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14. ABSTRACT The purpose is to identify the potential role of a novel protein NIBP in regulating the tumorigenesis of breast cancer. The scope covers human breast tissue, cancer cell lines and conditional knockout mice. During the first year of the funding period, we have profiled the expression pattern of NIBP at both mRNA and protein levels in various types and stages of breast cancer by qPCR and TMA immunostaining, showing strong correlation of NIBP overexpression with the progression, metastasis and prognosis of breast cancer. We have optimized ELISA for determination of NIBP in patient serum. We determined the important role of NIBP in breast cancer cell lines and initiated the xenograft animal model. Using various deletion and site-directed mutants of NIBP, NIK and IKK2, we identified the interacting domains among them and characterized their structural-functional correlations. We cloned and characterized lentiviral constitutive and Tet-On inducible NIBP expression vectors in HEK293T cells but failed to obtain high efficiency of lentivirus packaging that limited the application to breast cancer cell line and xenograft studies. We obtained two lines of NIBP floxed mouse but failed to induce efficient knockout of NIBP in cell-specific manner. A third line of NIBP floxed mouse is being developed.					
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Introduction:

Breast cancer continues to be a major cause of cancer death in women. For the development of reliable biomarkers and efficient therapeutics, it is important to identify the signaling pathways and molecular mechanisms for the development and progression of breast cancer. We identified a novel protein NIBP (for **N**IK and **I**KK2 **b**inding **p**rotein)(Hu et al., 2005). NIBP forms a unique complex with IKK2 and NIK and enhances NF κ B activation in breast cancer cells. The purpose of this study is to test a central hypothesis that a high level of NIBP expression in breast cancer cells may retain the basal activation of IKK2 and subsequent NF κ B, and contribute to the proliferation, tumor-formation and drug-resistance of breast cancer cells. The long term goal is to understand the molecular mechanisms for NIBP/NF κ B signaling in cancer cells that play a pivotal role in promoting tumor development and invasion. The experimental studies are designed to profile the expression pattern of NIBP in patients with various types and stages of breast cancer so as to establish NIBP as a novel biomarker to guide preventive interventions and improve survival and quality of life for breast cancer patients. Then, we will use various cell lines, athymic nude mice and mammary cell-specific NIBP knockout mice to investigate the key role of NIBP in regulating or mediating the tumor formation and metastatic invasion of breast cancer cells. We anticipate that overexpression of NIBP may promote the proliferation and survival of the cancer cells, and subsequently protect the cells from death leading to the drug-resistance. However, stable knockdown of NIBP may increase the vulnerability of cancer cells to chemotherapy and thereby reduce the drug-resistance. Mammary epithelial cell-specific knockout of endogenous NIBP may inhibit the incidence or progression of mammary tumor formation induced by chemical carcinogen or cancer genes. These studies will demonstrate that NIBP is essential for the growth, proliferation, migration and invasion of breast cancer cells. Therefore, intervention of NIBP function would be a potential target for drug development and clinical application.

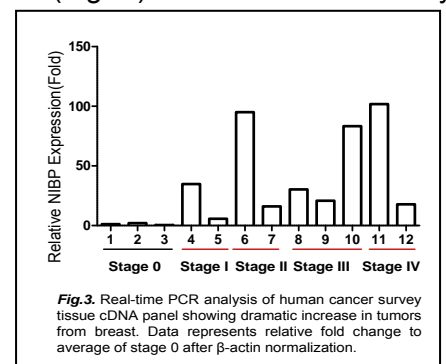
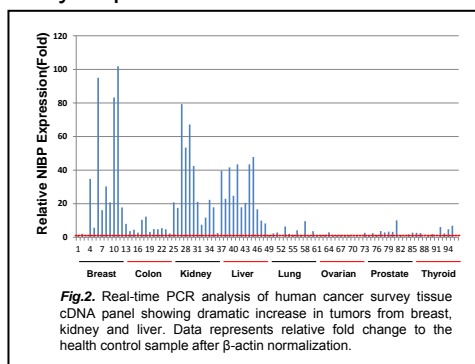
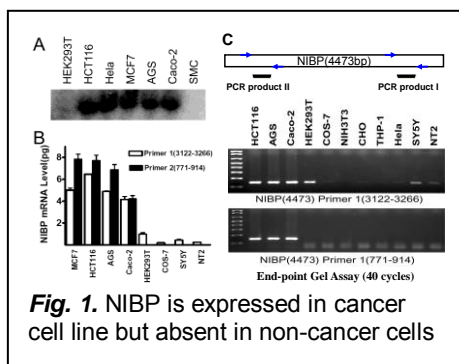
Body:

This funding covers two years' period starting May 1, 2011. During the first year, we have accomplished Aim I, partially Aim II and Aim III. We also initiated preliminary experiments that are essential for further accomplishment of all the proposed tasks.

Task 1. Characterize the expression pattern of NIBP in breast cancer.

1a. High expression of NIBP mRNA in cancer cell lines and tumor tissues.

Northern blot and quantitative RT-PCR analysis demonstrated that NIBP mRNA is highly expressed in selected cancer cell lines but not or weakly expressed in non-cancer cell lines (Fig. 1). Human cancer survey



tissue-scan quantitative RT-PCR analysis demonstrated extensive expression of NIBP mRNA in tumor tissues from several organs with the highest increase in tumors from breast, kidney and liver (Fig. 2). The expression level of NIBP mRNA is closely correlated with the invasive stages of breast cancer (Fig. 3).

1b. High expression of NIBP protein in human breast cancer tissues.

