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PRINCIPAL INVESTIGATOR: Thomas F. Deuel, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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Thomas F. Deuel, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: tdeuel@caregroup.harvard.edu

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Progress

Objective I

Progress has been made to meet Objective I.

1.) The Preliminary Results described above have been confirmed.

2.) We have now altered our strategy relative to the library to be used in these experiments. We now plan to use a cDNA library obtained from the National Cancer Institute. It is a cDNA library derived from four independent primary human breast cancers. This library is an exceedingly well-made library by individuals interested in breast cancer and experienced in making cDNA libraries. It is a composite of four separate tumors, providing the potential for discovery of additional genes that will cooperate with pleiotrophin to convert the MCF 10A cells expressing the *Ptn* gene into a more aggressive growth phenotype. Furthermore, the library is not derived from a cell line, but rather is derived from primary tissues, thereby avoiding the problems of mutations introduced in cell lines through prolonged passage. This library has been obtained in the laboratory. We also have altered our strategy to now attempt to place this library in a retrovirus system. The transfection efficiency of this library will be considerably higher than the transforming efficiency or the system previously planned and, potentially, may bypass the need for selection.

3.) We have transfected the angiogenic domains of PTN (69-136) and MK (1-3,59-121), both and full-length PTN and MK, into the MCF10A cells, a human cell line derived from spontaneous immortalization of non-malignant breast epithelium (Miller *et al.* 1993). Selection and characterization is ongoing. We anticipate moving ahead with completing clonal selection, expansion, and evaluation of the different levels of gene expression by Northern and Western analysis. Clones that show high-level expression will be retained, and cells will be tested for any changes in cell-cycle profiles, growth curves, and doubling times. Particularly important will be confirmation of previous preliminary results establishing that these cells do not form colonies in soft agar. This control is essential to ensure the effectiveness of the assay system for screening.

Objective II

1.) We have completed preliminary studies with the subtractive hybridization strategy originally outlined. We have not confirmed differential expression in the cells themselves. Nevertheless, in response to the critiques submitted to us at the time our project was awarded for funding, we have used alternative approaches to determine downstream affectors of PTN as well; this we believe has been most fruitful and is outlined below.

2.) We used a modification of the recently described yeast two-hybrid system to identify genes whose protein products associate with the receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ). RPTPβ/ζ is the (a) receptor for pleiotrophin. It is expressed in MCF7 cells.

A yeast 2-hybrid design has several advantages for determining protein-protein interactions. First, it is an *in vivo* system that should maintain the native conformation of

expressed proteins, thereby revealing authentic protein-protein interactions. Second, the genetic selection allows rapid screening of a large number of candidate proteins. Furthermore, this system facilitates the cloning and sequencing of cDNAs that encode interactive proteins with RPTPβ/ζ. Finally, this lab has used this technique successfully to demonstrate other protein-protein interactions, and thus has expertise that will facilitate the analysis.

The full-length intracellular domain at RPTPβ/ζ was used as "bait." After a yeast 2-hybrid screen using the Clontech Matchmaker System^{tm} (Clontech, USA), 292 colonies were seen under moderate selection (Trp^-/His^-). These 292 colonies were rescreened under the most stringent selective conditions ($Trp^-/His^-/Ade^-$). Of these 292 colonies, 137 grew under the most stringent selective conditions. These 137 colonies were than assayed for β-galactosidase activity. Eighty-nine of the 137 colonies demonstrated β-galactosidase activity, as described above. The intensity of X-gal coloration was measured with a densitometer on the filter lifts and 31 of the colonies had moderate to intense blue staining (reflectance greater than 0.1). Twelve of the cDNA inserts have been sequenced and the remaining 19 clones are in the process of being purified and sequenced. After verification and characterization of these 31 clones, the clones with the lesser β-galactosidase activity were sequenced. No colonies were seen for the negative controls discussed previously.

From the 12 clones isolated that encode proteins identified as interactive with the intracellular domain of RPTP βζ, eight had greater than 95% nucleotide sequence identity to the previously characterized human t-complex polypeptide (TCP-1). The other clones identified were: histone deacetylase 2 (HDAC2), transmembrane 4 superfamily member 81 (CD81), microtubule associated protein 1B (MAP1B), heat shock protein C041 (HSP041), and human adducin 2β.

We have chosen to focus initially on 3 of these clones, which encode TCP-1, HDAC2, and adducin 2β. The other clones encoding proteins of potential interest will be addressed subsequently as validation of their association with RPTPβ/ζ by repeated sequence analysis and their criteria indicate their potential to participate in PTN signaling. The rationale for the initial focus follows.

