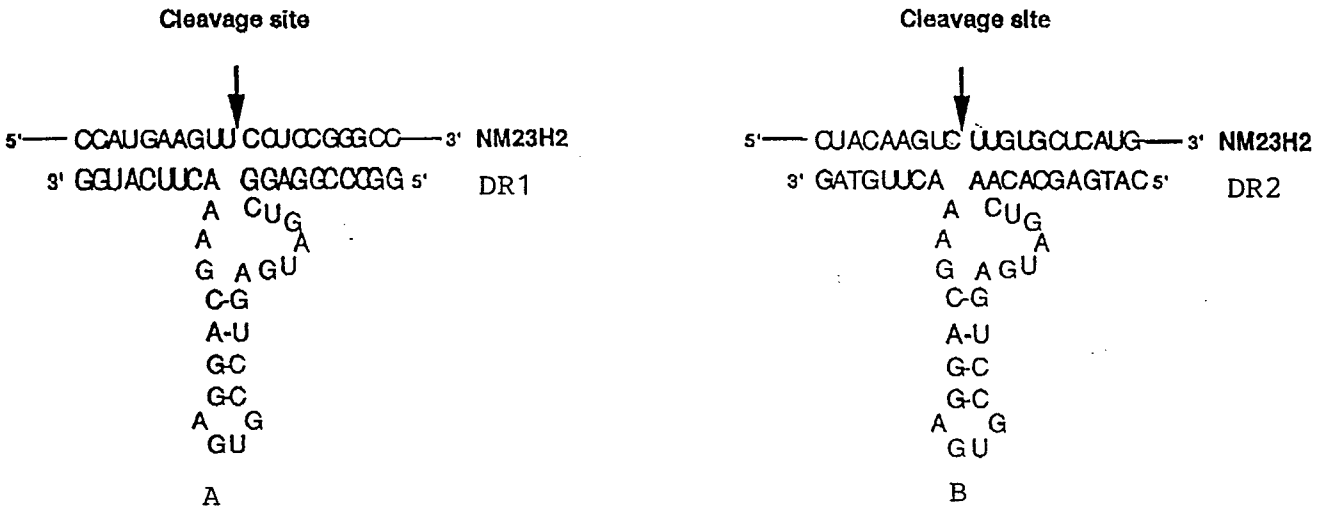
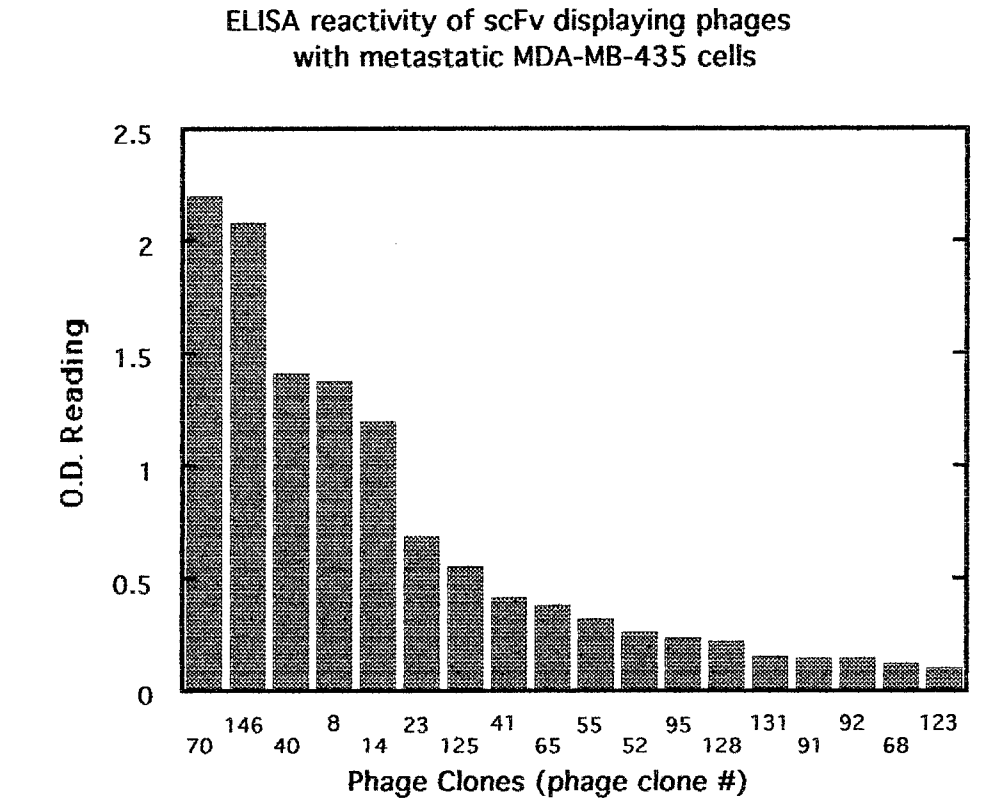