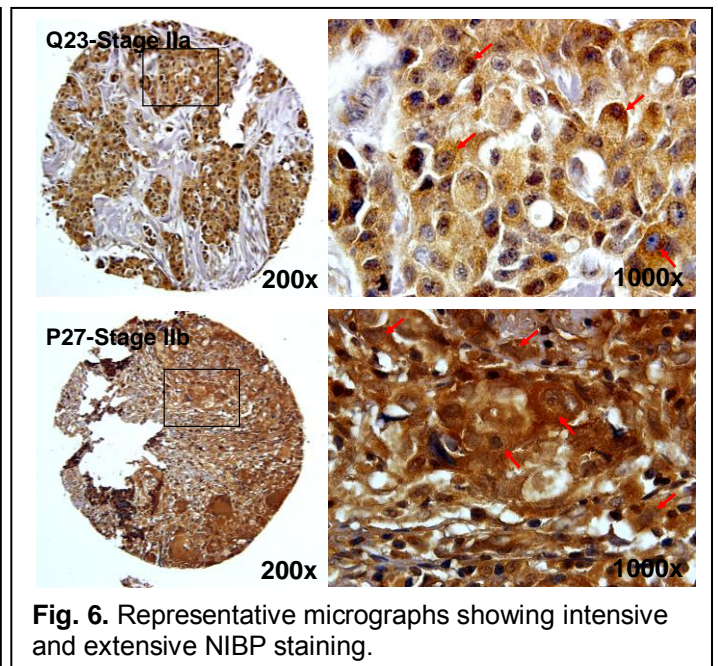
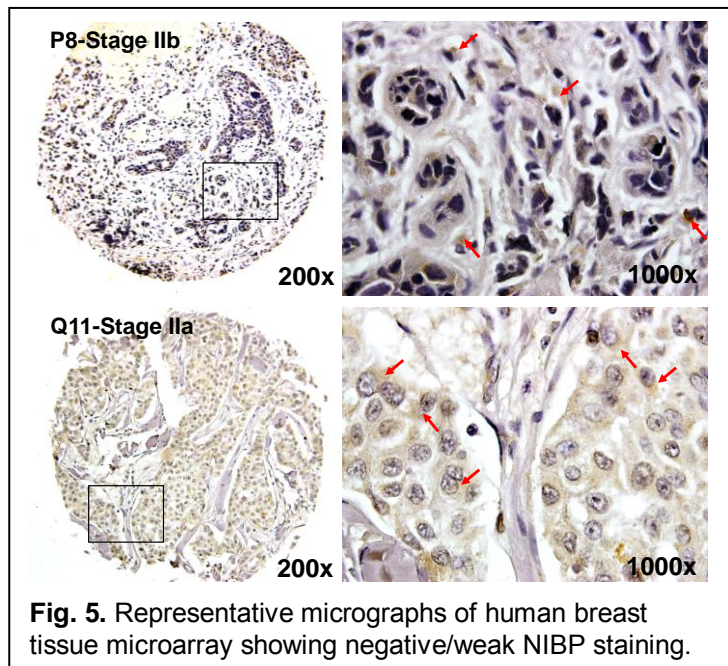
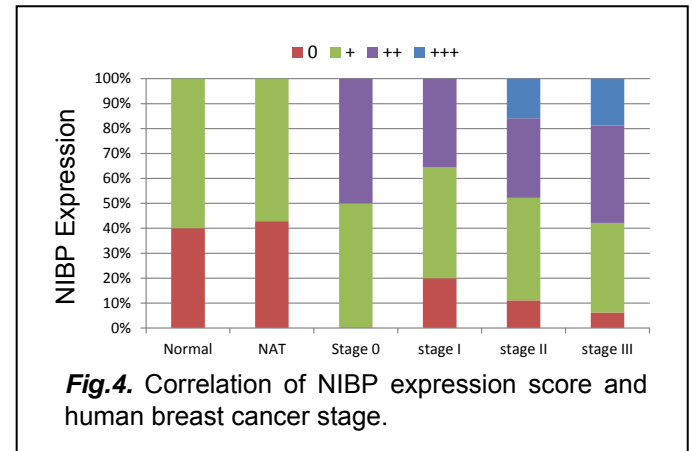
To obtain the specific staining for NIBP expression in human breast cancer tissues and cell lines, as well as to optimize NIBP ELISA, we generated several NIBP antibodies using synthesized peptides against different regions and recombinant GST-NIBP fusion proteins. These antibodies were named as NIBP(417), NIBP(401B), NIBP(ND-56), NIBP(ND-161), PTG-A, PTG-B, PTG-C and PTG-D. We also purchased NIBP

antibodies from Santa Cruz (NIBP-N14 and NIBP-D19) and Sigma (HPA025225). Most antibodies did not work for immunohistochemistry or Western blot. Our NIBP(417) antibody works best for both immunohistochemistry and Western blot. Thus, we used NIBP(417) antibody to analyze the expression pattern of NIBP immunoreactivity in high-density breast cancer tissue array, with stage, grade and normal breast tissue, carrying 322 cases with 616 cores (BR6161 from US Biomax). Immunohistochemical staining was quantified independently and in a blinded manner by two observers using a weighted histological score. In normal breast tissues (9 cases) or cancer adjacent normal breast tissues (21 cases), NIBP-like immunoreactivity was absent or weakly stained (Fig. 4, 5 and Table 1). Of 294 patients, 262 (89.1%) were histologically diagnosed as invasive ductal carcinoma, 21 (7.1%) have invasive lobular carcinoma and 11 (3.8%) suffered other types of breast cancer. The expression level of NIBP immunoreactivity is significantly increased as the breast cancer progresses in both ductal and lobular breast cancers (Fig. 4-6, Table 1). This is consistent with the result from real-time RT-PCR microarray (Fig. 3). Invasion is fundamental for tumor malignance. These studies indicate that NIBP expression could discriminate the benign from malignant diseases and distinguish the early stage from late stage of breast cancer. We are currently requesting the approval from NIH/NCI cancer diagnosis program to obtain the progression TMA contains 679 cases of breast cancer and the prognostic TMA collects 600 Stage I, 400 Stage II, and 200 Stage III breast tissue specimens. More detailed analysis of NIBP expression in these cohort TMAs as well as ELISA screening of patient serum (see below) will identify NIBP as a novel biomarker for the diagnosis, prognosis and treatment of breast cancer.

Table 1. NIBP expression is significantly correlated with late stage and grade

Characteristics	Cases	NIBP(417) immunoreactivity				
		0	+	++	+++	Missing
Normal	9	4	5			
NAT	21	16	5			
Grade						
G1	14	0	6(42.9%)	3(21.4%)	4(28.6)	1(7.1%)
G2	172	18(10.5%)	61(35.5%)	61(35.5%)	22(12.8%)	10(5.8%)
G3	48	7(14.6%)	14(29.2%)	12(25%)	8(16.7%)	7(14.6%)
Gx	60	3(5%)	18(30%)	6(10%)	2(3.3%)	31(51.7%)
AJCC Staging						
0	2	0	1(50%)	1(50%)	0	0
I	28	5(17.9%)	10(35.7%)	8(28.6%)	0(0%)	5(17.9%)
II	221	21(9.5%)	78(35.3%)	60(27.1%)	30(13.6%)	32(14.5%)
III	42	2(4.8%)	11(26.2%)	13(31%)	6(14.3%)	10(23.8%)

Note: Missing indicates no tissues or no tumors in the core. NAT: Normal adjacent breast tissue



1c. ELISA setup.