Since eight of the independent, high affinity binding clones are t-complex polypeptide 1, the likelihood that this interaction is significant is high. The protein is a member of a hetero-oligometric chaperone complex localized to the cytosolic face of the Golgi apparatus. It is involved in maintaining the correct folding of
cytosolic skeletal proteins, including actin and tubulin (Brown *et al.* 1996), and is involved in microtubule nucleation (Brown *et al.* 1996). Little is known about the regulation of TCP-1 mediated microtubule nucleation activity. It may also be an important step in the PTN signaling pathway and regulation of cell migration.

Another RPTPβ/ζ binding protein uncovered is HDAC2. HDAC2 is potentially of major importance because of its role in silencing (Nagy *et al.* 1997). The HDAC family of enzymes deacetylates the histone 4 core protein, making the deacetylated region of nuclear chromatin inaccessible to transcriptional machinery. HDAC4 and 5 are sequestered within the cytoplasm by tyrosine phosphorylation at three sites (McKensey *et*

al. 2000), allowing acetylated histones to remain in localized chromosome regions and "open" for gene
activation. Since HDAC2 shares 60% amino acid sequence identity with HDAC4, it is possible that HDAC2 activity is also regulated by phosphorylation. If the RPTPβ/ζ/HDAC2 interaction is shown to be valid *in vivo,* it is exciting to conjecture that RPTPβ/ζ may be active in the regulation of the steady state levels of tyrosine phosphorylation of HDAC2, potentially regulating its translocation to the nucleus and the "shut down" of transcription of selective genes. Thus, it is speculated that sequestration of HDAC2 may be regulated through RPTPβ/ζ phosphatase activity.

The other potential RPTPβ/ζ binding partner selected is adducin 2β. Adducin 2β is localized at spectrin-actin junctions at the inner cell membrane and at sites of cell-cell contact in epithelial cells (Kaiser *et al.* 1989). Three reasons support its potential importance as a binding partner for RPTPβ/ζ. It is localized to the inner cell membrane in cell types in which RPTPβ/ζis expressed. Adducin 2β also has a 13 amino acid tyrosine phosphorylation consensus motif and thus its phosphorylated form may be a substrate for RPTPβ/ζ. Finally, adducin 2β is a cytoskeletal organizing protein and is a substrate for phorbol 12-myristate 13-acetate (PMA)-activated kinases, which function to activate adducin 2β's ability to recruit spectrin and recruit actin filaments to the cell membrane (Kimwa *et al.* 1998). RPTPβ/ζ may thus function in regulating these cytoskeletal elements in response to PTN signaling.

In support of the importance of these results, we have treated Hela cells with 50ng/mL PTN. Cells were probed with an antibody directed against phospho-adducin. The effect of PTN is clear; there is a striking loss of phospho-adducin associated with the nucleus and concomitant increase in cell membrane associated phospho-adducin.

We also now have data to show that HD1C2 migrates to the nucleus of cells in response to PTN. Loss of membrane localization has been found to occur within five minutes of the addition of PTN to the cultured cells.

These results are particularly exciting. Each of the proteins identified is expressed in MCF7 cells. Each of the proteins is involved either with cytoskeletal regulation and thus cytoskeletal stability, critical to metastasis or cell adhesion in breast cancer or, with gene transcription. The use of the different approaches thus is of major importance and seems likely to be very helpful in understanding roles of PTN in progression of human breast cancer.

We have initiated manuscripts to describe results of experiments in which PTN and different mutant PTNs have been introduced into MCF7 cells and studied as tumors in the nude mouse. These data have made clear both MK and PTN as well as the mutant proteins promote the growth of these tumors in the nude mouse.

We have also used "feeder cells" of NIH 3T3 cells co-mingled with MCF7 cells and showed the potential of these feeder cells to dramatically enhance the aggressiveness of the tumors at site of implantation of MCF7 cells. These preliminary studies are directed to understanding the roles of non-tumor cells and extracellular matrix in promoting the growth of breast cancer cells *in vivo.*

Key Research Accomplishments

Objective I

1. The achievement of an appropriate cDNA library from primary human breast cancers as a source of

genes potentially able to use PTN to effectively enhance MCF 10A cell growth in the nude mouse.

2. The development of MCF 10A-PTN, -PTN mutant cell lines.

Objective II

1. The identification of downstream interactive proteins with the receptor protein RPTPβ/ζ and thus of

the PTN signaling pathway in human breast cancer cells.

Reportable Outcome

Manuscripts (in preparation) Pleiotrophin, Midkine and their Mutant Proteins Promote the Angiogenic Phenotype and Rapid Tumor Growth of Human MCF7 Breast Cancer Cells in Nude Mice.

Conclusions

We are pleased with progress thus far. We have established the base for obtaining the results we sought in submitting the proposal. We have advanced understanding of pleiotrophin and midkine in the promotion of human breast cancer cells. We have identified and initiated understanding of the functions of pleiotrophn in these cells.

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