Figure 5. Nucleotide sequences of anti-nm23-H2 ribozymes annealed to target regions in nm23-H2 mRNA. Ribozymes were designed to cleave nm23-H2 mRNA after the GUU triplet in diversity region 1 (A, Ribozyme DR1), bases 189-191, and after the GUC triplet in diversity region 2 (B, Ribozyme DR2), bases 501-503. Gene Bank accession number X58965.

Figure 6.


ELISA reactivity of scFv displaying phages
with metastatic MDA-MB-435 cells
O.D. Reading
2.5
2
1.5
1
0.5
0
70
146
40
8
14
23
125
41
65
55
52
95
128
131
91
92
68
123
Phage Clones (phage clone #)

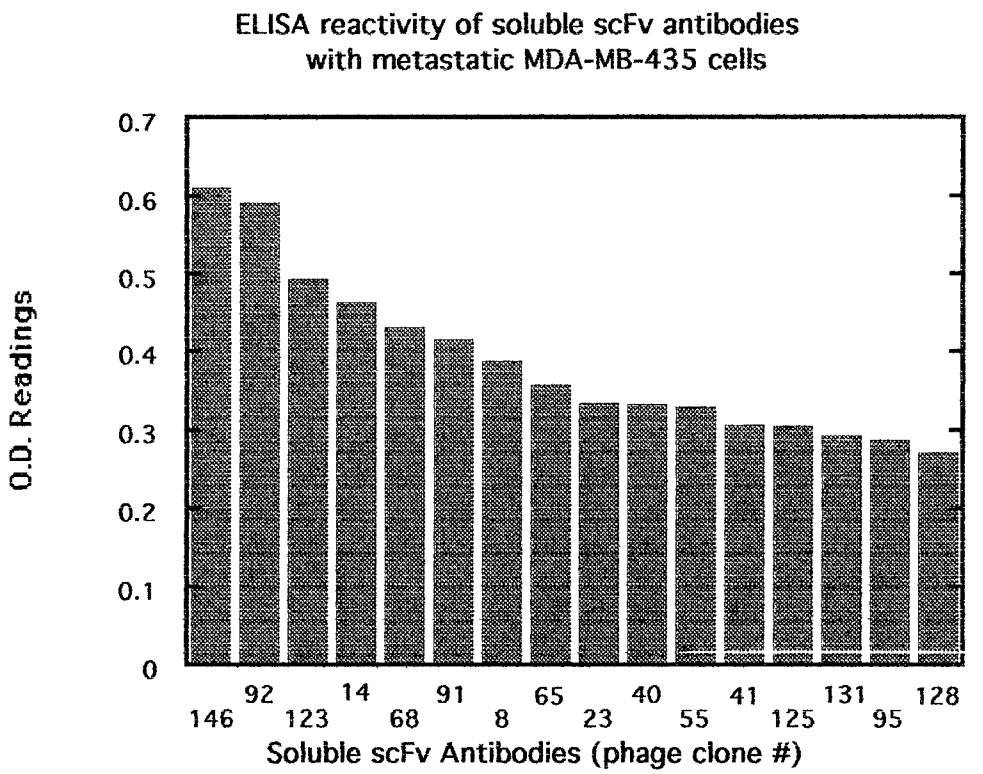

ELISA reactivity of soluble scFv antibodies
with metastatic MDA-MB-435 cells
O.D. Readings
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
146
92
123
14
68
91
8
65
23
40
55
41
125
131
95
128
Soluble scFv Antibodies (phage clone #)

Figure 6. Specific antigens are recognized on highly metastatic MDA-MB-435 human breast carcinoma cells by scFv expressed on the surface of filamentous phages.

The combinatorial scFv library (a gift from Dr. G. Winters, MRC Centre for Protein Engineering, Cambridge, UK) is described by Nissim et al. (Nissim, et al., 1994). This library utilizes 50 human germline V_H segments assembled *in vitro* with random synthetic 4-12 residue long CDR3. The V_H segments were cloned into phagemid vector pHEN1 carrying a human anti-BSA Vλ3 light chain to generate a repertoire of $>10^8$ clones expressing different scFv fragments. The scFv fragments are expressed with a c-myc tag recognized by anti-c-myc mAb9E10 either on a phage surface, or in soluble form when overproduced after induction of expression with IPTG. Library is amplified and phage particles according to Nissim, et al. (1994).

Cell panning with combinatorial antibody library. Highly metastatic and weakly metastatic MDA-MB-435 cells were grown in 6 well plates and fixed with 0.25% gluteraldehyde. To enrich phage library with scFv that recognized highly metastatic cells the phage library was panned over highly metastatic cells, unbound phages were removed, and bound phages were eluted and reamplified. After fourth round of panning, bound phages were eluted and panned over weakly metastatic cells. Unbound phages were collected and panned again over highly metastatic cells. Phages bound to highly metastatic cells were eluted and amplified. After three round of depletion 185 individual phages that were able to bind to highly metastatic cells and did not bind to weakly metastatic cells were independently amplified. Selected phages were tested in ELISA against highly metastatic and weakly metastatic cells using antiphage antibody. None of the selected phages reacted with weakly metastatic cells. The distribution of ELISA plate readings for phages reacted with highly metastatic cells is shown in Figure 6A and distribution of ELISA plate readings for soluble scFv produced by these clones in shown in Figure 6B. It is interesting that there is no strong correlation between reactivities of scFV antibody expressed on the phage surface or in solution