As shown above, NIBP expression is hugely increased in most of invasive breast cancer. Based on our preliminary observation that NIBP is secreted from mucosa into the lumen of the gut after colitis and colorectal

cancer, we predicted the appearance of NIBP in the blood of patients with invasive breast cancer, which might be used as a novel biomarker for diagnosis and prognosis of breast cancer. To this end, we have tested several NIBP antibodies for the optimization of NIBP ELISA. We first used direct ELISA by coating various concentration of recombinant NIBP protein (full-length, 1139aa) to screen the efficiency of various NIBP antibodies. As shown in Table 2, all the tested antibodies efficiently detected the coated antigen NIBP(1139) in a dose-dependent manner. We then tested various combinations of these antibodies for sandwich ELISA. Since our home-made NIBP(417) antibody was well characterized for immunohistochemistry (as above), immunoprecipitation, and Western blot (Hu et al., 2005), we further characterized the Sandwich ELISA using NIBP(ND161) chicken antibody as capture antibody and NIBP(417) rabbit antibody as detection antibody. The final protocol for Sandwich NIBP ELISA was summarized as: chicken NIBP(ND161) antibody coating (1:100) → Sample or Standard → rabbit NIBP(417) antibody (1:500) → anti-rabbit HRP-linked secondary antibody (1:400) → TMB color development. This protocol has been validated by the efficient detection of NIBP expression in brain lysate and the NIBP-shRNA knockdown in breast cancer cell line MDA-MB231 (Fig. 6). Preliminary trial using this protocol did not find the presence of NIBP in human serum from normal subject and Alzheimer's disease. We are currently collecting serum samples from breast cancer patients.

Task 2. Determine the role of NIBP in breast cancer cell lines.

2a Stable knockdown of NIBP inhibits the proliferation and colony formation of breast cancer cell lines.

Using lentivirus-mediated shRNA knockdown system, we established stable NIBP-knockdown breast cancer cell lines. Cell sorting using the internal EGFP marker was performed to ensure highly pure NIBP knockdown cell lines. Control empty vector and insufficient NIBP-shRNA were used as negative controls. The efficient knockdown of NIBP expression in the stable cell lines was validated by Western blot and Real-time RT-PCR. The efficient blockade of NFκB signaling was validated too (see task 5).

The proliferation and viability of the stable cell line were examined using BrdU incorporation assay and CellTiter-Glo(R) luminescent cell viability assay (Promega). The colony formation of the cells was determined by counting the number of colonies after plating equal number of cells and culturing for 2-3 weeks. As shown in Fig. 7, NIBP stable knockdown significantly inhibited cell proliferation under normal culture conditions (10% FBS). Colony formation was also significantly reduced in NIBP knockdown cells (Fig. 8).

Table 2. NIBP direct ELISA for antibody screening

	NIBP(417)	NIBP(401B)	PTG-B	NIBP-N14	NIBP-D19	NIBP(ND161)
	1:1k	1:1k	1:0.2k	1:0.2k	1:0.2k	1:0.2k
Blocking Buff	0.068	0.058	0.058	0.044	0.041	0.094
	0.072	0.055	0.059	0.047	0.043	0.098
1139-0.8ng/ml	0.22	0.102	0.48	0.049	0.05	0.193
	0.22	0.077	0.468	0.051	0.056	0.184
1139-8ng/ml	0.665	0.275	1.567	0.065	0.072	0.458
	0.703	0.32	1.675	0.065	0.09	0.453
1139-80ng/ml	2.439	1.967	3.037	0.097	0.202	1.996
	2.359	2.308	3.04	0.103	0.194	1.913
Fold Changes						
Blocking Buff	0.971	1.027	0.991	0.967	0.976	0.979
	1.029	0.973	1.009	1.033	1.024	1.021
1139-0.8ng/ml	3.143	1.805	8.205	1.077	1.190	2.010
	3.143	1.363	8.000	1.121	1.333	1.917
1139-8ng/ml	9.500	4.867	26.786	1.429	1.714	4.771
	10.043	5.664	28.632	1.429	2.143	4.719
1139-80ng/ml	34.843	34.814	51.915	2.132	4.810	20.792
	33.700	40.850	51.966	2.264	4.619	19.927

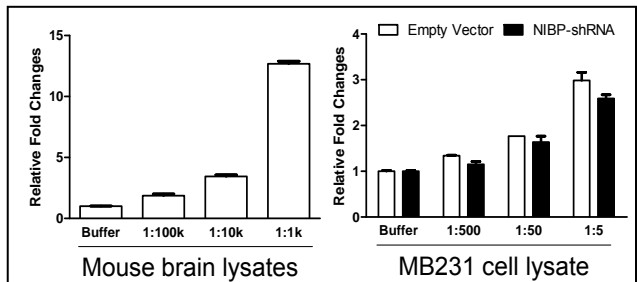


Fig. 6. Sandwich ELISA validated NIBP expression in mouse brain and NIBP knockdown by lentiviral NIBP-shRNA in breast cancer cell line.

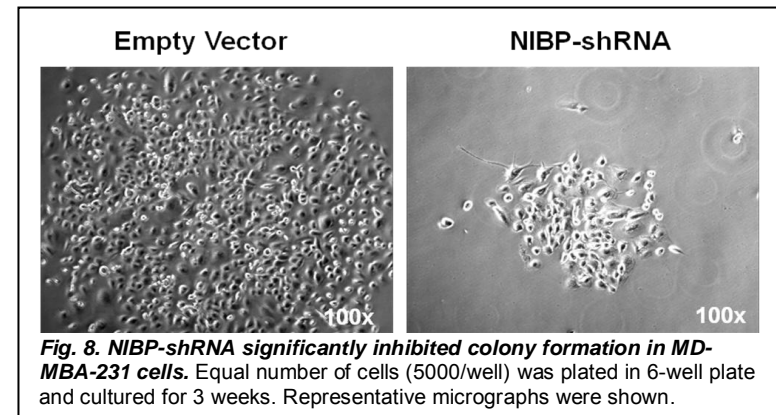


Fig. 8. NIBP-shRNA significantly inhibited colony formation in MD-MBA-231 cells. Equal number of cells (5000/well) was plated in 6-well plate and cultured for 3 weeks. Representative micrographs were shown.

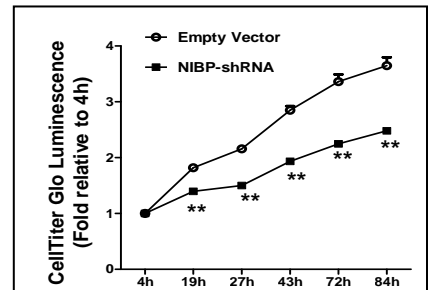
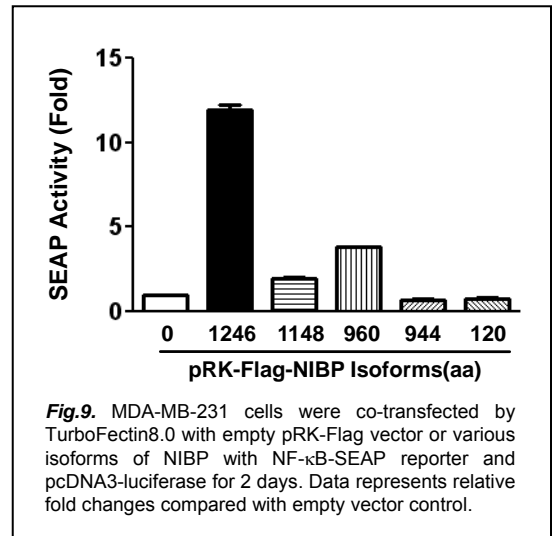


Fig. 7. NIBP-shRNA significantly inhibited cell proliferation in MD-MBA-231 cells. Equal number of cells (5000/well) was plated in 96-well plate and cultured for indicated time for Cell-titer assay. **p<0.01 indicates significant reduction compared with corresponding empty vector control time points by Student's t test.

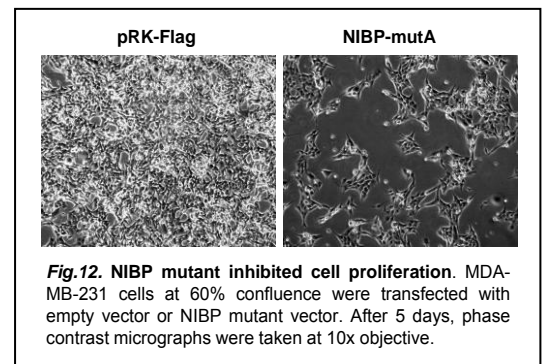
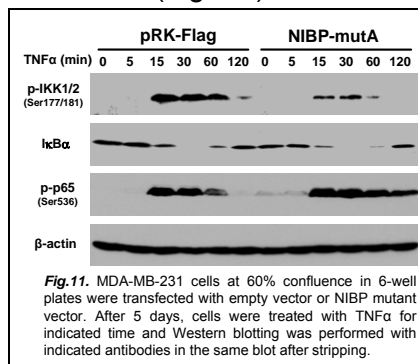
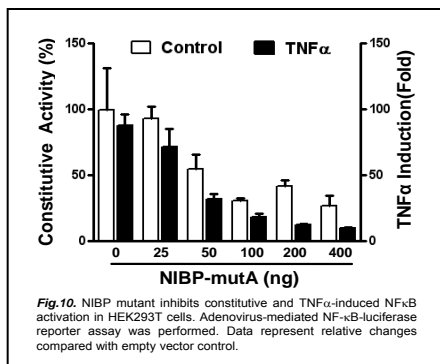
2b. Lentiviral overexpression of NIBP full-length in breast cancer cells.

By regular transfection, we found that the full-length of human NIBP (1246) dramatically increased NF κ B activation in MDA-MB-231 cells. However, the low transfection efficiency led to the difficulties in identifying the phenotypic difference between NIBP overexpression and the control cells. To increase the gene delivery efficiency, we proposed to use pLVX-Tet-On advanced lentiviral expression system (Clontech). We have cloned human NIBP(1246) into pLVX-Tight-Puro vector and validated the cloned sequence and the functional expression in HEK293T cells via regular transfection. Unfortunately, we were unable to package the pLVX-NIBP(1246) transfer vector into lentivirus, perhaps due to the big size of insert or unknown NIBP-related events. Then we purchased pReceptor-LV-NIBP-EGFP vector from Genecopoeia and encountered the same problem for lentivirus packaging. Currently, we are using AdMax-Hi-Q system (Microbix Biosystems) to overexpress NIBP full-length in breast cancer cell lines.



2c. The effects of NIBP mutants on the proliferation of breast cancer cell lines.

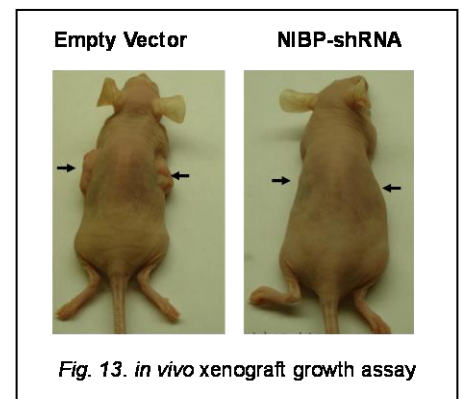
To identify the functional domain within NIBP and IKK2/NIK, we have prepared various deletion mutants and site-directed mutants (see task 5). One of the NIBP mutants inhibited constitutive and TNF α -induced NF κ B activation in HEK293T cells (Fig.10) and MDA-MB-231 cells (Fig.11). This mutant inhibited the proliferation of MDA-MB-231 breast cancer cell (Fig. 12).



Taken together, our studies identified the important role of NIBP in promoting cell proliferation and colony formation of breast cancer cells in vitro.

Task 3. Identify the biological role of NIBP in tumorigenic cell in nude mice.

After obtaining IACUC approval, we performed preliminary experiments to test the tumorigenic properties of MDA-MB231 cells in Nu/Nu nude mice. As shown in Fig 13. the cells can form tumors under skin. Then we performed formal experiments using lentivirus-infected breast cancer cell line being injected into the mammary fat pads of nude mice. Empty and NIBP-insufficient vectors were used as control. After 2-3 months, we failed to observe xenograft formation. Currently, we are working on the troubleshooting in collaboration with Jackson Lab in vivo xenograft service.



Task 4. Explore the preventive and therapeutic role of MEC-specific NIBP knockout in adult mice.

We have established floxed NIBP transgenic mice by making conditional Cre-LoxP NIBP knockout construct, screening positive ES clones, generating chimera mice and breeding for germline transmission. The heterozygote and homozygote of floxed NIBP mice are healthy and fertile. We have characterized 2 lines of

floxed NIBP mice by cross-breeding them with universal/cell-specific constitutive or inducible Cre mice. Unfortunately, we encountered the difficulties in validating the efficient knockout of NIBP in various cell types from these mice. We are currently working on the troubleshooting by making another line of floxed NIBP mouse in Fox Chase Cancer Center.