Figure 7. Sequence alignment of human NDPK A and B. Elements of secondary structure in the NDPK B are indicated. Sequences of peptides 1-5 are indicated.

```
NDPK A          C                                                GL    MQ    DL   E
NDPK B          MANLERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKF LRASEEHLKQ
                      β1                  α1                  β2
                                  peptide 1 EIIKR----PGGGP--AMKFL

NDPK A          V          A          H
NDPK B          HYIDLKDRPF FPGLVKYMNS GPVVAMVWEG LNVVKTGRVM LGETNPA
                           α2                  β3                  α3       [ -kpn--

NDPK A                                          E        G    H          T     QN  I
NDPKB DSKPGTIRGDFCI QVGRNIIHGS DSVKSAEKEI SLWFKPEELV DYKSCAHDWVYE
      ---------- α4------- loop]      β4              α5
peptide 2 DSKPG                                peptide 4    KPEELVDY
pep. 3 CGDSKPGGC                                peptide 5          YKSCAHDWVYE
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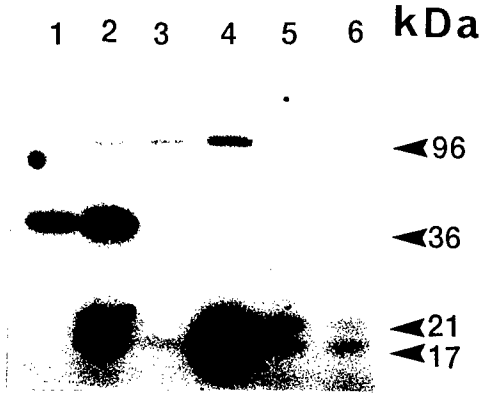

Figure 8. Charge fractionation of NDPK isoforms in MDA-MB-435 breast carcinoma cell lysates.

Cultured MDA-MB-435 breast carcinoma cells were lysed in PBS containing 1% NP-40. Lysates were clarified by centrifugation at 10,000 x g, dialyzed overnight at 4°C against 10 mM Tris-HCl, 1 mM Na Azide, pH 8.0 and clarified again by centrifugation at 10,000 x g for 10 min. Q-Sepharose beads were washed 3 times in dialysis buffer, pelleted and mixed with a volume of cell lysate sufficient to make a 25% (v/v) suspension of beads. The mixture was incubated for 1 hr at 4°C, spun at 2,000 x g and the supernatant was recovered. Unfractionated cell lysates, supernatants from Q-Sepharose treated lysates and Q-Sepharose bound fractions were assayed for NDPK autophosphorylation as follows. Aliqouts containing 10 million cell equivalents were incubated with 25 μCi of γ-[^{32}P]-ATP in kinase buffer (10 mM Tris-HCl, 1 mM Na Azide, 50 μM $MgCl_2$, 5 μM cold ATP, pH 8.0) for 30 sec. Kinase reactions were stopped by adding 0.25 volumes of 4X SDS sample loading buffer (8% SDS, 0.25M Tris-HCl, 40% glycerol, pH 6.8) and proteins were resolved under reducing conditions with 5% 2-mercaptoethanol by SDS PAGE through 15% acrylamide gels. Immediately following SDS PAGE the gels were wrapped in plastic and exposed to X-ray film (Kodak XRP) to detect phosphorylated proteins. NDPK A and B appear as 21 kDa and 17 kDa bands (arrows), respectively, of approximately equal intensity in unfractionated cell lysates (lane 2). The identities of the NDPK bands were