As alternative strategy, we have initiated the generation of MEC-specific IKK2 overexpression mice by crossbreeding MMTV-Cre mouse with Rosa26-Floxed-Stop-IKK2CA (constitutively active form) mouse. We have successfully generated GFAP-Cre-IKK2CA and Nestin-Cre-ERT2-IKK2CA mice for neurogenesis studies. We also initiated the cloning of Rosa26-Floxed-Stop-NIBP-IRES-tdTomato to generate floxed NIBP transgenic mouse line for further studies by Cre-induced cell-specific overexpression of NIBP.

Task 5. Determine the role and mechanisms of NIBP in regulating NFκB signaling in breast cancer cells.

5a. Endogenous NIBP interacts with phosphorylated IKK2 in breast cancer cells:

In HEK293T cells, PC12 cells and brain tissues, NIBP interacts with IKK2 but not IKK1 (Hu et al., 2005). Similar interaction was validated in breast cancer cell line MCF7 (Fig. 14A). The IKK2 co-immunoprecipitated with NIBP was phosphorylated as shown by the band shift compared to the input (Fig. 14C) and the immunoblotting with anti-phosphor IKK1/2(Ser-177/181) antibody (Fig.14B).

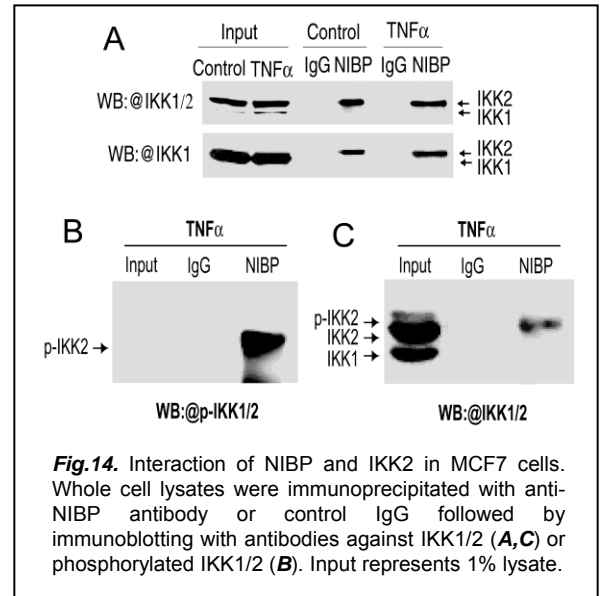


Fig.14. Interaction of NIBP and IKK2 in MCF7 cells. Whole cell lysates were immunoprecipitated with anti-NIBP antibody or control IgG followed by immunoblotting with antibodies against IKK1/2 (A,C) or phosphorylated IKK1/2 (B). Input represents 1% lysate.

5b. NIBP regulates classical IKK2/NFκB signaling: In HEK293T cells, NIBP enhances TNFα and IL-1β-induced NFκB activation evidenced by the increases in NFκB reporter, DNA-binding, IκBα degradation and p65 phosphorylation (Hu et al., 2005). Further studies showed that NIBP enhances IKK2 kinase activity by increasing IKK2 phosphorylation (Hu et al., 2005). Like NIK and IKK2, NIBP is required for NGF-induced neuronal differentiation in PC12 cells(Hu et al., 2005). In this funding, we demonstrated that overexpression of NIBP upregulated but NIBP mutant inhibited constitutive and TNFα-induced NFκB activation in breast cancer cells (Fig. 10, 11 and 15). Lentivirus-mediated NIBP shRNA also inhibited TNFα-induced phosphorylation of IKK1/2 and p65 and degradation of IκBα (Fig. 16). These data suggest that NIBP is required for the constitutive and inducible activation of NFκB signaling in breast cancer cell line. Recent studies from other labs support that NIBP enhances NFκB activation (Philippe et al., 2009; Zahoor et al., 2010).

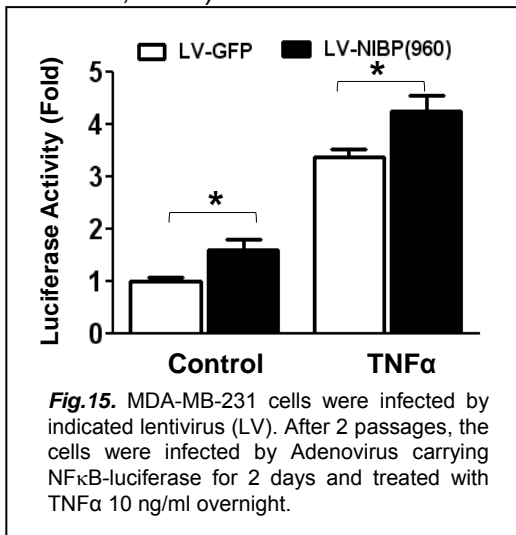


Fig.15. MDA-MB-231 cells were infected by indicated lentivirus (LV). After 2 passages, the cells were infected by Adenovirus carrying NFκB-luciferase for 2 days and treated with TNFα 10 ng/ml overnight.

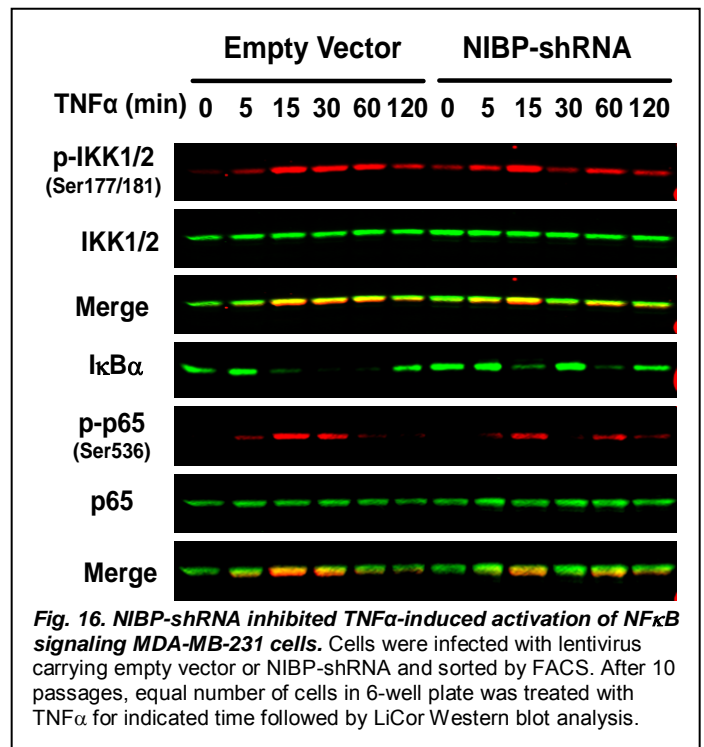


Fig. 16. NIBP-shRNA inhibited TNFα-induced activation of NFκB signaling MDA-MB-231 cells. Cells were infected with lentivirus carrying empty vector or NIBP-shRNA and sorted by FACS. After 10 passages, equal number of cells in 6-well plate was treated with TNFα for indicated time followed by LiCor Western blot analysis.

5c. NIBP has no effect on the non-canonical pathways of NFκB activation.

In MDA-MB-231 cells, treatment with BAFF induced the activation of non-canonical NFκB pathway as determined by the phosphorylation of p100 and the processing of p100 into p52. NIBP mutants or shRNA knockdown did not affect BAFF-stimulated activation of non-canonical NFκB pathway.

5d. Characterize structural-functional relationship between NIBP and IKK2/NIK.

The published NIBP has 960 amino acid residues encoded from mouse NIBP isoform I, designated NIBP(960) according to the number of amino acids. Various isoforms or mutants of human NIBP were prepared and expressed in mammalian expression vector (Fig. 17).

It was previously demonstrated that both NIBP(960) and NIBP(211) interact with IKK2 and NIK(Hu et al., 2005). Here we characterized the structural-functional relationship between various regions of NIBP and NIK/IKK2. As shown in Fig. 18, both A(1-865) and C(603-1148) mutants interacted with NIK and IKK2, whereas B(1-430) and D(1-210) did not interact with either NIK or IKK2, suggesting that the overlapped sequence (603-888) between mutant A and C is responsible for the interaction between NIBP and NIK/IKK2. This region matches the majority of the conserved domain TRS 120 within NIBP, implying that TRS 120 domain (665-888) may interact with NIK/IKK2. Thus, the TRS120 domain was cloned into the pRK-Flag vector, and designated NIBP120 or NIBP-mutE (Fig. 17). The mutE(665-888) has strong interaction with NIK (Fig. 18A) but not with IKK2 (Fig. 18B). This suggests that sequence (603-665) within NIBP contains the IKK2-binding site. Therefore, two regions (603-665 and 937- 1148) within NIBP(1148) interact with IKK2.

Further deletion studies on NIBP-mutF (equal to NIBP(211)) showed that both sub-mutant Fa(1-74) and Fb(1-120) of NIBP-mutF did not interact with NIK (Fig. 19), implying that NIK-binding site is present in the sub-domain (121-211) of NIBP-mutF. This is consistent with the result from yeast two-hybrid screening showing that NIBP-mutF (133-211) interacts with NIK. To analyze the domains within

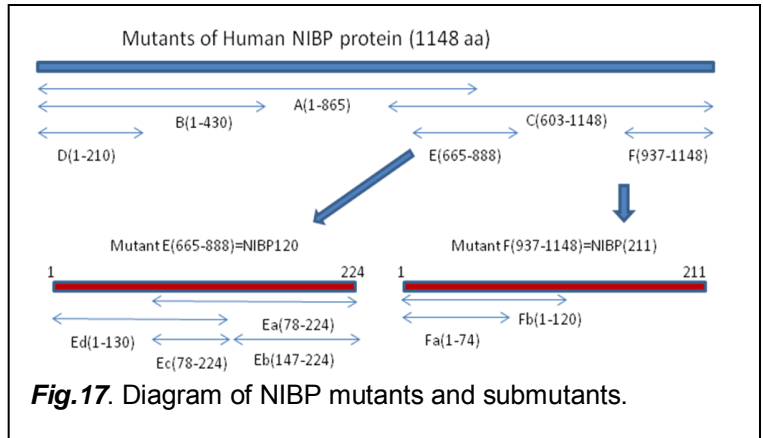


Fig. 17. Diagram of NIBP mutants and submutants.

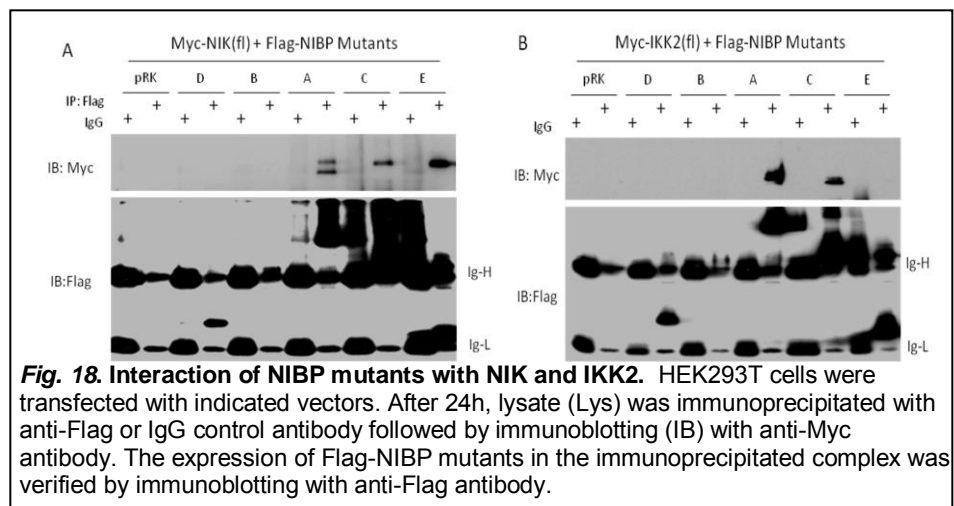


Fig. 18. Interaction of NIBP mutants with NIK and IKK2. HEK293T cells were transfected with indicated vectors. After 24h, lysate (Lys) was immunoprecipitated with anti-Flag or IgG control antibody followed by immunoblotting (IB) with anti-Myc antibody. The expression of Flag-NIBP mutants in the immunoprecipitated complex was verified by immunoblotting with anti-Flag antibody.