confirmed by autophosphorylating proteins immunoprecipitated from unfractionated cell lysates with a monoclonal antibody recognizing both NDPK A and B (lane 6). The high molecular weight bands represent different phosphoproteins since they remain phosphorylated after boiling (lane 1) while NDPK proteins whose phosphorylation is thermolabile were dephosphorylated. Positively charged NDPK B isoforms that do not bind Q-Sepharose are enriched in lysates treated with Q-Sepharose beads (lane 4). NDPK A and B proteins in Q-Sepharose treated lysates are dephosphorylated by boiling (lane3). NDPK A and B isoforms bound to Q-Sepharose beads show kinase activity of nearly equal intensity (lane 5). This result suggests that the Q-Sepharose bound complexes represent mixed multimers of NDPK A and B since NDPK B does not bind Q-Sepharose under these experimental conditions.

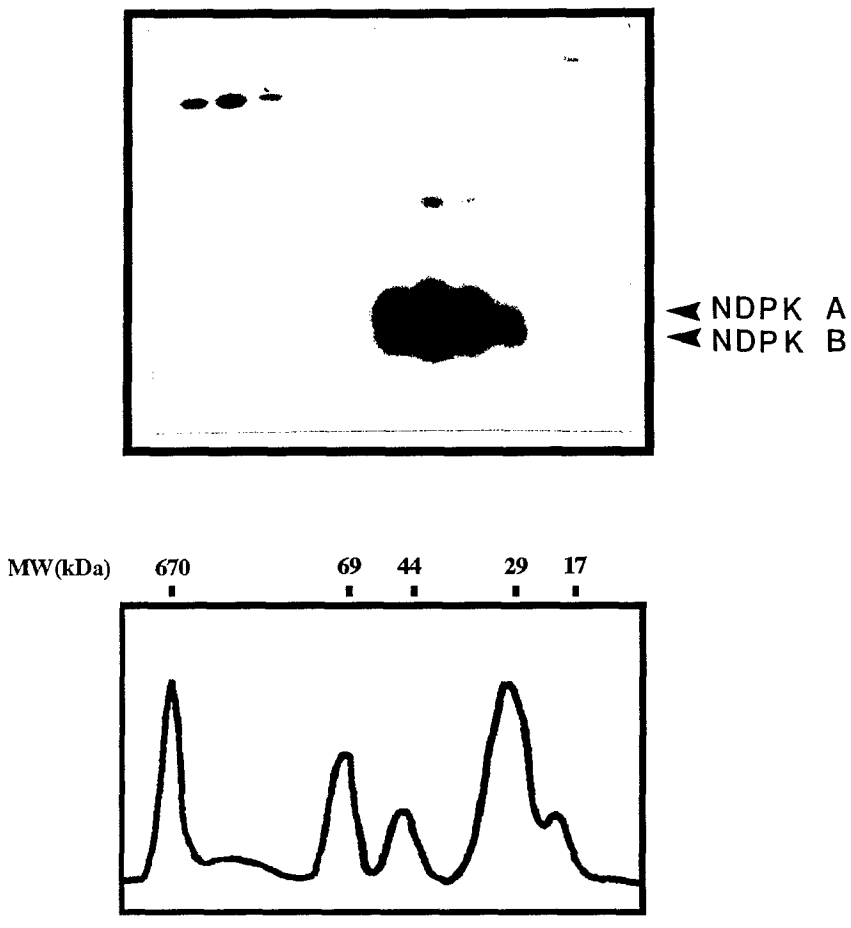

Figure 9. NDPK A and B isoforms exist in tetrameric, trimeric and dimeric complexes in MDA-MB-435 breast carcinoma cells.

MDA-MB-435 cells were lysed in Dulbecco's PBS (Life Technologies, Gaithersburg, MD) containing 1% NP-40. Lysates were clarified by centrifugation at 10,000 x g and dialyzed overnight at 4°C against 10 mM Tris-HCl, pH 8.0 containing 1 mM Na Azide. Lysates were concentrated in dialysis bags (3,500 kDa molecular cut-off) with polyethylene glycol (average M.W. 8,000) and cleared by centrifugation at 10,000 x g. Cell lysates containing approximately 3 x 10^7 cell equivalents in a volume of 20 µl were resolved by HPLC with Dulbecco's PBS containing 0.1% NP-40 at a flow rate of 0.5ml/min on an Ultropac TSK G3000SW (LKB, Bromma, Sweden), 7.5 x 600 mm, size exclusion column with a molecular cut-off of 600 kDa. After the void volume of buffer was eluted, a total of 12, 1 ml fractions were collected. These fractions covered a size range from 670 kDa to approximately 10 kDa (lower panel) based on calibration of the column with purified protein standards. Autophosphorylation assays were performed on each fraction as follows. A 40 µl aliquot from each fraction was mixed with 10 µl of 5X reaction buffer (250 µM $MgCl_2$) and reacted for 1 min with 2 µCi of γ-[^{32}P]-ATP. Reactions were stopped by addition of 4X SDS sample buffer containing 2-ME and samples were immediately loaded on 15% polyacrylamide gels. Following SDS PAGE, gels were exposed to X-ray film (XRP, Kodak, Rochester NY) at -80°C. Bands corresponding to NDPK A and B (arrows) were present in fractions covering the size range from 69-29 kDa. Tetrameric (~68 kDa), trimeric (~51 kDa) and dimeric (~34 kDa) NDPK complexes would elute within this size range.