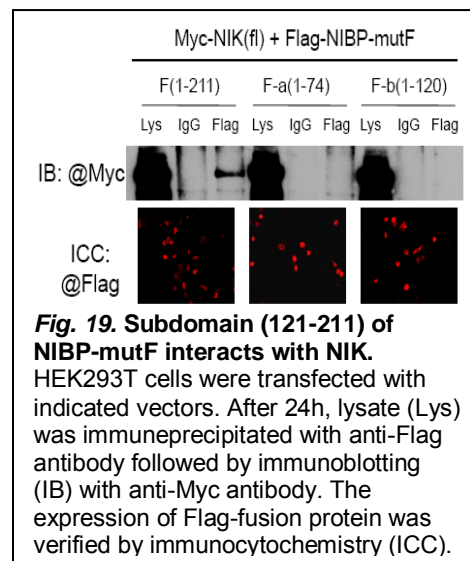


Fig. 19. Subdomain (121-211) of NIBP-mutF interacts with NIK. HEK293T cells were transfected with indicated vectors. After 24h, lysate (Lys) was immunoprecipitated with anti-Flag antibody followed by immunoblotting (IB) with anti-Myc antibody. The expression of Flag-fusion protein was verified by immunocytochemistry (ICC).

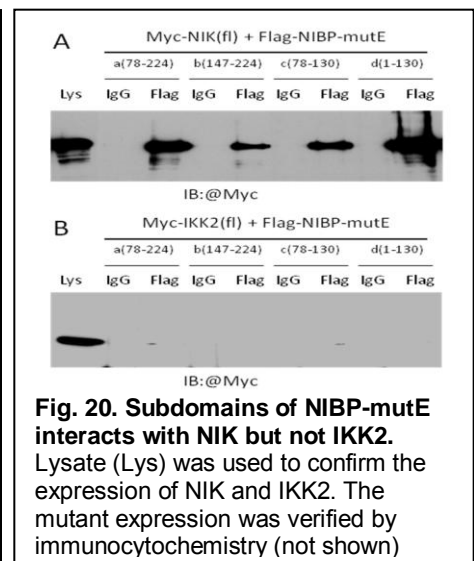
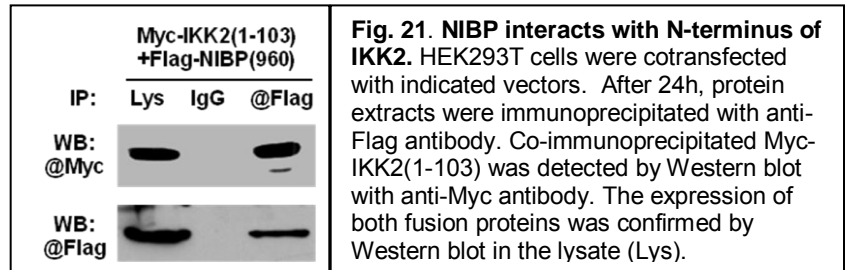


Fig. 20. Subdomains of NIBP-mutE interacts with NIK but not IKK2. Lysate (Lys) was used to confirm the expression of NIK and IKK2. The mutant expression was verified by immunocytochemistry (not shown)

NIBP-mutE(665-888) responsible for NIK binding, four sub-domain mutants were generated by PCR cloning (Fig. 20). These four sub-mutants had no interaction with IKK2, confirming the data as above. However, they all interacted with NIK to various extents (Fig. 20). MutE-a(79-224) and MutE-d(1-130) showed strong interaction with NIK, indicating the region 79-130 (MutE-c) is responsible for NIK binding, though the interaction is weaker than N-terminal region 1-130 (MutE-d). Taken together, the data show that at least three regions (Mut-F, Mut-Ec, and Mut-Eb) within NIBP are capable of interacting with full-length NIK.

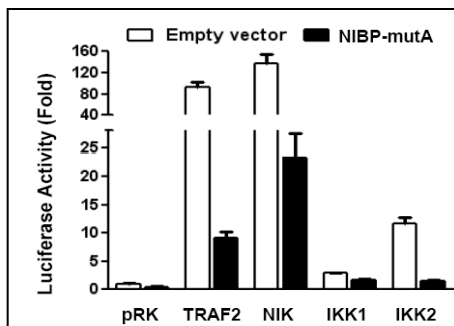
Yeast two-hybrid studies demonstrate that the N terminal region (1-145 aa) of NIK is the binding site for NIBP (Hu et al., 2005). To screen which region of IKK2 interacting with NIBP, various deletion mutants of Myc-IKK2 and IKK2-Flag were made and evaluated. The preliminary studies identified N-terminal region (1-103aa) of IKK2 interacting with NIBP (Fig. 21). These data are important for developing novel pharmaceutical targets.



Function of Selective NIBP Mutants

Since NIBP is a novel regulator of NF κ B signaling, the effects of various NIBP isoforms and mutants on cytokine-induced NF κ B activation were examined. As shown in Fig. 15, enhancing effect of NIBP(960) on the constitutive and TNF α -induced NF κ B activation was corroborated in breast cancer cell line MDA-MB-231. In addition, a similar enhancing effect of new isoforms of NIBP(1246) and NIBP(1148) was identified as shown in Fig. 9. Most interestingly, it was discovered that the mutant E (NIBP120) inhibited NF κ B activation in breast cancer cells MDA-MB-231 (Fig. 9). A similar effect of NIBP120 was validated in MCF7 and Hela cells.

To validate the effect of NIBP120 on NF κ B activation, the dose-response effect in HEK293T cells was evaluated. As shown in Fig. 10, 11, NIBP120 significantly inhibited constitutive and TNF α -induced NF κ B activation. NIBP120 also blocked NF κ B activation induced by over-expression of IKK2 and its upstream signaling components (Fig. 22).



TNF α -induced NF κ B activation is well known to be mediated through classical IKK2-I κ B α /p65 pathway. NIBP120 inhibited TNF α -induced phosphorylation of IKK1/2 (Fig. 11). Generally, IKK2 is phosphorylated by its upstream kinase NIK. Since NIBP120 strongly interacts with NIK but not IKK2, it is believed that NIBP120 may compete with endogenous NIBP (interacting with both NIK and IKK2) by binding to NIK and thus inhibits the activation of IKK2. Surprisingly, NIBP 120 increased TNF α -induced phosphorylation of p65 at Ser-536 (Fig. 11). Although the mechanisms and significance remain unknown, it may reflect the fact that p65 phosphorylation is activated by not only IKK2 but also several other kinases such as IKK1 and RSK1.

Another interesting finding was that NIBP120 increased the constitutive and TNF α -induced activation of MAPK signaling pathways as determined by the increased phosphorylation in JNK, p38 and ERK1/2 (Fig. 23). This suggests that NIBP120 may have wider

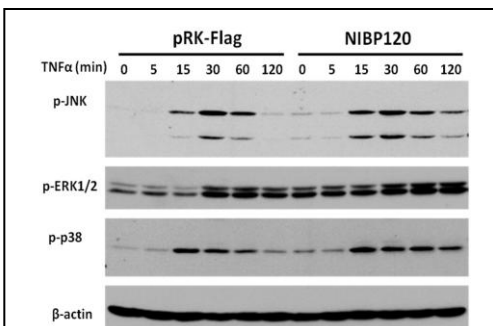


Fig. 22. NIBP120 inhibits IKK2-mediated NF κ B activation. HEK293T cells were cotransfected with indicated vectors and NF κ B firefly-luciferase and renilla-luciferase vectors. After 24 h, dual luciferase assay was performed. Data are expressed as relative change compared with empty vector control.

Fig. 23. NIBP120 enhances constitutive and TNF α -stimulated phosphorylation of JNK, ERK1/2 and p38 MAPK. MDA-MB-231 cells were transfected with empty or NIBP120 vector. After 5 days, cells were treated with TNF α for indicated time and Western blotting was performed with indicated antibodies after stripping.

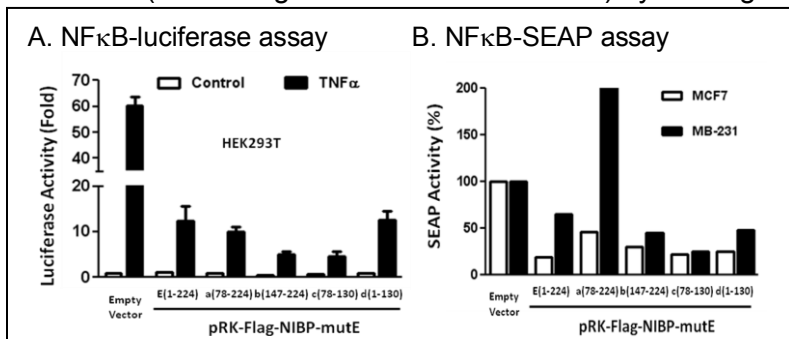


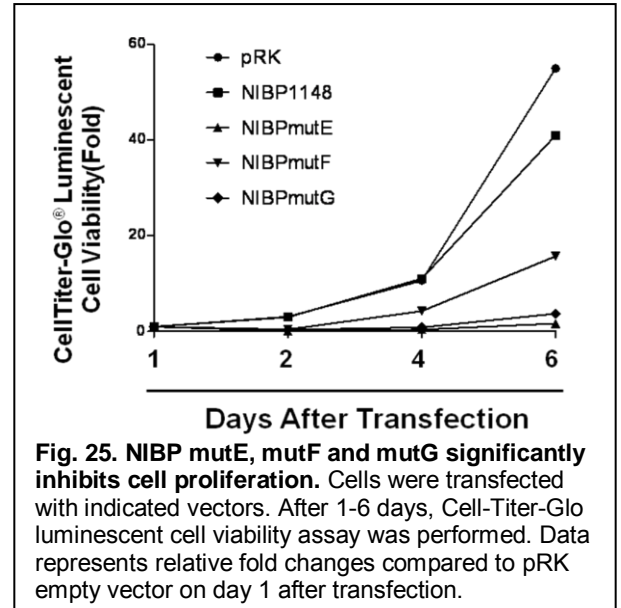
Fig. 24. Submutants of NIBP120 (mutE) inhibited constitutive and TNF α -stimulated NF κ B activation. HEK293T cells (A) or breast cancer cell lines (B) were cotransfected with empty vector or NIBP120 submutants plus NF κ B-firefly luciferase (A) or NF κ B-SEAP (secreted alkaline phosphatase) reporter (B) and pcDNA3-renilla luciferase reporter (for normalization). After 24 h, cells were treated with TNF α 10 ng/ml for 24 h and dual luciferase or SEAP activities were measured. Representative data in quadruplicate are expressed as relative change compared with empty vector control.

functions and applications in addition to NF κ B signaling.

To identify the subdomains of NIBP120 responsible for the inhibitory function, deletion mutants as shown in Fig. 17 were prepared and tested for their effect on NF κ B activation in HEK293T cells and MCF7 and MB-231 cancer cells. As shown in Fig. 24A, NIBP-mutE significantly blocked TNF α -induced NF κ B activation in HEK293T cells, while all four submutants retained the inhibitory effect with further inhibition by the mutEb and mutEc, implying that potential motif within mutEb and mutEc are present for the development of pharmaceutical inhibitors. In the breast cancer cell line, the constitutive activity of NF κ B reporter was significantly inhibited by all submutants in MCF7 and by mutEb, Ec and Ed (Fig. 24B) with the strongest inhibition by mutEc in both cell lines. Therefore, further identification of the motifs within mutEc(79-130) will be greatly valuable.

We also identified another peptide (64 amino acid) designated NIBPmutG (matching 604-668 residues of NIBP1148) that significantly inhibited the proliferation of cancer cells (Fig. 25).

We are still working on the effects of these mutants on other cancer cell line and primary tumor cells. We have also created various mutants of NIK and IKK2 in order to identify unique domain or peptides that may interact with NIBP and affect its functions.



Key Research Accomplishments

1. NIBP mRNA and protein were highly expressed in breast cancer cell lines and breast tumor tissues;
2. The expression level of NIBP was positively correlated to the stage of breast cancer;
3. ELISA to detect NIBP level in serum, stool and tissue lysate was optimized;
4. Lentivirus-mediated NIBP shRNA knockdown in breast cancer cell line inhibited the proliferation and colony formation of breast cancer cell lines;
5. Various NIBP mutants were identified and their functional significances were examined;
6. NIBP modulates canonical pathway of NF κ B activation;
7. Xenograft and NIBP conditional knockout animal studies are under way.

Reportable Outcomes:

Patents:

US Provisional Application No. 61/251,013 and the corresponding International Application No. PCT/US2010/052302, titled, 'Use of NIBP Polypeptides', published on April 21, 2011.

Conclusion:

During the first year of this funding period, we have completed most of the in vitro studies. We found very exciting data that NIBP is highly expressed in human breast cancer cell lines and tumor tissues. Such high level of NIBP leads to constitutive activation of classical NF κ B pathway that contributes to the development and progression of breast cancer. In the coming year, the major tasks will be the in vivo studies using xenograft and MEC-conditional knockout animals. The in vivo studies will further validate our in vitro data. NIBP could be a novel and important biomarker for the diagnosis and treatment of breast cancer.

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Appendices: N/A